Original Article

The Type and Frequency of Immunoregulatory CD4$^+$ T-Cells Govern the Efficacy of Antigen-Specific Immunotherapy in Nonobese Diabetic Mice

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Antigen-specific immunotherapy, an approach to selectively block autoimmune diabetes, generally declines in nonobese diabetic (NOD) mice as disease progresses. To define the parameters influencing the efficacy of antigen-specific immunotherapy once diabetes is established, plasmid DNA (pDNA) vaccination was used to suppress autoimmune-mediated destruction of syngeneic islet grafts in diabetic NOD recipients. pDNAs encoding a glutamic acid decarboxylase 65 (GAD65)-Ig molecule (pGAD65), interleukin (IL)-4 (pIL4), and IL-10 (pIL10) significantly delayed the onset of recurrent diabetes compared with pGAD65+pIL10-vaccinated recipients. Despite differences in efficacy, a similar frequency of GAD65-specific CD4$^+$ T-cells secreting IL-4, IL-10, or interferon-γ were detected in mice treated with pGAD65+pIL4+pIL10 and pGAD65+pIL10. However, the frequency of FoxP3-expressing CD4$^+$CD25$^+$CD62L^{hi} T-cells was increased in the renal and pancreatic lymph nodes of diabetic recipients vaccinated with pGAD65+pIL4+pIL10. These immunoregulatory CD4$^+$CD25$^+$ T-cells (CD4$^+$CD25$^+$ Treg) exhibited enhanced in vivo and in vitro suppressor activity that partially was transforming growth factor-β dependent. Furthermore, duration of islet graft protection in pGAD65+pIL4+pIL10-vaccinated diabetic recipients correlated with the persistence of CD4$^+$CD25$^+$ Treg. These data demonstrate that the frequency and maintenance of FoxP3-expressing CD4$^+$CD25$^+$ Treg influence antigen-induced suppression of ongoing β-cell autoimmunity in diabetic recipients. *Diabetes* 56:1395–1402, 2007

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GAD65, glutamic acid decarboxylase 65; HEL, hen egg lysozyme; IL, interleukin; IFN, interferon; pDNA, plasmid DNA; PLN, pancreatic lymph node; RLN, renal lymph node; Treg, regulatory T-cell; TGF, transforming growth factor.

Type 1 diabetes is characterized by the destruction of the pancreatic β-cells (1–3). The primary mediators of β-cell destruction are CD4$^+$ and CD8$^+$ T-cells, which typically exhibit a proinflammatory type 1 phenotype (1–3). Skewing toward type 1 differentiation by naive β-cell–specific T-cells is believed, in part, to be because of a deficiency in immunoregulatory CD4$^+$ T-cells. Reduced frequencies of β-cell–specific "adaptive" immunoregulatory CD4$^+$ T-cells, such as Th2 and Tr1 cells, have been reported in diabetic individuals and nonobese diabetic (NOD) mice (4–8). These CD4$^+$ T effectors regulate autoimmunity primarily through the bystander effects of interleukin (IL)-4 and -10 secretion. Diminished numbers and/or suppressor function of "natural" regulatory CD4$^+$CD25$^+$CD62L^{hi} T-cells (CD4$^+$CD25$^+$ Treg) also have been documented in diabetic NOD mice and patients (9–14). CD4$^+$CD25$^+$ Treg cells are characterized by a potent suppressor capacity mediated by cell-to-cell contact and/or expression of transforming growth factor (TGF)-β1 and possibly IL-10 (15–18). Whereas adaptive immunoregulatory effectors are established once naïve CD4$^+$ T-cells encounter antigen in the periphery, the phenotype and suppressor function of CD4$^+$CD25$^+$ Treg is induced in the thymus upon recognition of self-antigen and expression of the FoxP3 transcription factor (19–22).

In view of the functional imbalance between pathogenic type 1 and immunoregulatory β-cell–specific T-cells, various strategies of immunotherapy to prevent and treat type 1 diabetes have been devised to reestablish peripheral immunoregulation (23). Previous studies (24–27) have demonstrated that administration of β-cell autoantigens, such as glutamic acid decarboxylase 65 (GAD65), or insulin by various means induces Th2- and Tr1-like cells and prevents the onset of diabetes in NOD mice. Antigen-specific immunotherapy also provides a possible strategy to rescue residual β-cell mass and/or block autoimmune-mediated destruction of islet grafts in diabetic individuals. However, whether sufficient β-cell–specific immunoregulatory CD4$^+$ T-cells can be induced to suppress autoimmunity under overt diabetic conditions, in which the number of pathogenic effectors is high and only a limited pool of naïve β-cell–specific CD4$^+$ T-cells exists (28,29), remains largely untested.

We previously demonstrated that administration of plasmid DNA (pDNA) vaccines encoding a GAD65 immunoglobulin (Ig) chimeric molecule, IL-4, and/or IL-10 is an effective approach to suppress late preclinical type 1

diabetes in NOD mice via induction of immunoregulatory type 2 $CD4^+$ T-cells (26,30). In the current study, pDNA vaccination was used to define the parameters influencing the efficacy of antigen-based immunotherapy in diabetic NOD mice. Here, survival of syngeneic islet grafts implanted in diabetic recipients was used as a functional measure of suppression of β-cell autoimmunity. We demonstrate that suppressing β-cell autoimmunity in diabetic recipients is dependent on the frequency and persistence of FoxP3-expressing $CD4^+CD25^+$ Treg induced/expanded following pDNA vaccination.

RESEARCH DESIGN AND METHODS

NOD/LtJ, NOD.scid, and NOD.BDC2.5 (BDC2.5) (31) mice were housed under specific pathogen-free conditions. Mice were diagnosed as diabetic when urine glucose exceeded 0.25% for three successive measurements via Diastix (Bayer Corporation, Elkhart, IN). A urine glucose level of 0.25% is equivalent to a blood glucose value of 250 mg/dl (Autokit Glucose CII assay; Wako Chemicals, Richmond, VA). Mice were maintained in an American Association of Laboratory Animal Care–accredited facility, and all procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee.

pDNA preparation and administration. Construction and preparation of pDNAs encoding GAD65-IgFc (pGAD), hen egg lysozyme (HEL)-IgFc (pHEL), and murine IL4 (pIL4) have been previously described (10,30). The pNGVL3-IL10 construct (pIL10) encoding murine IL-10 was obtained from the National Gene Vector Laboratory at the University of Michigan. Diabetic NOD female mice received a total of four intramuscular (i.m.) injections in each quadricep with 50 μg of pDNA prepared in PBS on a weekly basis, in addition to daily intraperitoneal (i.p.) injections of 60 units human insulin.

