Functional Defects and the Influence of Age on the Frequency of CD4$^+$CD25$^+$ T-Cells in Type 1 Diabetes

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CD4$^+$CD25$^+$ T-cells appear to play a crucial role in regulating the immune response. Therefore, we evaluated the peripheral blood frequency and function of CD4$^+$CD25$^+$ T-cells in 70 type 1 diabetic patients and 37 healthy individuals. Interestingly, a positive correlation was observed between increasing age and CD4$^+$CD25$^+$ T-cell frequency in both subject groups. In contrast to previous studies of nonobese diabetic mice and type 1 diabetic patients, similar frequencies of CD4$^+$CD25$^+$ and CD4$^+$CD25$^{+Bright}$ T-cells were observed in healthy control subjects and type 1 diabetic patients of similar age. There was no difference between type 1 diabetic subjects of recent-onset versus those with established disease in terms of their CD4$^+$CD25$^+$ or CD4$^+$CD25$^{+Bright}$ T-cell frequency. However, type 1 diabetic patients were markedly defective in their ability to suppress the proliferation of autologous effector T-cells in vitro. This type 1 diabetes–associated defect in suppression was associated with reduced production of interleukin (IL)-2, γ-interferon, and transforming growth factor-β, whereas other cytokines including those of adaptive and innate immunity (IL-10, IL-1β, IL-6, IL-8, IL-12p70, and tumor necrosis factor-α) were similar in control subjects and type 1 diabetic patients. These data suggest that age strongly influences the frequency of CD4$^+$CD25$^+$ T-cells and that function, rather than frequency, may represent the means by which these cells associate with type 1 diabetes in humans. *Diabetes* 54:1407–1414, 2005

Multiple and largely redundant mechanisms in the immune system are present to regulate and suppress immune reactivity against self-antigens, thereby protecting the body from autoimmune disease (1). In one example, Sakaguchi et al. (2) identified a relatively small population of CD4$^+$ T-cells that appear important for control of self-reactive T-cells. Phenotypic identification indicated these CD4$^+$ T-cells coexpressed the α-chain (CD25) of the interleukin (IL)-2 receptor (3). Since that description nearly a decade ago, a plethora of studies have to a large extent supported the notion that CD4$^+$CD25$^+$ T-cells, often referred to as CD4$^+$CD25$^+$ regulatory T-cells (Tregs), play a major role in suppressing immune reactivity (4–6). Additional studies in animal models of human disease have indicated that CD4$^+$CD25$^+$ T-cells, either through decreased frequency or diminished function, may be related to autoimmune disease development (rev. in 7). Indeed, in certain disease-susceptible strains of mice, transfer of CD4$^+$CD25$^+$ T-cells prevents the development of autoimmune disease, whereas in other model systems, a variety of pathologies (e.g., gastritis, prostatitis, and thyroiditis) can be produced under situations wherein the development of CD4$^+$CD25$^+$ T-cells is delayed or outright removed (8–10). In one animal model of autoimmune type 1 diabetes, the nonobese diabetic (NOD) mouse, a role for CD4$^+$CD25$^+$ T-cells in the pathogenesis of disease has been inferred (but not universally supported [11]) by a variety of methods including but not limited to that of frequency (12–15).

In humans, a Treg population having the CD4$^+$CD25$^+$ phenotype has been identified, with the most amenable population being the so-called CD4$^+$CD25^{high} T-cells (16–19). In terms of functional properties, these Tregs demonstrated an ability to suppress the activities of co-cultured CD4$^+$CD25$^-$ responder T-cells as well as being hyporesponsive to non–antigen-specific T-cell stimulation. This population of cells has been intimately related with susceptibility to allergies (20), whereas for autoimmunity in humans, another study reported decreased frequencies of CD4$^+$CD25$^+$ T-cells in peripheral blood of patients with systemic lupus erythematosus but not in individuals with rheumatoid arthritis (21). In terms of type 1 diabetes, one report (22) suggested decreased CD4$^+$CD25$^+$ T-cell frequencies in human type 1 diabetes, findings similar to a second study investigating subjects with autoimmune polyglandular syndrome type 2 (23). Given the ever-increasing interest and potential importance of this Treg population as a means for therapeutic intervention (24), we sought to investigate a potential role for these cells in type 1 diabetes.

RESEARCH DESIGN AND METHODS

We assessed markers of cellular and humoral immunity from 61 children and adults with established type 1 diabetes (25 male/36 female; 53 Caucasian, 5 African American, 3 Hispanic; mean age 18.94 ± 7.26 years, range 5.44–44; mean disease duration 8.17 + 6.19 years) and 37 nondiabetic healthy individuals (21 male/16 female; 31 Caucasian, 3 African American, 2 Hispanic, 1 Asian; mean age 24.78 ± 11.46 years, range 10–56) from the general population. As part of this study, we sought to investigate CD4$^+$CD25$^+$ T-cells as a

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APC, allophycocyanin; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, γ-interferon; IL, interleukin; PE, phycoerythrin; Teff, CD4$^+$CD25$^-$ effector T-cell; TGF, transforming growth factor; TNF, tumor necrosis factor; Treg, CD4$^+$CD25$^+$ regulatory T-cell.

