Defective Suppressor Function in $CD4^+CD25^+$ T-Cells From Patients With Type 1 Diabetes

Shelley Lindley,[1] Colin M. Dayan,[2] Amanda Bishop,[2] Bart O. Roep,[3] Mark Peakman,[1] and Timothy I.M. Tree[1]

Type 1 diabetes is a T-cell–mediated disease that is associated with loss of immunological tolerance to self-antigens. The mechanisms involved in maintenance of peripheral tolerance include a specialized subset of regulatory T-cells (Treg) within the $CD4^+CD25^+$ T-cell population, but the function and phenotype of these cells in type 1 diabetes have not been investigated. We hypothesized that a deficiency in the $CD4^+CD25^+$ Treg population or its function could contribute to the lack of self-tolerance evident in patients with type 1 diabetes. We show that although levels of $CD4^+CD25^+$ T-cells are normal in patients with recent-onset adult type 1 diabetes, the ability of the Tregs in this population to suppress T-cell proliferation during in vitro cocultures is markedly reduced compared with control subjects ($P = 0.007$). Moreover, in patients with type 1 diabetes, these cocultures display a more proinflammatory phenotype, with increased secretion of interferon-γ ($P = 0.005$) and decreased interleukin-10 production ($P = 0.03$). These deficiencies may reflect a disturbance in the balance of the $CD4^+CD25^+$ population, because in patients with type 1 diabetes, a higher proportion of these cells coexpress the early activation marker CD69 ($P = 0.007$) and intracellular CTLA-4 ($P = 0.01$). These data demonstrate deficiency in function of the $CD4^+CD25^+$ Treg population that may influence the pathogenesis of type 1 diabetes. *Diabetes* 54:92–99, 2005

Type 1 diabetes is a chronic, T-cell–mediated autoimmune disease that results in the destruction of the insulin-secreting β-cells (1). The pathological mechanisms that lead to disease development are not known with certainty, but there is compelling evidence that the disease is associated with loss of immunological tolerance to self. For example, pancreatic β-cell destruction is associated temporally with the presence of islet cell autoantibodies directed against the autoantigens insulin, GAD65, and the islet tyrosine phosphatase IA-2 (2). Autoreactive T-cells that recognize these and other islet autoantigens have been identified and are thought to play a direct role in type 1 diabetes immunopathogenesis (3–6), a proposal supported by studies showing that administration of therapeutic agents that inhibit T-cell function delays disease progression (7,8).

Studies in animal models have indicated that loss of tolerance to β-cell autoantigens could be the result of numerous factors, including genetic and environmental influences (9–11). T-cell tolerance is established centrally in the thymus and further strengthened and maintained through multiple mechanisms of peripheral tolerance (12). The manifest lack of self-tolerance to β-cell autoantigens in patients with type 1 diabetes compared with their nondiabetic counterparts therefore could be due, at least in part, to a failure in one or more of these mechanisms. Recent interest has focused on a feature of tolerance that seems to bridge the central and peripheral processes, namely the $CD4^+CD25^+$ regulatory T-cell (13–16). These represent a naturally occurring CD4 T-cell population expressing CD25 that arises from the thymus and seeds into the periphery, creating a cohort of cells with profound T-cell immunosuppressive qualities. $CD4^+CD25^+$ cells can be detected in peripheral blood in humans and are able to suppress proliferation and cytokine production from both $CD4^+$ and $CD8^+$ T-cells in vitro in a cell contact–dependent manner (17–21). We therefore hypothesized that a relative defect, in terms of number or function, of these regulatory T-cells (Tregs) could contribute to the lack of self-tolerance seen in type 1 diabetes. To test this hypothesis, we enumerated and phenotypically and functionally characterized $CD4^+CD25^+$ T-cells from patients with newly diagnosed type 1 diabetes and a group of HLA- and age-matched control individuals.

In agreement with recently published studies in patients with the autoimmune diseases multiple sclerosis (22) and autoimmune polyglandular syndrome type II (23), we demonstrate a functional deficiency in the regulatory potential of $CD4^+CD25^+$ T-cells from patients with type 1 diabetes that may reflect an underlying susceptibility to autoimmune disease.