Islet transplantation. One week after the final pDNA vaccination, mice received syngeneic islet transplants. Pancreata from NOD.scid mice were perfused with 2 mg/ml collagenase P (Roche, Indianapolis, IN) and digested for 20 min at 37°C. Islets were purified via Ficoll gradient, handpicked, washed in PBS, and implanted under the kidney capsule. Mice were considered to be euglycemic posttransplantation after two successive negative glycosuric measurements. Graft failure was defined as glycosuric values >0.25% (250 mg/dl blood glucose) for two successive measurements.

Enzyme-linked immunosorbent spot. Enzyme-linked immunosorbent spot to determine the number of spot-forming cells producing interferon (IFN) γ, IL-4, and IL-10 was carried out as described (30). Splenocytes were prepared from individual mice, washed, resuspended at 5×10^6 cell/ml in HL-1 medium (BioWittaker, Wakersville, MD), and plated at 1×10^6 cells/well (200 μl/well). GAD65 was added at a final concentration of 10 μg/ml. The cloning and preparation of murine GAD65 previously have been described (24). An ImmunoSpot plate reader (Cellular Technology) was used to quantitate the number of spot-forming cells per well.

Flow cytometric analysis. Antibodies were purchased from BD Pharmingen and used for analysis of $CD4^+CD25^+$ T-cells: fluorescein isothiocyanate–rat anti-CD3 (17A2), PerCP-rat anti-CD4 (GK1.5), phycoerythrin-rat anti-CD62L (Mel-14), and allophycocyanin-rat anti-CD25 (PC61). $CD4^+CD25^+CD62L^{hi}$ and $CD4^+CD25^-$ T-cells were sorted using a MoFlo high-speed sorter (DakoCytomation, Fort Collins, CO) for single-cell real-time PCR analysis, suppression assays, and adoptive transfer experiments. Sorted bulk populations, >98% purity, were stained with the above antibodies as described (10).

Single-cell real-time PCR. Single $CD4^+CD25^+CD62L^{hi}$ or $CD4^+CD25^-$ T-cells were sorted from the pancreatic lymph node (PLN) and renal lymph node (RLN). These cells were reverse transcribed, and real-time PCR was performed using primers and probes specific for FoxP3, TGF-β1, IL-10, and β-actin as described (10). The lower limit of detection was 10 copies for all genes tested. Each gene was detected in independent real-time PCR reactions using 5 μl of a 25-μl total cDNA mixture. Data are represented as RNA expression normalized to β-actin RNA content. The normalized mRNA level for a gene was determined by the following: (raw transcript level derived from standard curve) × (β-actin corrective ratio). The β-actin corrective ratio was calculated as (lowest β-actin level within sample set)/(β-actin level for cell of interest).

In vitro $CD4^+CD25^+$ T-cell suppression assay. $CD4^+CD25^+CD62L^{hi}$ T-cells sorted from the PLN or RLN were cocultured in 96-well plates with 1×10^5 sorted $CD4^+CD25^-$ T-cells plus 1 μg/ml anti-CD3ε (145-2C11) (eBioscience, San Diego, CA) and 1×10^5 irradiated T-cell–depleted splenocytes as described (10). T-cells were cocultured for 72 h at 37°C and pulsed with 1 μCi/well of ^{3}H-thymidine for 18 h. ^{3}H-thymidine incorporation was measured by a scintillation counter (Beckman LS6500). Percent inhibition was determined by the following equation: $1 - \{[(CD4^+CD25^-CD62L^{hi}$ (or $CD62L^{lo}$) + $CD4^+CD25^+)$ cpm]/[$(CD4^+CD25^-$ alone) cpm]$\} \times 100$. Some studies included the addition of anti–TGF-β1, -2, or -3 (1D11) or IgG1 isotype control antibody (R&D Systems, Minneapolis, MN) at 100 μg/ml.

Measurement of TGF-β secretion via enzyme-linked immunosorbent assay. As described, a capture enzyme-linked immunosorbent assay was used to measure levels of TGF-β in supernatants harvested from individual wells prepared from in vitro suppression assays (10). Cytokine concentrations in culture supernatants were determined using standard curves of hTGF-β (BD Pharmingen). The lower limit of detection for TGF-β was 40 pg/ml.

In vivo $CD4^+CD25^+$ T-cell suppression assay. 1.2×10^5 $CD4^+CD25^+$ T-cells sorted from the PLN of diabetic NOD female mice vaccinated with pDNA were coinjected intravenously with 1.2×10^6 BDC2.5 $CD4^+$ T-cells into NOD.scid recipients. Splenic BDC2.5 $CD4^+$ T-cells were isolated with anti–CD4-coated magnetic microbeads (Miltenyi Biotec) and magnetic cell sorting selection. Mice were monitored daily for diabetes as described above.

Characterization of islet graft insulitis. Grafted kidneys from individual mice were flash frozen in Optimal Cutting Temperature compound (Sakura Finetechnical, Tokyo, Japan) and the entire kidney sectioned. Hematoxylin and eosin staining was performed on serial tissue sections 5 microns apart.

RESULTS

Survival of syngeneic islet grafts is enhanced in diabetic NOD recipients treated with pGAD65+pIL4+pIL10. To determine whether antigen-specific immunotherapy can suppress ongoing β-cell autoimmunity in diabetic NOD mice, pDNAs encoding GAD65, IL-4, and/or IL-10 were administered (26). As a functional readout for suppression of β-cell autoimmunity, survival of syngeneic islet grafts implanted under the kidney capsule of diabetic NOD females was used. Diabetic NOD female mice maintained on insulin received four intramuscular injections over a 4-week period of pGAD65+pIL4+pIL10 or pGAD65+pIL10. Control groups consisted of diabetic NOD mice left untreated or receiving pHEL+pIL4+pIL10. One week after the final pDNA vaccination, diabetic mice received 500–600 syngeneic islets under the kidney capsule and recurrent diabetes monitored once euglycemia was reestablished. All recipient mice vaccinated with control pHEL+pIL4+pIL10 or left untreated developed recurrent diabetes at a median onset of 13 and 11 days, respectively (Fig. 1*A*). Onset of recurrent diabetes was similarly detected in NOD recipients treated with pGAD65+pIL4 (Fig. 1*A*). In contrast, NOD recipients vaccinated with pGAD65+pIL4+pIL10 exhibited a significant delay ($P < 10^{-3}$) in recurrent diabetes compared with pHEL+pIL4+pIL10-treated and untreated recipients, with a median onset of 45 days (Fig. 1*A*). Grafts implanted in pGAD65+pIL4+pIL10-treated NOD recipient mice were free of cellular infiltration at 3 weeks posttransplantation (Fig. 1*B*). However, at 5 weeks posttransplantation significant infiltration of the islet grafts was detected, and upon the onset of recurrent diabetes much of the graft had been destroyed (Fig. 1*B*). pGAD65+pIL10 vaccination also resulted in a delay in recurrent diabetes relative to pHEL+pIL4+pIL10-treated and untreated recipients ($P < 0.02$), with a median onset of 19 days. However, the duration of protection was significantly shorter compared with pGAD65+pIL4+pIL10-vaccinated mice ($P < 10^{-3}$; Fig. 1*A*). These results demonstrate that pGAD65+pIL4+pIL10 vaccination significantly delays recurrent diabetes by preventing infiltration of grafted islets in an antigen-dependent manner.