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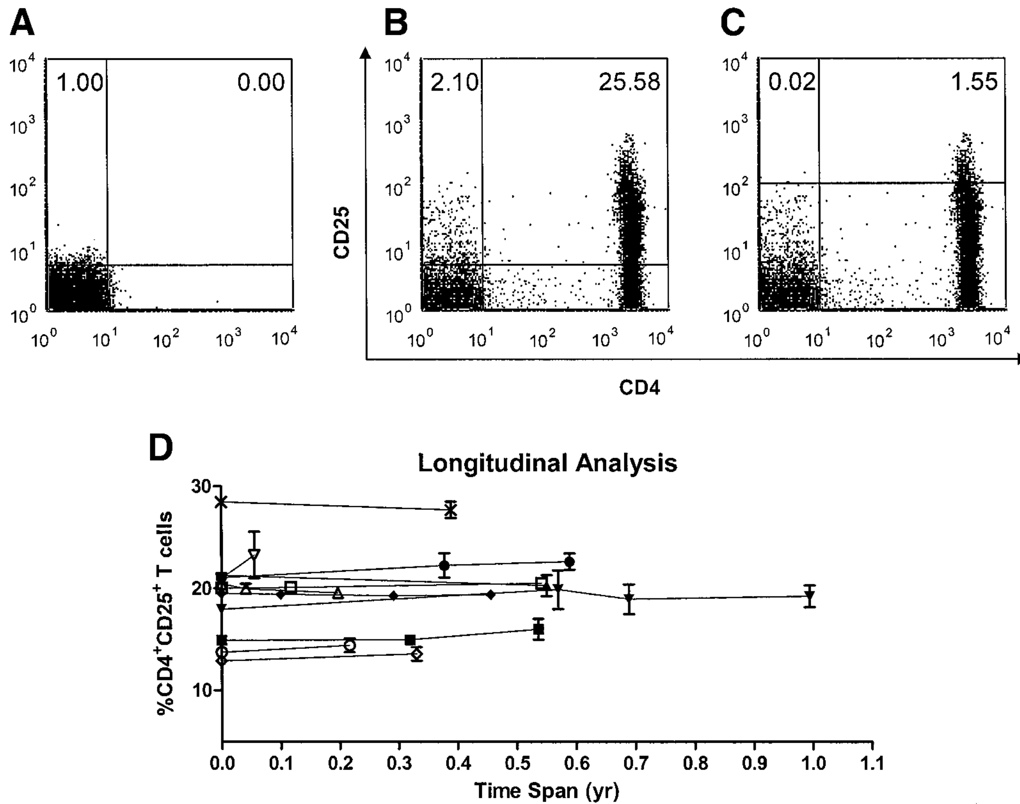

FIG. 1. Representative flow cytometric plots from a normal healthy control subject showing expression of CD4 and CD25. *A*: Regions were set individually for each subject by gating on CD3$^+$ lymphocytes with isotype background staining of 1% (5 × 10^4 events acquired). *B*: Lymphocyte and CD3$^+$ gated CD4$^+$CD25$^+$ T-cells. *C*: Plot showing gate for CD4$^+$CD25$^+$ T-cells with a fluorescence intensity of CD25 exceeding 100 defined as bright. Numbers within the *upper right quadrants* indicate percent of cellular events counted positive for the indicated markers. *D*: Stability of CD4$^+$CD25$^+$ T-cells over time. Whole blood samples from 11 healthy control subjects spanning a time period of up to 12 months were tested (*x*-axis), with initial and each reanalysis shown (bars shown represent mean and SE).

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function of disease duration. Therefore, both new-onset as well as established type 1 diabetic patients were included. A patient with type 1 diabetes was classified as new onset if collected within 4 months of the initial date of diagnosis. The new-onset type 1 diabetic patient group consisted of 9 individuals (6 male/3 female; 8 Caucasian, 1 African American; mean age 14.11 ± 6.49 years, range 8–26). Hence, a total of 70 type 1 diabetic patients were subject to investigation. All patients with type 1 diabetes were diagnosed according to American Diabetes Association criteria (25). Healthy control subjects lacked any autoimmune disorders or related probands with type 1 diabetes and were determined to be negative for the presence of either of the type 1 diabetes–associated autoantibodies anti-GAD (GADA) and anti-IA2 (IA2A) (26). An institutional review board approved informed consents, and assents were obtained for each study participant.

Flow cytometry. Heparinized whole blood was collected and immediately subjected to methods for cellular staining. Whole blood (100 μl) was aliquoted (per tube) along with each appropriate test antibody, fluorescein ishiocyanate anti-CD3 (clone HIT3a), allophycocyanin (APC) anti-CD4 (SK3), and phycoerythrin (PE) anti-CD25 (M-A251). An extended analysis was conducted on a subset of type 1 diabetic patients and control subjects with PerCP anti-CD4 (SK3), APC-labeled anti-CD62L (DREG-56), CD45RA (HI100), and CD45RO (UCHL1). The following isotype control antibodies were used: fluorescein ishiocyanate mouse IgG1 (MOPC-21), PE mouse IgG1 (MOPC-21), PerCP mouse IgG1 (MOPC-21), APC-labeled mouse IgG1 (MOPC-31C), mouse IgG_{2A} (G155–78), and mouse IgG_{2B} (clone 27–35). PE QuantiBRITE beads were included in certain runs to determine the levels of CD25 expression on gated cells. All antibodies and reagents for cytometric analysis were purchased from BD Biosciences (San Jose, CA). After staining for 30 min (4°C), erythrocytes were lysed and cells were fixed (BD FACS Lysing solution; 349202), followed by washing with stain buffer (BD Stain Buffer; 554657) according to the manufacturer's recommendations. The cells were then subjected to flow cytometric analysis (5 × 10^4 events acquired per test) on a four-color BD FACSCalibur cytometer. FCS Express (De Novo Software, version 2.200.0023; Thornhill, ON, CA) was used for analysis of cytometric data. Cells were gated based on scatter to remove large blasting cells and on CD3 to remove monocytes expressing CD4. Gates for each test marker were established from isotype control antibody staining (1%, Fig. 1*A*). The regulatory phenotype of the gated CD4$^+$CD25$^+$ population was confirmed through in vitro suppressive function and expression of the regulatory gene FOXP3 (4) by RT-PCR as previously described (data not shown).

Cell culture. Cells were cultured in RPMI 1640 medium (Cellgro, Herndon, VA) supplemented with 5 mmol/l HEPES, 2 mmol/l L-glutamine, penicillin (50 μg/ml)/streptomycin (50 μg/ml)/neomycin (100 μg/ml) (Invitrogen, Carlsbad, CA), 50 μmol/l 2-mercaptoethanol, and 5% human type AB serum (Sigma, St. Louis, MO) in U-bottom 96-well plates (Costar, Cambridge, MA).

Cell purification. Peripheral blood was collected in sodium-heparinized Vacutainer tubes (BD Biosciences). An accessory cell population (>98% T-cell depleted) was produced by incubating an aliquot of blood with a T-cell depletion antibody cocktail (StemCell, Vancouver, BC, Canada) followed by density gradient centrifugation according to the manufacturer's instructions (Cellgro). The CD4$^+$ T-cell population was purified by negative selection using a CD4$^+$ T enrichment cocktail (StemCell). After purification and washing in PBS containing 2% serum, the "untouched" CD4$^+$ population then underwent a positive selection for CD4$^+$CD25$^+$ regulatory T-cells (>90% pure) using CD25 microbeads (Mitenyi Biotech, Bergisch Gladbach, Germany) with separation on the AutoMACS sorter (Miltenyi). The unlabeled CD4$^+$CD25$^-$ population (>98% pure) provided the CD4$^+$CD25$^-$ effector T-cell (Teff) population for use in suppression assays.