RESEARCH DESIGN AND METHODS

Fresh peripheral blood samples were obtained from 21 patients with recent-onset type 1 diabetes (mean ± SD: age 32.3 ± 6.8 years) and 15 age-, sex-, and HLA-matched healthy nondiabetic control subjects with no family history of the disease (age 30.3 ± 6.8 years) for use in the frequency and phenotypic analysis studies and from 11 patients with recent-onset type 1 diabetes (age

From the [1]Department of Immunobiology, Guy's, King's & St Thomas' School of Medicine, King's College London, U.K.; the [2]Bristol Royal Infirmary, Bristol, U.K.; and the [3]Department of Immunohaematology & Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands.

Address correspondence and reprint requests to Timothy I.M. Tree, Department of Immunobiology, Guy's, King's & St Thomas' School of Medicine, 2nd Floor, New Guy's House, Guy's Hospital London SE1 9RT, U.K. E-mail: timothy.tree@kcl.ac.uk.

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IFN-γ, interferon-γ; MFI, mean fluorescence intensity; PBMC, peripheral blood mononuclear cell; PerCP, peridin-chlorophyll protein; Treg, regulatory T-cells.

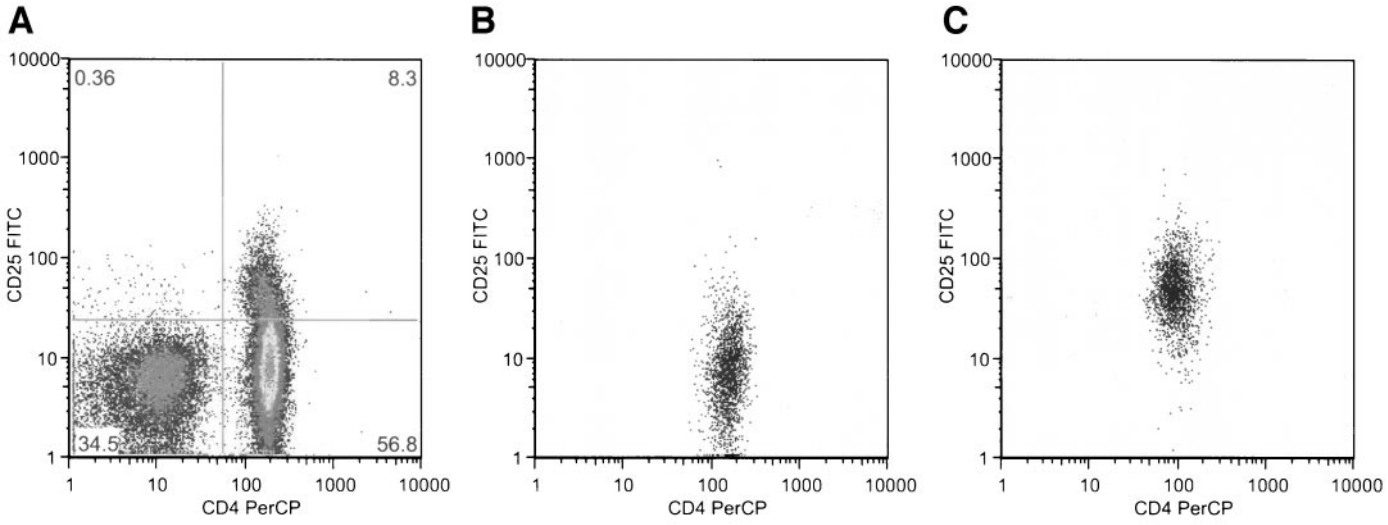

FIG. 1. Example of the gating used to define CD4 and CD25 status. Lymphocytes identified by their forward and side scatter properties are gated for CD3 expression and then examined for coexpression of CD4 and CD25 (*A*). Quadrants in *A* were drawn on the basis of 99.5th percentile staining by relevant isotype control antibodies. $CD4^+CD25^{hi}$ cells (boxed) were defined as the top 2% of CD25 staining $CD4^+$ T-cells. *B* and *C*: Representative examples of purified $CD25^-$ and $CD25^+$ CD4 T-cells, respectively.