pGAD65+pIL4+pIL10 and pGAD65+pIL10 vaccination induce similar GAD65-specific type 2 $CD4^+$ T-cell reactivity in diabetic NOD mice. To determine how vaccination with pGAD65+pIL4+pIL10 induced more effective islet graft protection than pGAD65+pIL10, the

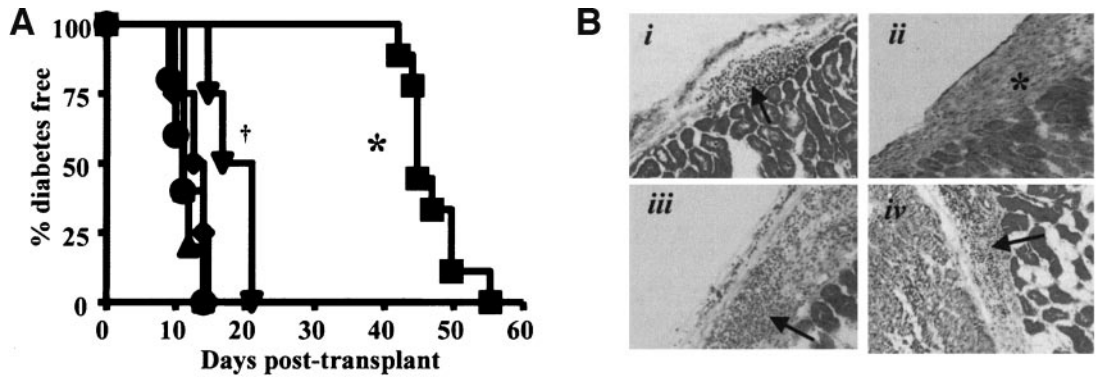

FIG. 1. pGAD65+pIL4+pIL10 prolongs syngeneic islet graft survival in diabetic NOD recipients. *A*: Recurrent diabetes was monitored in graft recipients treated with pGAD65+pIL4+pIL10 (n = 9), pGAD65+pIL10 (n = 5), pGAD65+pIL4 (n = 5), pHEL+pIL4+pIL10 (n = 5), or left untreated (n = 5). *P < 0.001, pGAD65+pIL4+pIL10 vs. other groups; †$P \leq 0.02$, pGAD65+pIL10 vs. pHEL+pIL4+pIL10, pGAD65+pIL4, and untreated. ▲, untreated; ♦, pHEL+pIL4+pIL10; ▼, pGAD65+pIL10; ■, pGAD65+pIL4+pIL10; ●, pGAD65+pIL4. *B*: Graft immunohistology for untreated and pGAD65+pIL4+pIL10 recipients. Arrows = infiltration; asterisk = infiltration free.

frequency of GAD65-specific IFNγ-, IL-4–, and IL-10–secreting $CD4^+$ T-cells in the spleen of pDNA-treated diabetic NOD mice was measured via enzyme-linked immunosorbent spot. pHEL+pIL4+pIL10-treated and untreated diabetic NOD mice exhibited a typical type 1 profile in response to GAD65 characterized by a high frequency of IFNγ- and no detectable IL-4– or IL-10–secreting $CD4^+$ T-cells (Fig. 2). In contrast, the frequency of IFNγ-secreting GAD65-specific $CD4^+$ T-cells was reduced, and a concomitant increase in IL-4– and IL-10–secreting GAD65-specific $CD4^+$ T-cells was detected in pGAD65+pIL4+pIL10-vaccinated diabetic NOD mice (Fig. 2). Interestingly, a similar frequency of IFNγ-, IL-4–, and IL-10–secreting GAD65-specific $CD4^+$ T-cells was detected in pGAD65+pIL10-treated diabetic NOD mice (Fig. 2). Analogous results were obtained in the PLN and RLN of diabetic NOD mice vaccinated with the respective pDNA and implanted with an islet graft (data not shown). These findings demonstrate that despite differences in islet graft protection, pGAD65+pIL4+pIL10 and pGAD65+pIL10 treatments induce similar GAD65-specific Th2- and Tr1-like cell responses in diabetic NOD mice.

The frequency of FoxP3 and TGF-β1 coexpressing $CD4^+CD25^+CD62L^{hi}$ T-cells is increased by pGAD65+pIL4+pIL10 vaccination. Because the disparity in islet graft protection between pGAD65+pIL4+pIL10 and pGAD65+pIL10 treatment did not correlate with a significant difference in GAD65-specific $CD4^+$ T-cell reactivity, a role for "natural" $CD4^+CD25^+$ Treg was examined. CD62L typically is used to differentiate between activated $CD4^+CD25^+$ T-cells ($CD62L^{lo}$) and natural $CD4^+CD25^+$ Treg ($CD62L^{hi}$) (32–34). Accordingly, the number of $CD62L^{hi}$ T-cells residing in the RLN and PLN was measured via flow cytometry in islet graft recipients treated with the pDNAs or left untreated. An increase in $CD62L^{hi}$ T-cells was detected in the RLN and PLN of islet graft recipients receiving pGAD65+pIL4+pIL10 compared with pHEL+pIL4+pIL10 (RLN: P = 0.003; PLN: P = 0.006) or no pDNA (RLN: P = 0.003) (Table 1). A significant increase in $CD62L^{hi}$ T-cells also was detected in the RLN (P = 0.007) and PLN (P = 0.01) of pGAD65+pIL4+pIL10- versus pGAD65+pIL10-vaccinated recipient mice (Table 1). No differences in the level of surface expression of CTLA-4, glucocorticoid-induced tumor necrosis factor receptor gene, CD45RB, and CD103 were detected among $CD62L^{hi}$ T-cells prepared from the respective groups of mice (data not shown). These findings demonstrate that the number/frequency of $CD62L^{hi}$ T-cells is increased in NOD recipient mice treated with pGAD65+pIL4+pIL10 compared with pGAD65+pIL10-treated recipients, which in turn have elevated $CD62L^{hi}$ T-cells relative to pHEL+pIL4+pIL10-treated or untreated recipients.