Suppression assay. A suppression assay was developed to test the capacity of CD4$^+$CD25$^+$ regulatory T-cells to suppress the proliferation of co-cultured Teffs. Regulatory T-cells were added in decreasing ratios (1:0, 1:1, ½:1, and 0:1) to a constant number of Teffs (5 × 10^3 cells/well). A combination of 5 μg/ml soluble anti-CD3 (clone HIT3a) and 2.5 μg/ml soluble anti-CD28 (clone CD28.2; eBioscience, San Diego, CA) provided the polyclonal stimulus for proliferation over a 6-day culture period. The 5 × 10^4 irradiated (3,300 rad) T-cell–depleted accessory cells were also added to each well in a total volume of 200 μl. Then 1 μCi ^{3}H-thymidine (Amersham Biosciences, Piscataway, NJ) was added at day 5 for the final 16 h of culture to assess proliferation. Supernatants from six replicate wells were collected for each condition at 24 and 48 h and at day 5 just before the addition of ^{3}H-thymidine to assess cytokine production. Suppression is determined by the reduction of ^{3}H-thymidine incorporation in the combination of cells and is calculated by the following equation: percent suppression = [1 − (mean cpm Treg + Teff)/(mean cpm Teff) × 100%].

Cytokine determination. Assessments of cytokine profiles from the suppression assay were performed using a commercially available multiplexed kit (Beadlyte Human Multi-Cytokine Detection System 3; Upstate Biotechnology, Waltham, VA) and the Luminex (100) LabMAP System (Austin, TX). Simultaneous measurement of 10 cytokines was performed: specifically IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12 (p70), tumor necrosis factor (TNF)-α, γ-interferon (IFN-γ), and granulocyte-macrophage colony-stimulating factor (GM-CSF). Transforming growth factor (TGF)-β1 levels were determined by standard enzyme-linked immunosorbent assay (BioSource, Camarillo, CA) and, for technical reasons, were only performed at 120 h. All assays were performed according to the manufacturer's protocols. For the 24- and 48-h time points, 5

μl was collected from six replicate wells and then diluted 1:2 in tissue culture media before analysis. At the 5-day time point, 20 μl was removed from each replicate well and assessed neat. Cytokine concentrations were determined utilizing SOFTmax PRO software (Molecular Devices, Sunnyvale, CA) with four-parameter data analysis.

Statistical analysis. Analyses were undertaken using GraphPad Prizm 4.00 software (GraphPad, San Diego, CA), and values at $P < 0.05$ were deemed significant. Mann-Whitney U tests were used to compare cellular frequencies, Treg-mediated suppression, and cytokine production results between healthy control subjects and type 1 diabetic subjects. Spearman's correlations were used to compare cellular frequencies with subject age.

RESULTS

$CD4^+CD25^+$ T-cell frequencies in peripheral blood are stable over short to intermediate periods of time. To establish the frequency of $CD4^+CD25^+$ T-cells, whole blood was stained with relevant phenotypic markers (CD3, CD4, and CD25) and analyzed by fluorescence-activated cell sorter. Lymphocytes were gated based on scatter and CD3 expression (a representative healthy control is shown in Fig. 1) and then analyzed for expression of CD4 and CD25 (Fig. 1*B*). To assess the so-called $CD4^+CD25^{+Bright}$ T-cells, cells exhibiting a fluorescence intensity of >100 units were considered positive (Fig. 1*C*), as previously described (21).

One parameter of interest for studies of $CD4^+CD25^+$ T-cells involved the questions of reproducibility and longitudinal variation within an individual. To assess this aspect, we performed an analysis of $CD4^+CD25^+$ T-cells on 11 healthy control subjects for various amounts of time spanning up to 1 year between the initial test and each subsequent reanalysis. The $CD4^+CD25^+$ T-cell frequency demonstrated a remarkable level of stability (Fig. 1*D*; median coefficient of variation 6.83%, range 1.69–13.91).

The frequency of $CD4^+CD25^+$ T-cells in peripheral blood associates with age. A historical caution for studies of type 1 diabetes, a disorder that often initially presents in children and adolescents, involves a determination of whether age influences the factor under assessment. Therefore, an association analysis was performed on all study subjects correlating age at time of testing versus the $CD4^+CD25^+$ frequency (Fig. 2*A*), an analysis that revealed increasing age was associated with an increase in total $CD4^+CD25^+$ frequency ($r = 0.60$, $P < 0.0001$).

To determine whether this association with age influenced healthy control subjects (Fig. 2*B*) or type 1 diabetic patients (Fig. 2*C*) uniquely, similar analyses were performed and indicated that in both study groups, age influenced the frequency of $CD4^+CD25^+$ T-cells ($r = 0.64$, $P < 0.0001$, and $r = 0.51$, $P < 0.0001$, for healthy control subjects and type 1 diabetic subjects, respectively) and that the two groups were not significantly different (NS) in terms of their age associations. In contrast to the age association observed with total $CD4^+CD25^+$ T-cells, no age association with the portion of $CD4^+CD25^{+Bright}$ T-cells was identified between healthy control subjects and subjects with type 1 diabetes. This finding suggests that the observed differences as a function of age are likely due to cells expressing low to intermediate levels of CD25.