27.7 ± 6.2 years) and 13 age- and HLA-matched healthy nondiabetic control subjects (27.45 ± 4.63) for the functional studies. Diabetes was diagnosed according to the criteria set out by the National Diabetes Data Group (24), and all patient blood samples were collected within 6 months of the onset of initial insulin therapy.

Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood by density gradient centrifugation (Lymphoprep; Axis-Shield PoC AS, Oslo, Norway). Ethical approval for this study was granted by the local ethics committee and informed consent obtained.

Monoclonal antibodies. Phycoerythrin-labeled monoclonal anti–HLA-DR (clone TU36), anti-CD69 (clone FN50), anti–CTLA-4 (clone BNI3), anti-CD45R0 (clone UCHL1), peridin-chlorophyll protein (PerCP)-labeled anti-CD4 (clone SK3), and allophycocyanin-labeled anti-CD3 (clone UCHT1) antibodies (BD PharMingen, San Diego, CA), as well as fluorescein isothiocyanate (FITC)-conjugated anti-CD25 (clone M-A251; Serotec, Oxford, U.K.) and relevant isotype- and fluorochrome-matched control antibodies, were used in this study. Antibody concentrations used in cell staining were based on the data supplied by the manufacturers and initial optimization studies.

Flow cytometric analysis. Immunofluorescence staining was performed after the PBMCs were washed twice with ice-cold wash buffer (PBS, 10% FCS; PAA Laboratories, Yeovil, U.K.). For each staining, 4×10^5 cells were resuspended in 200 μl buffer. Four-color staining (anti-CD4, anti-CD25, and anti-CD3 plus one of anti-CD69, anti-HLA-DR, anti-CTLA-4, or anti-CD45R0) for surface marker expression was carried out on ice in the dark for 30 min. For investigation of intracellular CTLA-4 expression, cells were surface stained as described above with anti–CD3-allophycocyanin, anti–CD4-PerCP, and anti–CD25-FITC, fixed and permeabilized according to the manufacturer's instructions (Fix and Perm kit; Caltag Laboratories, Burlingame, CA), stained with anti–CTLA-4-phycoerythrin, and washed twice in wash buffer and analyzed.

Background fluorescence was assessed using the appropriate isotype- and fluorochrome-matched control monoclonal antibody to determine the percentage of positive cells. PBMCs were gated on their forward and side scatter properties, and $CD3^+CD4^+$ cells were gated using the relevant labeled antibodies. For identification of $CD25^+$ cells, dot plot quadrants were set so that the lymphocytes that were stained with the isotype control were within the 99.5th percentile. For indicating the intensity of HLA-DR, CTLA-4, CD45R0, or CD69 expression on $CD4^+CD25^+$ T-cells, mean fluorescent intensities were calculated using FlowJo software.

Antibody staining analysis was performed on a FACSCalibur (Becton Dickinson, Mountain View, CA) using CellQuest (Becton Dickinson) and FlowJo software. Instrument settings were optimized using single-stained tubes. A minimum of 100,000 events were acquired and analyzed.

Allelic discrimination of the CTLA-4 gene +49 A/G dimorphism. Amplification of the CTLA-4 gene +49 locus was performed in a total volume of 11 μl that contained ~10 ng of genomic DNA, 200 μmol/l dNTPs, 5% glycerol, 0.2 units of *Taq*DNA polymerase (Promega, Leiden, The Netherlands), and 11 pmol/l of both outer primers (outer forward 5′-CCT TGA TTC TGT GTG GGT TC-3′, outer reverse 5′-CCT TTA ACT TCT GGC TTT GC-3′). The amplification buffer consisted of 50 mmol/l KCl, 10 mmol/l Tris (pH 8.4) at room temperature, 1.5 mmol/l $MgCl_2$, and 0.06 mg/ml BSA (Promega). Amplification refractory mutation system PCR was performed in a total volume of 21 μl, using 1 μl of the amplification product as template, 25 pmol/l of each allele specific inner primer (inner forward "A" allele 5′-GCT CAG CTG AAC CTG GCT A-3′, inner reverse "G" allele 5′-CAG GGC CAG GTC CTG GC-3′), 6.25 pmol of each outer primer, and 0.4 units of *Taq* polymerase. Touchdown PCR was performed at 56°C annealing temperature. Products (317 bp for the control, 189 bp for the A allele, and 165 bp for the G allele) were separated on a 4% (wt/vol) agarose gel stained with ethidium bromide, and alleles were mean determined by comparison with a 25-bp ladder as reference.