To further define the population of RLN and PLN $CD62L^{hi}$ T-cells induced in islet graft recipients via pDNA vaccination, mRNA expression of FoxP3, TGF-β, and IL-10 was quantitated. For this purpose, a real-time PCR assay

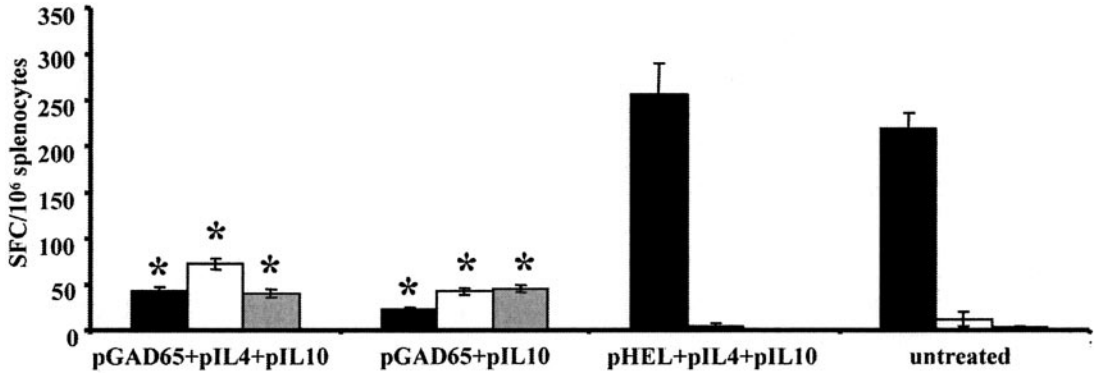

FIG. 2. pGAD65+pIL4+pIL10 and pGAD65+pIL10 induce similar GAD65-specific responses in diabetic NOD female mice. Splenocytes were prepared 3 weeks postimmunization, and the frequency of GAD65-specific IFNγ-, IL-4–, and IL-10–secreting T-cells measured via enzyme-linked immunosorbent spot. Spot-forming cells from medium only (0–18) were subtracted. Data are an average of two separate experiments (four mice per group per experiment). *P < 0.001, pGAD65+pIL4+pIL10 and pGAD65+pIL10 vs. untreated and control mice. ■, IFNγ; □, IL-4; ▨, IL-10.

TABLE 1
Increased RLN and PLN $CD4^+CD25^+CD62L^{hi}$ T- cells are detected in pGAD65+pIL4+pIL10 immunized diabetic NOD recipient mice

	pGAD65+pIL4+pIL10	pGAD65+pIL10	pHEL+pIL4+pIL10	Untreated
RLN $CD4^+$	7,784 ± 418*	5,351 ± 225	5,942 ± 232	5,700 ± 248
RLN $CD25^+$	2,020 ± 120* (26.0)†	1,005 ± 101‡ (18.8)	623 ± 59 (10.5)	564 ± 45 (9.9)
RLN $CD25^+CD62L^{hi}$	1,577±105* (78.1)§	633 ± 30‡ (63.0)	211 ± 22 (33.9)	191 ± 10 (33.9)
PLN $CD4^+$	7,625 ± 398†	5,268 ± 245	5,632 ± 198	5,544 ± 321
PLN $CD25^+$	1,852 ± 112* (24.3)†	916 ± 98‡ (17.4)	518 ± 64 (9.2)	543 ± 52 (9.8)
PLN $CD25^+CD62L^{hi}$	1,211 ± 110* (65.4)§	420 ± 24‡ (46.0)	164 ± 34 (31.8)	180 ± 21 (33.3)

Data are means ± SD (%) for the average of two experiments; number of T-cells is per 10,000 cells analyzed. *$P \leq 0.032$, pGAD65+pIL4+pIL10 vs. all groups. †Percentage of CD4+ T cells. ‡$P < 0.04$, pGAD65+pIL10 vs. pHEL+pIL4+pIL10 and untreated. §Percentage of CD4+CD25+ T-cells.

was used to measure RNA expression in individual cells (10). Single $CD62L^{hi}$ T-cells were sorted from the RLN and PLN prepared from pools of three mice in each treatment group and FoxP3 expression measured in the individual T-cells. Between 10 and 22% of $CD62L^{hi}$ T-cells prepared from the RLN and PLN of untreated and pHEL+pIL4+pIL10-vaccinated recipient mice expressed FoxP3 (Fig. 3*A*). In contrast, FoxP3 expression was detected in >50% of RLN and PLN $CD62L^{hi}$ T-cells in pGAD65+pIL4+pIL10 vaccinated recipient mice (Fig. 3*A*). Furthermore, the frequency of FoxP3-expressing RLN and PLN $CD62L^{hi}$ T-cells sorted from pGAD65+pIL10-vaccinated recipients was reduced more than twofold compared with recipients treated with pGAD65+pIL4+pIL10 (Fig. 3*A*). As a control, FoxP3 also was assessed in sorted $CD4^+CD25^-$ T-cells. Consistent with previous findings (10), only a small percentage (<3%) expressed low levels of FoxP3 mRNA per cell.

Next, TGF-β1 and IL-10 expression was assessed in sorted $CD62L^{hi}$ FoxP3-expressing RLN and PLN $CD4^+$ T-cells. Seventy to 90% of FoxP3-expressing $CD62L^{hi}$ T-cells prepared from the RLN and PLN expressed TGF-β1 mRNA independent of the experimental group (Fig. 3*B* and *C*). Strikingly, the average level of TGF-β1 mRNA in RLN and PLN $CD62L^{hi}$ T-cells was increased in pGAD65+pIL4+pIL10-vaccinated recipient mice relative to the other experimental groups (Fig. 3*B* and *C*). For example, a 23- and 10-fold increase in the average level of TGF-β1 mRNA was detected in RLN and PLN $CD62L^{hi}$ T-cells of pGAD65+pIL4+pIL10- and pGAD65+pIL10-vaccinated recipients, respectively (Fig. 3*B* and *C*). No IL-10 expression was detected in FoxP3-expressing RLN and PLN $CD4^+$ T-cells sorted from the experimental groups. Together, these results demonstrate that enhanced islet graft survival in pGAD65+pIL4+pIL10-vaccinated recipient mice correlates with a significantly increased frequency of FoxP3-expressing RLN and PLN $CD4^+CD25^+CD62L^{hi}$ T-cells, which exhibit elevated levels of TGF-β1 mRNA expression relative to the pGAD65+pIL10 and control groups.