The frequencies of $CD4^+CD25^+$ T-cells do not differ in healthy control subjects and in patients with type 1 diabetes of similar age. Healthy control subjects and patients with type 1 diabetes demonstrated similar percentages of lymphocyte gated $CD3^+$ T-cells (68.41 ± 7.98 vs. 70.04 ± 7.75, respectively; NS) and lymphocyte and $CD3^+$ gated $CD4^+$ T-cells (62.00 ± 8.21 vs. 60.35 ± 7.62; NS). When the frequency of $CD4^+CD25^+$ T-cells was plotted, type 1 diabetic patients exhibited lower frequencies than control subjects (Fig. 3*A*, 15.21 ± 5.54 vs. 19.03 ± 5.54, respectively; $P = 0.0006$). This trend was even more pronounced in the new-onset type 1 diabetic group, which averaged only 12.80 ± 2.97 (data not shown, $P = 0.0007$). However, as indicated previously, a strong association exists between age of a subject at the time of testing and the frequency of $CD4^+CD25^+$ T-cells. Hence, we then performed comparisons between subjects below the age of 20 (a common age limit for studies of age and type 1 diabetes). Interestingly, the frequency of $CD4^+CD25^+$ cells among these two populations, once limits of age were applied, was very similar (Fig. 3*B*, healthy control subjects [$n = 14$], 14.89 ± 2.32, and type 1 diabetic subjects [$n = 49$] 13.46 ± 4.26; NS). The percentages of $CD4^+CD25^+$ T-cells as a function of total cells (without CD3 gating) are

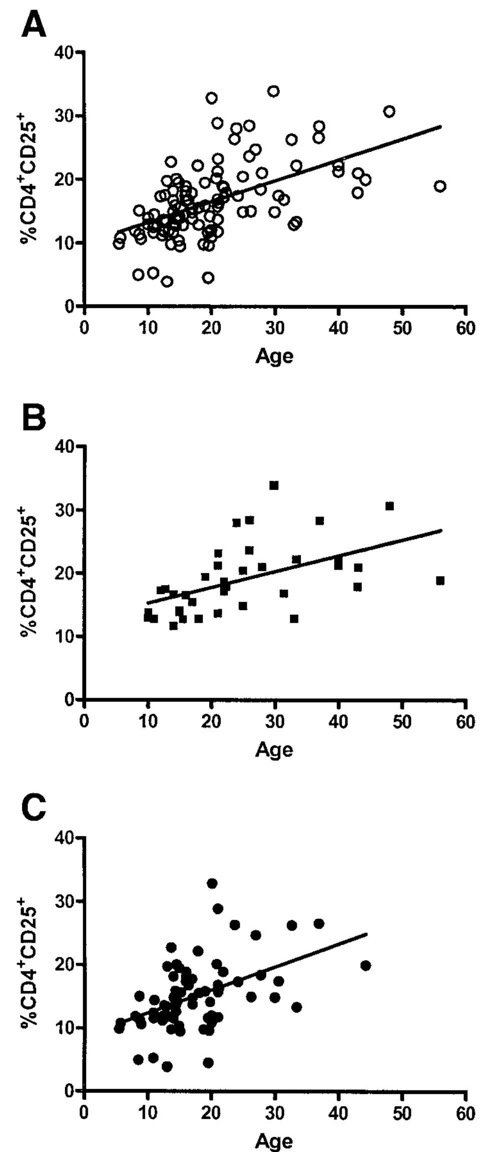

FIG. 2. Relationship between $CD4^+CD25^+$ T-cell frequency and age at time of testing. Frequency versus age studies in all subjects analyzed (*A*) ($n = 107$; ○), normal healthy control subjects (*B*) ($n = 37$; ■), and patients with new-onset and established type 1 diabetes (*C*) ($n = 70$; ●).

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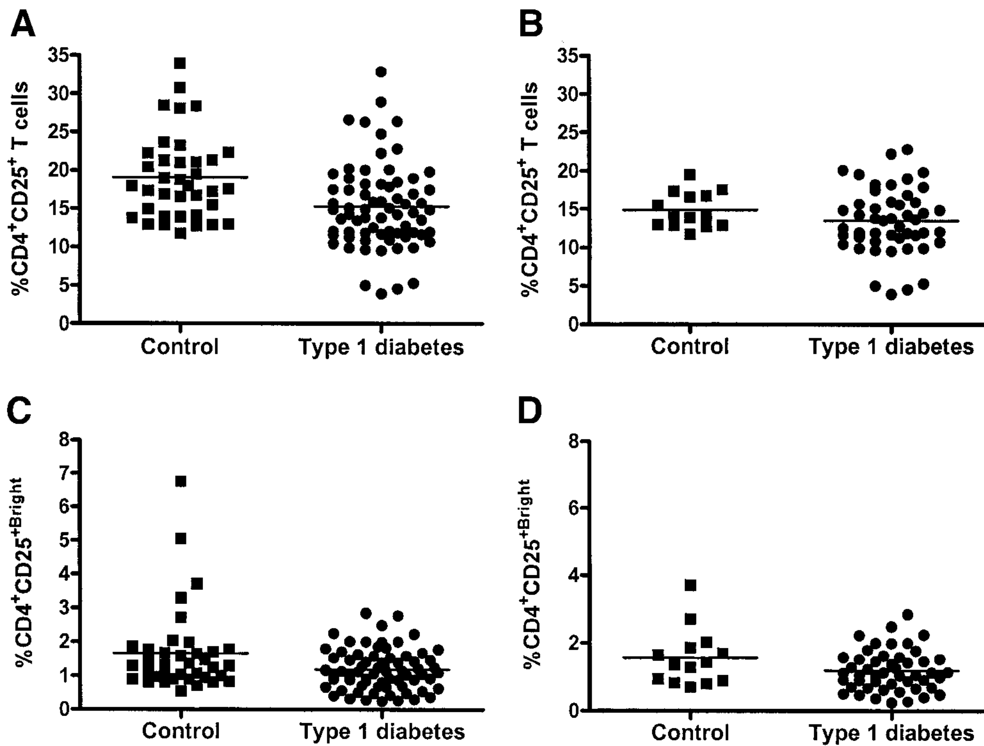

FIG. 3. Frequencies of CD4$^+$CD25$^+$ T-cells and CD4$^+$CD25$^{+\text{Bright}}$ T-cells do not differ significantly between control subjects and patients with type 1 diabetes when corrected for age. Frequencies of CD4$^+$CD25$^+$ T-cells (*upper panels*; *A* and *B*) and CD4$^+$CD25$^{+\text{Bright}}$ T-cells (*lower panels*; *C* and *D*) are shown. Cellular frequencies for all control subjects (n = 37; ■) and patients with type 1 diabetes (n = 70; ●) (*left panels*; *A* and *C*) are shown. Data for cellular frequencies for normal healthy control subjects (n = 14; ■) and patients with type 1 diabetes (n = 49; ●) under the age of 20 years old (*right panels*; *B* and *D*) are presented.

in line with the published "normal" range of ~5–10%. Specifically, for healthy control subjects, it is 5.22 ± 2.41%, and for type 1 diabetic patients, it is 4.68 ± 1.84%.