T-cell isolation and culture. $CD4^+$ T-cells were isolated from PBMCs by negative selection using magnetic cell sorting technology (MACS; Miltenyi Biotech, Bisley, U.K.). $CD4^+$ T-cells were separated into $CD25^+$ and $CD25^-$ populations by MACS, using anti-CD25 microbeads (Miltenyi Biotech). In preliminary studies, we optimized the preferential isolation of $CD25^{hi}$ cells by varying the duration, temperature, and bead concentration during purification. In our study, the highest expression of CD25 on $CD4^+CD25^+$ cells was achieved by reducing by 50% the manufacturer's recommended bead concentration. T-cell–depleted accessory cells were isolated by negative selection of PBMCs with anti-CD3 microbeads (Miltenyi Biotech) followed by irradiation at 3,000 rad. The purity of all cell populations was assessed by flow cytometry, using anti-CD3, anti-CD4, and anti-CD25 antibodies as described above.

T-cells were cultured in RPMI-1640 media supplemented with 2 nmol/l L-glutamine, 5 mmol/l HEPES, 100 μg/ml penicillin/streptomycin, 0.5 mmol/l sodium pyruvate, and 0.05 mmol/l nonessential amino acids (all from Invitrogen, Paisley, U.K.), and 5% AB serum (Harlan Sera Labs, Loughborough, U.K.) in 96-well U-bottom plates.

Lymphocyte stimulation assays. Lymphocyte stimulation assays were performed by culturing $CD4^+CD25^-$ (5×10^3/well) with $CD4^+CD25^+$ T-cells at various ratios (0:1, 1:0, and 1:1) in the presence of 5×10^4 irradiated accessory cells. All T-cells in these cultures were stimulated using plate-bound anti-CD3 (clone UCHT1) and soluble anti-CD28 (clone CD28.2) antibodies (BD Pharmingen, Oxford, U.K.). Briefly, plates were incubated with 50 μl/well PBS that contained 5 or 10 μg/ml anti-CD3 antibody for 4 h at 37°C and then washed twice in PBS.

All T-cell culture conditions were carried out in triplicate. On day 5 of culture, 100 μl of supernatant was removed from each well (and stored at −80°C) and 100 μl of fresh medium that contained 0.5 μCi of [^{3}H] thymidine was added for the final 16 h of culture before harvesting. Percentage suppression was calculated as 100 − [(cpm in cocultures/cpm in $CD4^+CD25^-$ cultures) × 100]. Cytokine production was measured on a subset of individuals (eight patients with type 1 diabetes and nine control subjects) using the Th1/Th2 Cytometric Bead Assay Kit (BD Pharmingen) according to the manufacturer's instructions.

Statistical analysis. The normality of the distributions of levels of lymphocytes expressing the defined markers in patients with type 1 diabetes and nondiabetic control subjects were determined using the Kolmogorov-Smirnov goodness-of-fit test. Percentage levels of lymphocyte populations and mean