$CD4^+CD25^+CD62L^{hi}$ T-cells in the RLN and PLN of pGAD65+pIL4+pIL10-vaccinated recipient mice have increased suppressor function. The above findings suggested that $CD4^+CD25^+$ Treg induced/expanded by pGAD65+pIL4+IL10 versus pGAD65+pIL10 had a greater capacity to suppress β-cell autoimmunity. To examine this possibility, PLN $CD4^+CD25^+$ T-cells were sorted from diabetic NOD female mice vaccinated with pGAD65+pIL4+IL10 or pGAD65+pIL10 and tested for the capacity to suppress the transfer of diabetes by BDC2.5 $CD4^+$ T-cells in NOD.scid recipients. No significant difference in the onset and frequency of diabetes was detected in NOD.scid recipients injected with $CD4^+CD25^+$ T-cells prepared from pGAD65+pIL10-treated donor mice plus BDC2.5 $CD4^+$ T-cells compared with BDC2.5 $CD4^+$ T-cells alone (Fig. 4*A*). In contrast, onset of diabetes was significantly delayed ($P \leq 0.0016$) in NOD.scid mice coinjected with $CD4^+CD25^+$ T-cells isolated from pGAD65+pIL4+

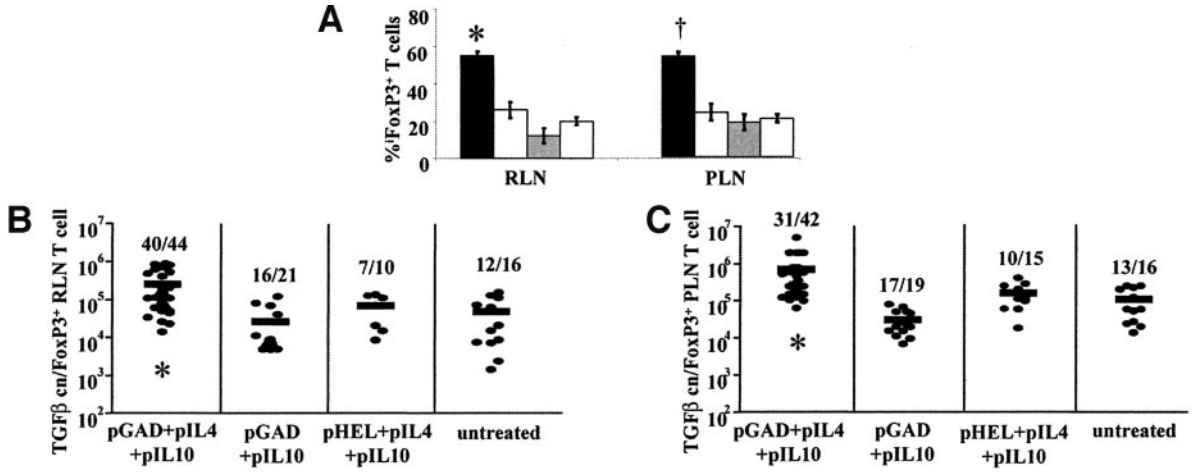

FIG. 3. pGAD65+pIL4+pIL10 induces an increased frequency of $FoxP3^+$ and TGF-β^+ $CD62L^{hi}$ T-cells in NOD recipients. Single $CD62L^{hi}$ T-cells harvested 3 weeks posttransplantation were sorted from RLN and PLN. *A*: FoxP3 mRNA expression was measured via reverse transcriptase real-time PCR. *B* and *C*: TGF-β1 expression was measured in $FoxP3^+$ T-cells identified in *A*. The number of $CD4^+CD25^+CD62L^{hi}FoxP3^+$ T-cells that are TGF-$\beta1^+$ is provided in each column. ■, average level of mRNA expression per cell. *A–C*: *$P \leq 0.003$, pGAD65+pIL4+pIL10 vs. all groups; †$P \leq 0.002$, pGAD65+pIL4+pIL10 vs. all groups. Data are an average of two experiments (40 cells per experiment).

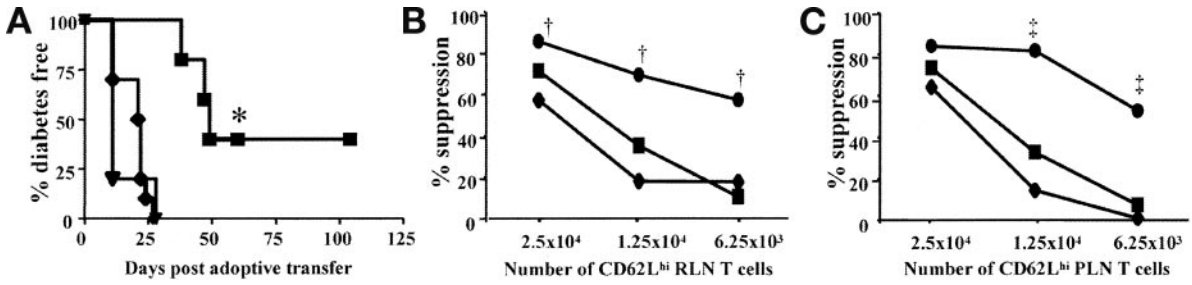

FIG. 4. Increased suppression by CD4$^+$CD25$^+$ and CD62L^{hi} T-cells sorted from pGAD65+pIL4+pIL10-vaccinated NOD recipients. *A*: Groups of five NOD.scid mice received CD4$^+$ BDC2.5 T-cells either alone or with sorted PLN CD4$^+$CD25$^+$ T-cells from treated groups. ♦, BDC2.5 only; ▼, pGAD65+pIL10+BDC2.5; ■, pGAD65+pIL4+pIL10+BDC2.5. *B* and *C*: Varying numbers of sorted RLN or PLN CD62L^{hi} T-cells were tested for suppression of CD4$^+$CD25$^-$ T-cell proliferation. Data are an average of two experiments performed in triplicate. SD for data points ± 12%. Average cpm of stimulated CD4$^+$CD25$^-$ T-cells only in RLN and PLN was 92,101 ± 2,941 and 85,012 ± 1,427, respectively. *$P \leq 0.0016$, pGAD65+pIL4+pIL10 CD4$^+$CD25$^+$ T-cells plus CD4$^+$ BDC2.5 T-cells vs. all groups; †$P < 0.001$, pGAD65+pIL4+pIL10 vs. all groups; ‡$P \leq 0.004$, pGAD65+pIL4+pIL10 vs. all groups. ●, pGAD5+pIL4+pIL10; ■, pGAD5+pIL10; ♦, control.

pIL10-vaccinated NOD mice plus BDC2.5 CD4$^+$ T-cells (Fig. 4*A*).