A major focus of attention in immune regulation as it pertains to individuals with type 1 diabetes has been directed at a population of Treg known as CD4$^+$CD25$^{+\text{Bright}}$ T-cells. Their definition in terms of gating has not been subjected to universal acceptance, but for our studies, we used a uniform gate across all samples dependent on units of fluorescence intensity. Specifically, we used a definition of those CD4$^+$CD25$^+$ T-cells with fluorescence intensity of CD25 expression exceeding 100 units as CD4$^+$CD25$^{+\text{Bright}}$ T-cells. Under these conditions, no significant difference in frequency was observed between healthy control subjects and the type 1 diabetic population for the percentage of CD4$^+$CD25$^{+\text{Bright}}$ T-cells either when all subjects were analyzed (Fig. 3*C*, 1.66 ± 1.25 vs. 1.19 ± 0.61, control and type 1 diabetes, respectively) or when these analyses were restricted to subjects under 20 years of age (Fig. 3*D*, 1.57 ± 0.84 vs. 1.19 ± 0.59, control and type 1 diabetes, respectively). Furthermore, quantitation of the number of PE molecules per CD4$^+$CD25$^+$ gated T-cell using standardized PE-labeled beads showed slightly higher levels of CD25 per gated cell in the type 1 diabetic population compared with control subjects (949.7 ± 135.9 vs. 834 ± 103.2, P = 0.01). In an extended analysis of cell markers in a subset of study participants (n = 12 type 1 diabetic patients, n = 8 healthy control subjects), no significant difference was detected in the percentage of cells expressing the markers CD62L (90.08 ± 2.89 vs. 90.13 ± 3.22), CD45RA$^+$ (53.49 ± 10.54 vs. 55.66 ± 17.17), and CD45RO$^+$ (82.88 ± 7.38 vs. 70.36 ± 18.09) on Tregs between healthy control subjects and type 1 diabetic patients, respectively.

Deficient suppression by Treg in patients with type 1 diabetes. The ability to suppress the proliferation of responding Teffs is a hallmark of Tregs. A suppression assay was used to determine the suppressive capacity of peripheral blood CD4$^+$CD25$^+$ T-cells from healthy control subjects (n = 9; mean age 23.75 ± 7.03 years) and patients with type 1 diabetes (n = 10; 18.11 ± 7.77 years) of similar age. These studies indicated that Tregs from patients with type 1 diabetes were functionally deficient in their ability to suppress Teffs in vitro. Specifically, at a ratio of 1 Treg to 1 Teff, type 1 diabetic patients suppressed proliferation less than healthy control subjects (median 14.99, interquartile range −12.8 to 47.5 and median 63.30, interquartile range 49.36 to 67.05, respectively; P = 0.002) (Fig. 4*A*, *left plots*). This trend continued in type 1 diabetic patients and healthy control subjects at a ratio of ½ Treg to 1 Teff (*right plots*, median 7.06, interquartile range −0.75 to 36.04 and median 46.22, interquartile range 28.47–54.91, respectively; P = 0.008). Under all cell ratios of Treg to Teff (1:0, 1:1, ½:1, and 0:1), the mean cpm in type 1 diabetic patients and healthy control subjects did not differ significantly (Fig. 4*B*, NS). Furthermore, Teffs from patients with type 1 diabetes proliferated similarly to healthy control subjects when stimulated by the mitogen phytohemagglutinin (Fig. 4*B*, $9.7 \times 10^4 \pm 2.2$ vs. $11.3 \times 10^4 \pm 3.7$ cpm, NS). Interestingly, in certain patients with type 1 diabetes, Tregs not only failed to suppress the proliferation of Teffs, but also acted in synergy, leading to increased proliferation over Teffs alone (Fig. 4*A*).

Altered cytokine profile from stimulated cultures in patients with type 1 diabetes. To identify mechanistic factors that may contribute to this defect in suppression, supernatants from these suppression assay cultures from type 1 diabetic patients and healthy control subjects were analyzed for the production of a variety of cytokines at periods of 24, 48, and 120 h. Specifically, multiplexed cytokine detection was conducted to measure levels of IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12 (p70), TNF-α, IFN-γ, and GM-CSF, whereas TGF-β1 was determined by convention-

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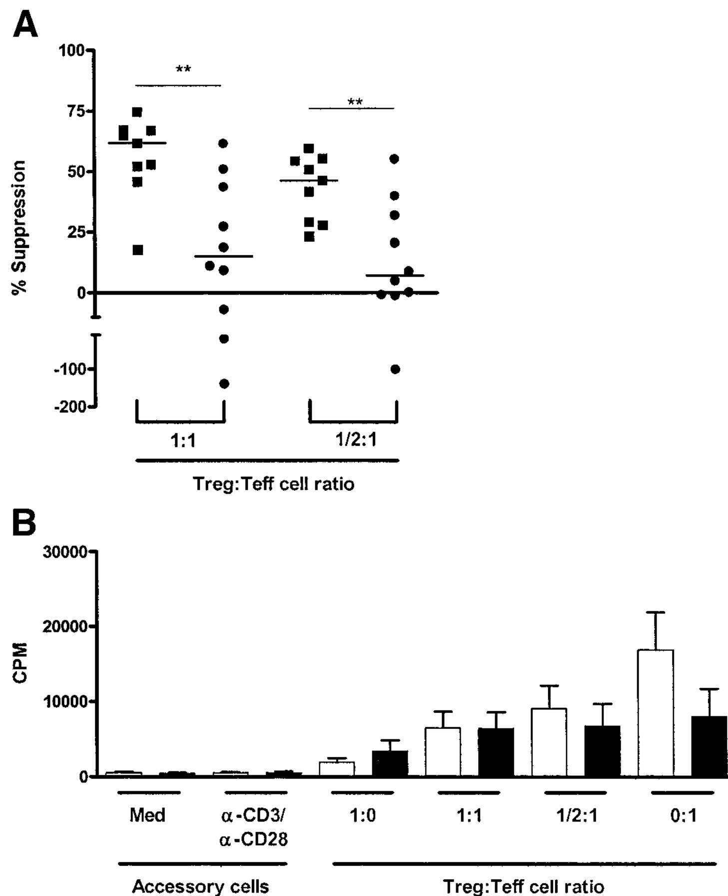