Next, the in vitro suppressor function of RLN and PLN CD62L^{hi} T-cells isolated from islet graft recipients injected with pDNA 3 weeks posttransplant was measured. RLN and PLN CD62L^{hi} T-cells sorted from pGAD65+pIL10- or pHEL+pIL4+pIL10-vaccinated recipients inhibited CD4$^+$ CD25$^-$ T-cell proliferation similarly (Fig. 4*B* and *C*). In comparison, the suppressor activity of CD62L^{hi} T-cells isolated from the RLN (Fig. 4*B*) or PLN (Fig. 4*C*) of pGAD65+pIL4+pIL10 was significantly increased. This difference was most notable at the lowest number (6.25×10^3) of CD62L^{hi} T-cells tested (Fig. 4*B* and *C*).

Earlier work demonstrated that TGF-β1 was required to mediate suppression by CD62L^{hi} T-cells isolated from NOD mice (10). To determine whether this also was the case for CD62L^{hi} T-cells sorted from the experimental groups, the level of TGF-β secretion was measured in supernatants harvested from the above suppression assays. Consistent with the in vitro suppression data, similar levels of TGF-β were detected in cultures containing CD62L^{hi} T-cells sorted from pGAD65+pIL10- and pHEL+pIL4+pIL10-vaccinated recipients, whereas the amount of TGF-β was significantly increased in cultures of RLN and PLN CD62L^{hi} T-cells prepared from pGAD65+pIL4+pIL10 NOD recipients (Fig. 5*A* and *B*). Furthermore, the suppressor activity displayed by RLN and PLN CD62L^{hi} T-cells sorted from the three experimental groups was effectively blocked by the addition of anti–TGF-β but not by an isotype antibody control (Fig. 5*C* and *D*). Together, these data demonstrate that the pool of CD62L^{hi} T-cells induced/expanded by pGAD65+pIL4+IL10 versus pGAD65+pIL10 treatment exhibit increased suppressor activity that is (at least) partially TGF-β dependent in vitro.

Recurrent diabetes in pGAD65+pIL4+pIL10-vaccinated NOD recipients corresponds with a loss of FoxP3-expressing CD4$^+$CD25$^+$CD62L^{hi} T-cells. Although more effective than pGAD65+pIL10 treatment, recurrent diabetes nevertheless developed in pGAD65+pIL4+pIL10-vaccinated islet graft recipients. One explana-

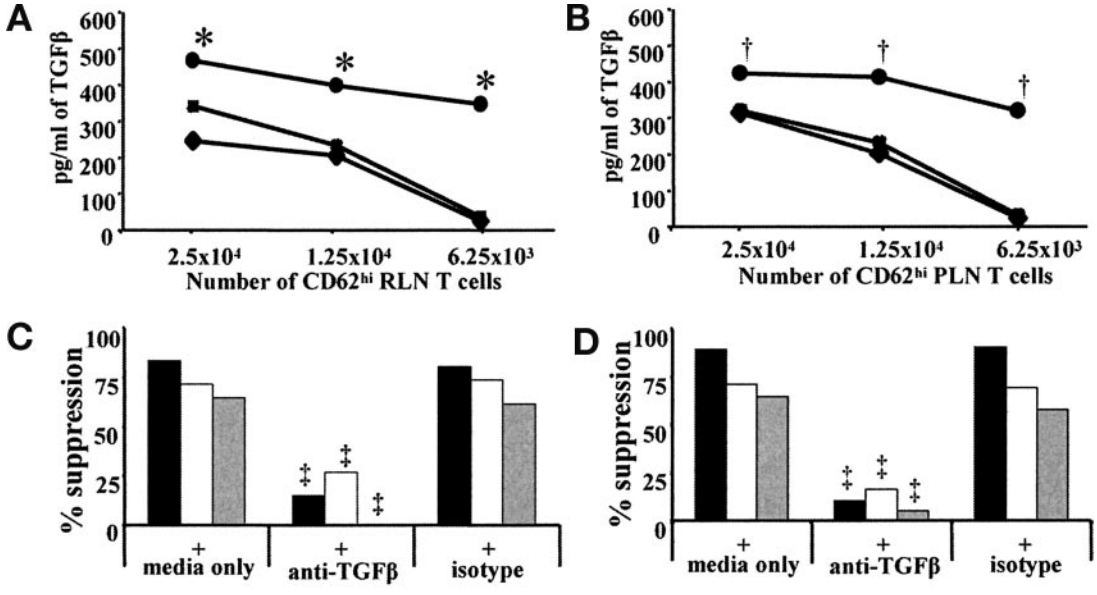

FIG. 5. In vitro suppression mediated by CD62L^{hi} T-cells involves TGF-β. *A* and *B*: TGF-β was measured via enzyme-linked immunosorbent assay in supernatants from suppression assays in Fig. 4*B* and *C*. Data are an average of two experiments performed in triplicate. SD for data points ± 46.8 pg/ml. *$P < 0.001$, pGAD+pIL4+pIL10 vs. all groups; †$P < 0.004$, pGAD+pIL4+pIL10 vs. all groups. ●, pGAD5+pIL4+pIL10; ■, pGAD5+pIL10; ♦, control. *C* and *D*: Sorted RLN (*C*) or PLN (*D*) CD62L^{hi} T-cells were coincubated with CD4$^+$CD25$^-$ T-cells and anti–TGF-β1, -2, -3, or isotype control antibody. Data are an average of two experiments performed in triplicate. SD for data points ±15.6%; average cpm of stimulated CD4$^+$CD25$^-$ T-cells only in RLN and PLN was 75,553 ± 8,861 and 81,167 ± 8,339, respectively; ~$P < 10^{-3}$, anti–TGF-β vs. no anti–TGF-β for all groups. ■, pGAD+pIL4+pIL10; □, pGAD65+pIL10; ▨, control.

TABLE 2
A temporal decline in the number of $CD4^+CD25^+CD62L^{hi}$ T-cells is detected in $pGAD65^+pIL4^+pIL10$-treated NOD recipient mice

	3 weeks	5 weeks	Diabetic
RLN $CD4^+$	2,121 ± 121	1,845 ± 102	1,932 ± 114
RLN $CD25^+$	544 ± 25* (27.1)†	373 ± 28 (20.2)	332 ± 31 (17.2)
RLN $CD25^+CD62L^{hi}$	358 ± 29* (62.3)‡	170 ± 23§ (45.7)	97 ± 25 (29.3)
PLN $CD4^+$	2,454 ± 133*	2,105 ± 109	1,989 ± 103
PLN $CD25^+$	615 ± 18* (25.1)†	417 ± 24 (19.8)	370 ± 26 (18.6)
PLN $CD25^+CD62L^{hi}$	369 ± 15* (59.8)‡	171 ± 21§ (41.2)	78 ± 9 (21.1)

Data are means ± SE (%) for the average of three experiments from pools of three to four mice per group; number of T-cells is per 3,000 cells analyzed. *$P \leq 0.025$, 3 vs. 5 weeks and diabetic. †Percentage of $CD4^+$ T-cells. ‡Percentage of $CD4^+$ $CD25^+$ T-cells. §$P \leq 0.02$, 5 weeks vs. diabetic.