FIG. 4. Functional suppression by CD4$^+$CD25$^+$ regulatory T-cells is deficient in patients with type 1 diabetes. Data plotted represent the percent inhibition of proliferation by Tregs from normal healthy control subjects (n = 9; ■) and patients with type 1 diabetes (n = 10; ●). Tregs were plated alone (1:0; 5 × 10^3/well) and in decreasing ratios (1:1, ½:1, 0:1) to a constant number of Teffs. Cells were stimulated with soluble α-CD3 (5 μg/ml) and α-CD28 (2.5 μg/ml) in the presence of 5 × 10^4 accessory cells. Percent inhibition was calculated from the mean cpm of six replicate wells at a Treg-to-Teff ratio of 1:1 (*left plots*) and ½:1 (*right plots*), respectively, with **P < 0.01 (*A*). Proliferation measured by ^{3}H-thymidine incorporation during suppression assays by indicated cell populations from patients with type 1 diabetes (■) and normal control subjects (□) (*B*) is shown. Bars represent the mean ± SE.

al enzyme-linked immunosorbent assay. Significant alterations in the levels of IFN-γ were identified in supernatants from type 1 diabetic patients compared with healthy control subjects at nearly all time points (120 h shown in Fig. 5*A*), with diminished production in Teff populations (i.e., 0:1) from type 1 diabetes being the most significant. Patients with type 1 diabetes also displayed a deficiency in the production of TGF-β1 (Fig. 5*B*; P = 0.03) in Treg cultures (i.e., 1:0). The production of IL-2 and IL-4 in cultures from subjects with type 1 diabetes was also clearly diminished, but in the case of IL-4, such variances were uniform and independent of Treg-to-Teff ratios (Table 1). At 120 h, a trend toward reduced levels of IL-10 was seen in type 1 diabetic patients (6.9 ± 6.77 vs. 17.1 ± 16.6 pg/ml; Treg:Teff of 1:0), yet the difference was not statistically significant nor were there significant differences at any other time point. Only at the 120-h time point, Teff cultures from healthy control subjects produced higher levels of GM-CSF when compared with type 1 diabetic patients (319.55 ± 190.86 vs. 66.61 ± 35.46 pg/ml, respectively; Treg:Teff 0:1). It is important to note that with one exception (Table 1), significant differences in production of the innate mediators IL-1β, IL-6, and TNF-α were not detected at any time point or at any Treg-to-Teff ratio (i.e., differences were not observed with every analyte evaluated). IL-8 levels were at or above the upper limit of detection for the assay (10 ng/ml) in both subject groups, and IL-12 (p70) levels were undetectable at all time points.

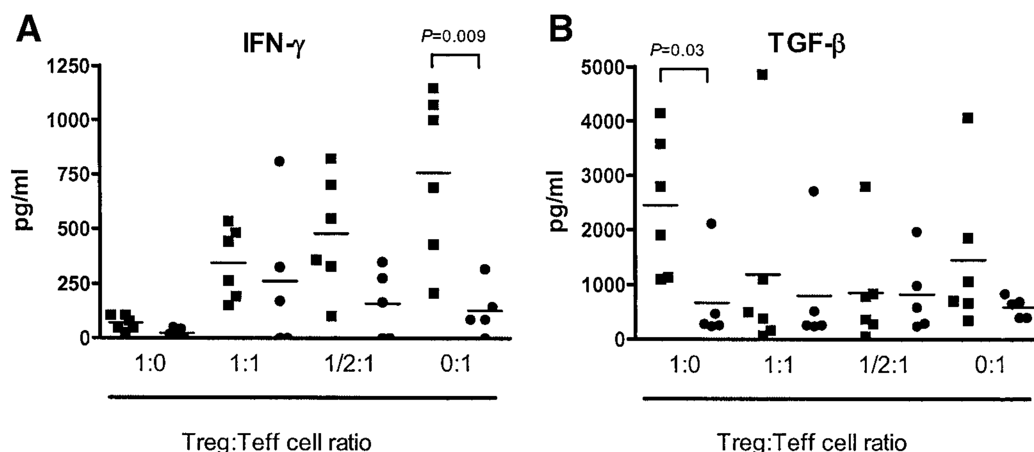

FIG. 5. Altered cytokine profile from stimulated cultures in patients with type 1 diabetes. Cytokine profiles from soluble α-CD3 (5 μg/ml) and α-CD28 (2.5 μg/ml) stimulated cultures (Treg-to-Teff ratios 1:0, 1:1, ½:1, and 0:1) from healthy control subjects (n = 6; ■) and patients with type 1 diabetes (n = 5; ●) are shown. Supernatants were collected at 24, 48, and 120 h and pooled from six replicate wells. Only 120-h data are shown. Graphs represent the mean ± SD with situations identifying statistical significance indicated. The results for IFN-γ (*A*) and TGF-β (*B*) are shown.

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TABLE 1
Cytokine elaborations monitoring regulatory and effector T-cell function

	Treg-to-Teff ratio	24 h		48 h		120 h	
		Control	Type 1 diabetes	Control	Type 1 diabetes	Control	Type 1 diabetes
GM-CSF	1 to 0	35.14 ± 29.21	40.75 ± 50.65	48.84 ± 33.98	69.28 ± 84.23	66.73 ± 41.41	98.43 ± 121.57
	1 to 1	37.78 ± 26.16	42.84 ± 44.52	84.89 ± 31.91	83.99 ± 78.56	194.10 ± 101.98	138.90 ± 108.04
	1/2 to 1	25.31 ± 17.75	39.95 ± 49.92	71.02 ± 22.75	63.13 ± 64.33	200.27 ± 122.95	122.81 ± 120.51
	0 to 1	9.19 ± 1.79	11.31 ± 8.53	46.48 ± 34.6	21.45 ± 10.88	319.55 ± 190.86	66.60 ± 35.46*
IFN-γ	1 to 0	40.18 ± 25.47	ND	97.26 ± 42.01	ND	68.28 ± 33.58	22.45 ± 23.55
	1 to 1	107.84 ± 59.33	12.86 ± 28.76*	197.86 ± 121.44	85.66 ± 102.7	343.28 ± 162.32	261.50 ± 335.51
	1/2 to 1	116.01 ± 47.77	ND	247.49 ± 134.41	56.94 ± 66.84†	476.87 ± 265.31	157.82 ± 158.32
	0 to 1	92.27 ± 35.97	ND	244.30 ± 174.00	33.2 ± 53.31†	757.61 ± 381.46	127.01 ± 118.18*
IL-10	1 to 0	4.55 ± 3.61	5.55 ± 5.63	7.35 ± 6.70	6.18 ± 6.75	17.14 ± 16.68	6.09 ± 6.72
	1 to 1	5.13 ± 4.34	5.89 ± 5.23	12.66 ± 10.03	7.29 ± 6.47	19.43 ± 15.55	7.48 ± 7.00
	1/2 to 1	2.42 ± 2.63	5.64 ± 4.93	10.84 ± 11.03	6.77 ± 6.10	20.03 ± 18.87	7.82 ± 8.01
	0 to 1	ND	2.38 ± 0.60	6.30 ± 7.61	3.09 ± 2.38	20.52 ± 17.93	4.02 ± 3.03
IL-1β	1 to 0	73.2 ± 79.47	268.04 ± 494.87	56.69 ± 66.57	212.48 ± 389.24	22.16 ± 27.22	101.41 ± 193.56
	1 to 1	85.89 ± 95.90	297.73 ± 538.58	64.40 ± 74.75	196.73 ± 357.26	23.71 ± 26.27	88.10 ± 161.87
	1/2 to 1	54.05 ± 69.41	216.42 ± 393.42	38.76 ± 52.13	123.32 ± 214.84	15.87 ± 20.58	69.66 ± 130.27
	0 to 1	3.06 ± 3.69	20.95 ± 40.99	1.83 ± 2.34	14.36 ± 26.03	1.06 ± 1.28	8.04 ± 12.68
IL-2	1 to 0	37.00 ± 17.42	4.09 ± 6.19*	33.70 ± 16.77	9.15 ± 14.44†	7.76 ± 2.44	ND
	1 to 1	77.32 ± 42.25	23.18 ± 35.93	76.47 ± 45.35	23.96 ± 30.25	10.62 ± 6.33	ND
	1/2 to 1	72.71 ± 41.55	14.25 ± 19.65	76.96 ± 45.13	15.10 ± 17.37	9.01 ± 1.93	ND
	0 to 1	57.87 ± 29.74	7.85 ± 11.49†	71.48 ± 39.90	7.42 ± 10.25*	12.59 ± 5.83	1.34 ± 2.99*
IL-4	1 to 0	16.94 ± 1.17	ND	17.79 ± 2.30	ND	9.82 ± 1.99	ND
	1 to 1	17.94 ± 2.09	ND	21.53 ± 7.03	ND	12.55 ± 4.12	ND
	1/2 to 1	17.46 ± 1.78	ND	21.55 ± 5.31	ND	12.06 ± 3.36	ND
	0 to 1	16.62 ± 1.24	ND	20.20 ± 3.10	ND	13.73 ± 4.32	ND
IL-6	1 to 0	554.82 ± 399.42	546.20 ± 558.80	604.70 ± 478.87	636.99 ± 640.94	556.07 ± 484.87	676.42 ± 779.87
	1 to 1	568.93 ± 389.64	580.77 ± 515.13	630.01 ± 468.02	588.81 ± 547.11	580.88 ± 463.23	654.74 ± 695.63
	1/2 to 1	370.04 ± 287.4	548.08 ± 611.06	435.21 ± 369.68	522.26 ± 559.58	319.55 ± 378.46	628.53 ± 842.72
	0 to 1	68.36 ± 34.91	173.09 ± 183.77	72.90 ± 36.33	182.10 ± 184.35	65.52 ± 34.94	191.56 ± 221.22
TNF-α	1 to 0	335.91 ± 220.69	233.85 ± 268.28	184.03 ± 134.90	113.04 ± 123.22	78.80 ± 88.72	64.59 ± 112.71
	1 to 1	375.45 ± 176.35	240.45 ± 211.47	270.34 ± 61.80	127.76 ± 93.14†	130.87 ± 50.46	75.33 ± 104.31
	1/2 to 1	288.04 ± 135.38	200.50 ± 186.64	250.15 ± 53.81	112.71 ± 104.08	125.48 ± 42.28	75.69 ± 113.62
	0 to 1	116.87 ± 34.11	68.59 ± 58.36	170.03 ± 128.39	58.33 ± 45.73	136.88 ± 81.80	33.53 ± 26.03
TGF-β	1 to 0					2,443.54 ± 1,276.65	672.64 ± 812.22†
	1 to 1					1,182.04 ± 1,838.65	798.82 ± 1,076.81
	1/2 to 1					853.12 ± 999.01	815.04 ± 708.84
	0 to 1					1,450.45 ± 1,380.20	594.47 ± 191.30

Supernatants were collected from in vitro suppression assays in subjects with type 1 diabetes or healthy control subjects at 24, 48, and 120 h and at various ratios of regulatory T- to effector T-cells. *$P < 0.01$; †$P < 0.05$; ND, not detected.

DISCUSSION

Despite an abundant interest in $CD4^+CD25^+$ T-cells as they relate to the pathogenesis and perhaps more importantly susceptibility to type 1 diabetes, only limited studies of these cells in humans with the disorder have thus far been reported (22,23). To that literature we add our findings that the population of Treg defined by phenotypic expression of the CD4 and CD25 markers does not appear to differ in individuals with type 1 diabetes versus healthy control subjects. In addition, our studies portend that individuals with type 1 diabetes express normal frequencies of $CD4^+CD25^{+Bright}$ T-cells. Key to these descriptions regarding "normal" frequencies in most patients with type 1 diabetes was our identification that age-related differences in the percentage of peripheral blood $CD4^+CD25^+$ T-cells exist. However, at a functional level, meaning the ability of Tregs to suppress activities associated with Teff action, key differences were observed between individuals with type 1 diabetes and healthy control subjects. Those differences were associated with deficiencies in production of a number of cytokines.