tion is an inability of induced/expanded $CD4^+CD25^+$Treg to persist. To test this hypothesis, RLN and PLN $CD62L^{hi}$ T-cells of pGAD65+pIL4+pIL10-vaccinated recipient mice were examined at 3 and 5 weeks posttransplantation and at the onset of recurrent diabetes. Flow cytometric analyses showed a progressive decline in $CD62L^{hi}$ T-cells in RLN and PLN harvested from pGAD65+pIL4+pIL10-vaccinated recipients (Table 2), which corresponded with a concomitant increase in the frequency of $CD4^+CD25^+$ $CD62L^{lo}$ T-cells (data not shown). Furthermore, the frequency of FoxP3-expressing RLN and PLN $CD62L^{hi}$ T-cells also decreased with time (Fig. 6*A*). An approximately fourfold reduction in FoxP3-expressing $CD62L^{hi}$ T-cells was detected in RLN harvested at the time of recurrent diabetes onset versus 3 weeks posttransplantation (Fig. 6*A*). In addition, a temporal decline in the level of TGF-β1 RNA was detected in RLN and PLN $CD62L^{hi}$ T-cells (Fig. 6*B* and *C*), which correlated with reduced levels of TGF-β secretion (Fig. 6*E*). An ~10-fold reduction in TGF-β was seen in cultures containing 6.25×10^3 RLN or PLN $CD62L^{hi}$ T-cells prepared 5 vs. 3 weeks posttransplantation (Fig. 6*E*). Consistent with a reduced frequency of FoxP3-expressing cells and TGF-β production, the suppressor activity of RLN and PLN $CD62L^{hi}$ T-cells also declined from 3 to 5 weeks postimplantation (Fig. 6*D*). These results demonstrate that decreasing numbers of induced/expanded FoxP3-expressing $CD4^+CD25^+$ Treg correlate with the onset of recurrent diabetes in pGAD65+pIL4+pIL10-vaccinated islet graft recipients.

DISCUSSION

Defining the "rules" of antigen-specific immunotherapy is critical for the development of rational immunotherapeutic approaches to suppress ongoing β-cell autoimmunity and treat diabetic patients. For this purpose, we exploited a pDNA vaccination protocol to block autoimmune-mediated destruction of syngeneic islet grafts implanted in diabetic NOD recipients. Two general conclusions can be made from this study.

First, suppression of β-cell autoimmunity in diabetic NOD recipients was dependent on the nature and frequency of the immunoregulatory T-cells induced/expanded via pDNA vaccination. Induction of Th2- and Tr1-like cells alone appeared to be insufficient to mediate effective islet graft protection. For example, a similar frequency of GAD65-specific $CD4^+$ T-cells secreting IL-4 and IL-10 (and IFNγ) was detected (Fig. 2) despite differences in efficacy of the pGAD65+pIL4+pIL10 and pGAD65+pIL10 treatments (Fig. 1*A*). On the other hand, there was a strong correlation between the frequency and suppressor activities of $CD4^+CD25^+$ Treg elicited via the respective pDNA treatments and the onset of recurrent diabetes. $CD62L^{hi}$ T-cells were increased (Table 1), and

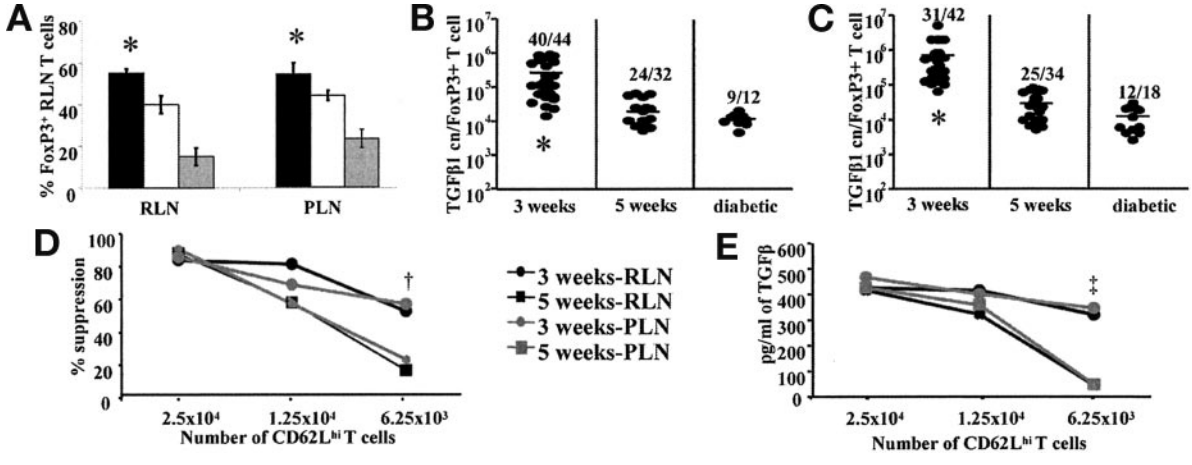

FIG. 6. $FoxP3^+$ $CD62L^{hi}$ T-cells decline in pGAD65+pIL4+pIL10-vaccinated recipients. *A* and *C*: Single T-cells were sorted from RLN and PLN of pGAD65+pIL4+pIL10-vaccinated recipients and FoxP3 (*A*) or TGF-β1 (*B* and *C*) mRNA measured via reverse transcriptase real-time PCR. *$P < 0.01$, 3 weeks vs. diabetic mice. *A*: ■, 3 weeks; □, 5 weeks; ▨, diabetic. *D*: Sorted RLN or PLN $CD62L^{hi}$ T-cells from pGAD65+pIL4+pIL10-vaccinated recipients were tested for suppression of $CD4^+CD25^-$ T-cell proliferation. SD of data points ±10%. Average cpm of stimulated $CD4^+CD25^-$ T-cells only in RLN and PLN was 23,469 ± 2,914 and 19,976 ± 1,886, respectively. †$P \leq 0.03$, RLN/PLN 3 weeks vs. RLN/PLN 5 weeks. *E*: TGF-β was measured via enzyme-linked immunosorbent assay in supernatants from suppression assays in *D*. SD of data points ±34.2 pg/ml. ‡$P \leq 0.002$, RLN/PLN 3 weeks vs. RLN/PLN 5 weeks. Data are an average of two experiments performed in triplicate.