Although these studies support the potential for abnormal immune regulation in type 1 diabetes, it must be emphasized that, to date, there remains no definitive surface marker for Tregs. An additional complicating factor for these types of analyses is the notion that the α-chain of the IL-2R (i.e., CD25) is expressed across a continuum of cells, with the most potent suppressor function purportedly attributed to Tregs comprising the bright population using flow cytometric techniques (27). Such limitations, along with differences in methodology, may explain some of the lack of concordance between our studies with the published studies of Kukreja et al. (22), which indicated differences in Treg frequency between type 1 diabetic patients and healthy control subjects. In those studies, dramatically lower $CD4^+CD25^+$ T-cells were reported for subjects with type 1 diabetes versus healthy control subjects, with fairly distinct separations of Treg frequencies between the type 1 diabetic and healthy control groups. In our studies reported herein, such differences were not seen, but rather a broad degree of overlap was often observed, suggesting (but not concluding) that heterogeneity may exist within the type 1 diabetic population with respect to any deficiencies associated with these cells. Indeed, some type 1 diabetic patients did present with a remarkably limited number of $CD4^+CD25^+$ T-cells. In addition, we suspect another major difference in findings, and those of Kukreja et al. (22) reside in our inclusion of

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age matching. We note that in the studies of Kukreja et al., the mean age of the new-onset type 1 diabetic population (therein, having the lowest frequency of $CD4^+CD25^+$ T-cells) was 9.4 ± 2.1 years, whereas their healthy control population was 37 ± 5.6 years. As shown in our studies herein, if one does not perform studies on populations of similar age, frequency differences can be observed independent of disease state. These associations with age also mask any effects that disease duration may have on Treg frequencies.

In the end, perhaps the definitive answer on the exact frequency of regulatory populations in cases of autoimmune disorders including type 1 diabetes will only come when populations under scrutiny are matched for age with a definitive marker or set of phenotypic markers identified—a facet that currently does not exist. Yet, even with such an advance, studies of Treg in type 1 diabetes, unlike those of investigating juvenile idiopathic arthritis (28), would involve extrapolation of results away from the actual site of inflammation (i.e., the pancreatic islets). It is possible that tissue- or lymph node–specific tropisms of Treg could mask important clues as to the pathogenic role of such cells in situations like type 1 diabetes.

The inability to categorically identify a regulatory population of T-cells also points to the heterogeneous nature and natural course of Treg development. A subset of centrally derived regulatory T-cells likely originates from the thymus, whereas others may be generated from the latter stages of effector T-cell differentiation under tolerogenic conditions (3). For our studies, to diminish the possibility that recently activated T-cells expressing CD25 were aberrantly categorized as Treg, we maintained a tight lymphocyte gate across all study populations to reduce the possibility of including large blasting cells but did not exclude cells based on other cellular activation markers. Indeed, a recent article by Fisson et al. (29) showed in a series of in vivo experiments that a subset of Tregs in mice express multiple activation markers. Hence, this type of study would call into question the use of markers of cellular activation to exclude activated nonregulatory cells.

Unfortunately, the mechanisms conferring the suppressive capabilities of Treg are poorly understood, and much of the data that do exist have been derived from murine systems. Most models suggest a requirement for direct cell-to-cell contact between Treg and Teff (7). In terms of soluble factors, direct suppressive functions of Treg appear to act independent of IL-10 and TGF-β (30,31). Other studies suggest an important role for TGF-β based on the association between a reduction of suppression with blockade of TGF-β (32,33). A role for IFN-γ in the process has also been suggested by studies involving indoleamine 2,3-dioxygenase and Treg (34,35). We would note our desire to have these associations between cytokine elaboration and Treg function confirmed through analysis of larger populations of control subjects and type 1 diabetic patients because our studies were performed on a limited number of subjects.

One potential limitation of these studies is the potential for overinterpretation of a defect lying solely in the $CD25^+$ population over the $CD25^-$ population because our studies presented herein used CD25 cells from type 1 diabetic patients as responders. Whereas we did not observe significant differences between proliferation of the type 1 diabetic patient and control Teffs, the type 1 diabetic patients' responses as well as IFN-γ responses were lower. To address the issue of whether the lack of observed suppression did not result from an artifact due to low T effector responses, a series of "crossover" suppression assays were performed in parallel that involved autologous suppression assays and co-incubation of Treg from type 1 diabetic patients with Teff from age-matched control subjects, as well as Treg from control subjects with Teff from type 1 diabetic patients. Preliminary results from those studies (data not shown) do continue to support the notion that the defect resides within the Treg population of patients with type 1 diabetes.

In terms of our providing novel contribution to this literature, our studies would support a clear association between the production of IFN-γ and TGF-β in pathways of suppression by Treg. The production of IL-2, while noted as being reduced in subjects with type 1 diabetes, has for decades been noted as a defect associated with their cellular immune reactivity. In this context, if a dose-dependent influence of IL-2 was required for the maintenance of the suppressive capacity of Treg, differences in the functional assays such as those in this study might be observed. However, with this collective listing of cytokine variances, we must emphasize a notion mentioned previously that direct cell contact between Treg and Teff is thought, by a majority of published studies, to be a key facet to this process. Despite this, it remains plausible to speculate that the production of these cytokines may influence Treg function through effects on antigen-presenting cells and Teff populations.

In terms of future directions and outstanding issues, these studies identified a strong association between age and the frequency of $CD4^+CD25^+$ T-cells. Yet to be investigated are the interesting questions of whether age influences the regulatory functions of these cells in individuals with type 1 diabetes. Indeed, a study by Tsaknaridis et al. (36) indicates that the suppressive capacity of Treg may actually decline with age. This issue was not addressed as part of the current study because our primary purpose was to determine whether "age matching" was a vital issue for comparisons of frequency in studies of type 1 diabetes. Yet serendipitously, our findings have opened an interesting agenda for future studies that would address the question of whether increases in $CD4^+CD25^+$ T-cells populations with time could, in part, be a physiological response to a decrease in functional capacity or if this increase simply results from a maturation of T-cell populations from increasing antigen exposures. We will also obtain patient samples from both young and old(er) type 1 diabetic patients, matched for age, who have similar disease durations for the purpose of addressing potential issues related to frequency as a function of disease duration. An additional issue not yet addressed here, but will be the subject of future efforts, will examine the potential contributions of various type 1 diabetes susceptibility genes (e.g., HLA, Insulin VNTR, CTLA-4, and SUMO-4) to the observed defects in suppression. Indeed, genetic factors may, like we have seen with age, underlie some of the heterogeneity observed with both function and frequency.

Finally, despite the conflicting reports and substantial

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overlap in Treg frequencies between type 1 diabetic patients and control subjects, the therapeutic potential of this cell population holds great promise. This study reveals functional defects in a population of cells critical for the maintenance of peripheral tolerance. The future employment of effective treatment modalities using Treg should consider the functional defects outlined herein along with the mechanisms of control underlying them.

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