within this pool the frequency of FoxP3-expressing cells was elevated in pGAD65+pIL4+pIL10- versus pGAD65+pIL10-treated mice (Fig. 3*A*). Furthermore, in vivo and in vitro suppressor activity of either $CD4^+CD25^+$ or $CD62L^{hi}$ T-cells, respectively, was enhanced in pGAD65+IL4+pIL10-vaccinated mice compared with the pGAD65+pIL10 and/or pHEL+pIL4+pIL10 treatment groups (Fig. 4). In addition to frequency, a qualitative difference within the pool of $CD62L^{hi}$ T-cells was detected depending on the pDNA vaccination protocol. For instance, >10-fold increase in TGF-β1 mRNA expression (Fig. 3*B* and *C*) coupled with elevated levels of TGF-β secretion (Fig. 5) were detected for RLN and PLN $CD62L^{hi}$ T-cells prepared from pGAD65+pIL4+pIL10- versus pGAD65+pIL10- and pHEL+IL4+pIL10-vaccinated diabetic recipient mice. Consistent with previous work by our group and others (10,35,36), the in vitro suppressor activity of sorted $CD62L^{hi}$ T-cells was associated with TGF-β secretion (Fig. 5), although cell-to-cell contact also contributes to in vitro suppression (S.M.P., R.T., unpublished results). The relative contribution of TGF-β in $CD62L^{hi}$ T-cell–mediated suppression in vivo, however, is not clear. Furthermore, in vitro suppressor activity was only detected for $CD62L^{hi}$ but not $CD4^+CD25^+CD62L^{lo}$ T-cells sorted from the same RLN and PLN of pGAD65+pIL4+pIL10-vaccinated NOD mice (S.M.P., R.T., unpublished results). This latter finding, similar to results made in unimmunized NOD mice (10), correlated with a reduced frequency of FoxP3-expressing cells (30–35%) in $CD62L^{lo}$ versus $CD62L^{hi}$ T-cells. Currently, it is unclear whether the pDNA-mediated increase in $CD4^+CD25^+$ Treg was due to the induction of FoxP3 expression in "conventional" naïve $CD4^+$ T-cells and/or expansion of an established pool of $FoxP3^+$ $CD62L^{hi}$ T-cells (37). Work by Chatenoud et al. (36) suggests that "conventional" $CD4^+CD25^-$ T-cells serve as precursors for TGF-β–expressing $CD4^+CD25^+$ Treg induced in diabetic NOD mice by anti-CD3 Ab treatment, although expression of FoxP3 was not directly examined. Others (38) suggest that contact of $CD4^+CD25^+$ Treg with conventional $CD4^+$ T-cells can induce an immunoregulatory phenotype characterized by the secretion of anti-inflammatory cytokines. Collectively, these findings demonstrate that effective suppression of ongoing β-cell autoimmunity in diabetic NOD recipients is dependent on the frequency of FoxP3-expressing $CD62L^{hi}$ T-cells induced and/or expanded via pDNA vaccination.

The second general conclusion made in this study is that duration of the suppressive effect on β-cell autoimmunity in diabetic recipients is dependent on the persistence of $CD4^+CD25^+$ Treg. The onset of recurrent diabetes in islet graft recipients vaccinated with pGAD65+pIL4+pIL10 corresponded with a temporal decline in the frequency of $CD62L^{hi}$ T-cells (Table 2) and a concomitant increase in pathogenic T-cells in the RLN and PLN. Furthermore, there was a progressive decline in the frequency of FoxP3-expressing cells within the pool of $CD62L^{hi}$ T-cells and the in vitro suppressor activity and levels of TGF-β mRNA expression and secretion by sorted RLN and PLN $CD62L^{hi}$ T-cells (Fig. 6). The diminishing frequency of $CD62L^{hi}$ T-cells also was reflected by the progression of islet graft insulitis. At 3 weeks postimplantation, when the frequency of FoxP3-expressing $CD62L^{hi}$ T-cells was high, islet transplants were free of insulitis (Fig. 2), indicating that protection was mediated by suppression of graft infiltration. However, infiltrates were readily detected as the frequency of $CD4^+CD25^+$ Treg was reduced (Fig. 1*B*). Studies have demonstrated time-dependent defects in the frequency and/or function of subsets of immunoregulatory $CD4^+$ T effectors in NOD mice (6–9,11–14). Zaghouani et al. (11) reported a temporal decrease in TGF-β expression by splenic $CD4^+CD25^+$ Treg induced in 8-week-old nondiabetic NOD mice injected with GAD-Ig protein. Therefore, properties intrinsic to $CD4^+CD25^+$ Treg and/or the extracellular environment found in NOD female mice may promote the decline of FoxP3-expressing $CD62L^{hi}$ T-cells detected in pDNA vaccinated recipients. Initial attempts to maintain the pool of $CD4^+CD25^+$ Treg with subsequent injections of pGAD65+pIL4+pIL10 or pIL4+pIL10 exacerbated the onset of recurrent diabetes (S.M.P., R.T., unpublished results), suggesting that the dynamics of the in vivo milieu that promote an immunoregulatory versus pathogenic response are highly complex. Because similar defects appear to exist in type 1 diabetic patients (14), establishing conditions to maintain sufficient $CD4^+CD25^+$ Treg numbers and function may prove to be a key parameter for long-term suppression of β-cell autoimmunity in the clinic.

Importantly, islet graft protection was found to be GAD65 dependent because treatment with pHEL+pIL4+pIL10 (Fig. 1) or pHEL+pIL10 (data not shown) failed to delay recurrent diabetes or mediate an increase in the frequency of $CD4^+CD25^+$ Treg. However, whether the increased $FoxP3^+CD62L^{hi}$ T-cells are indeed GAD65-specific still needs to be elucidated. Because thymic expression of GAD65 is detected (39), it is plausible that GAD65-specific $CD4^+CD25^+$ Treg are selected in the thymus and subsequently expanded in the periphery following pGAD65+pIL4+pIL10 vaccination. Another mutually nonexclusive scenario is that induction of GAD65-specific Th2- and Tr1-like effectors establish an extracellular milieu that in the context of exogenous IL-4 and IL-10 preferentially promotes induction/expansion of $CD4^+CD25^+$ Treg specific for other β-cell or islet antigens.

In summary, we demonstrate that under overt diabetes conditions, antigen-specific immunotherapy can be used to establish β-cell–specific type 2 immunoregulatory $CD4^+$ T-cells and induce/expand FoxP3-expressing $CD4^+CD25^+$ Treg. Furthermore, the degree of suppression of ongoing β-cell autoimmunity is dependent on the frequency and persistence of $CD4^+CD25^+$ Treg. How to achieve long-term maintenance of immunoregulatory effectors needs further investigation. Accordingly, combining antigen-dependent and -independent immunotherapy strategies may prove effective in enhancing and extending suppression of ongoing β-cell autoimmunity in diabetic individuals (40,41).

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