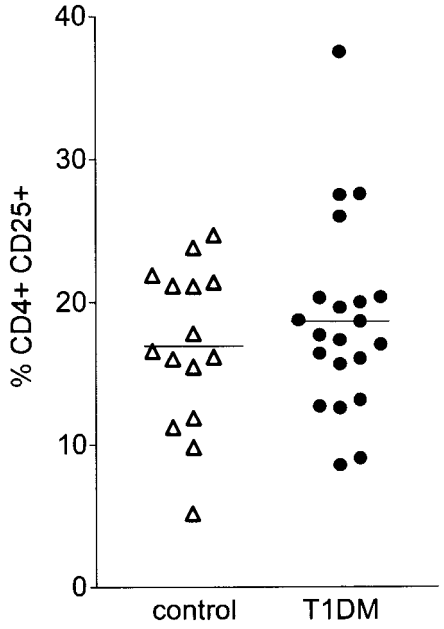

FIG. 2. Percentage of CD4$^+$CD25$^+$ T-cells in patients with type 1 diabetes (T1DM) and nondiabetic control individuals. Results are expressed as the percentage of CD4$^+$ T-cells coexpressing CD25, and mean values for each group are indicated by a horizontal line.

fluorescence intensities of the cell staining in the two subject groups were compared using the Student's *t* test for normally distributed populations and Mann-Whitney *U* test for nonnormally distributed samples. Relationships between parameters were examined using calculation of the Pearson's correlation coefficient and Spearman's rank correlation test. Results of lymphocyte stimulation assays were compared using the Mann-Whitney *U* test. Differences in the genotype and allele frequencies of the CTLA-4 + 49 A/G dimorphism were examined using χ^2 and Fisher's exact test, respectively. All statistical analyses were performed using GraphPad Prism (GraphPad Software), and $P < 0.05$ was considered significant.

RESULTS

Results of flow cytometry analyses are expressed as the percentage of CD4$^+$ T-cells coexpressing CD25 and the percentage of CD4$^+$CD25$^+$, CD4$^+$CD25^{hi}, or CD4$^+$CD25$^-$ cells coexpressing HLA-DR, CD45R0, CD69, or CTLA-4. An example of the gating used to define CD4 and CD25 status is shown in Fig. 1.

Percentage of lymphocytes expressing CD4$^+$CD25$^+$ and levels of CD25 expression in patients with type 1 diabetes and control subjects. There was no significant difference in the percentage of CD4 lymphocytes that express CD25 in patients with type 1 diabetes (mean ± SD: 18.7 ± 6.7%) and control subjects (16.9 ± 5.6%; Fig. 2). Furthermore, there was no significant difference between the two groups in the level of CD25 expression per cell, when expressed as the mean fluorescence intensity (MFI) of the CD4$^+$CD25$^+$ cell population (mean ± SD MFI in patients with type 1 diabetes, 10.2 ± 2.1; control subjects, 10.3 ± 2.3). Some studies have suggested that the regulatory cells among the CD4$^+$CD25$^+$ population predominantly express high levels of CD25 (CD25^{hi}) (25,26). In addition to analyzing the phenotype of all CD4$^+$CD25$^+$ cells, therefore, CD25^{hi} cells were defined as the top 2% of CD25$^+$CD4$^+$ T-cells. The MFI of these cells was not significantly different between patients with type 1 diabetes and control individuals (MFI, 95.4 ± 16.7 and 93.8 ± 22.4, respectively). In addition, the use of a specific threshold to define CD25^{hi} cells (defined as a fluorescence intensity of >100) did not reveal any differences between patients and control individuals. In summary, therefore, we did not find evidence of either an alteration in the frequency of CD4$^+$CD25$^+$ cells in patients with type 1 diabetes or any change in the amount of CD25 expressed per cell.

Expression of activation and differentiation markers on CD4$^+$CD25$^+$ and CD4$^+$CD25^{hi} T-cells. Because the CD4$^+$CD25$^+$ population is known to be a heterogeneous mixture of cells with effector, regulatory, and other functions, we next examined whether patients with type 1 diabetes and nondiabetic control subjects differed in the relative distribution of activation and differentiation markers among the CD4$^+$CD25$^+$ cells. Patients with recent-onset type 1 diabetes had a significantly higher percentage of CD4$^+$CD25$^+$ and CD4$^+$CD25^{hi} T-cells coexpressing the early activation marker CD69 in comparison with control subjects ($P = 0.007$ and $P = 0.002$, respectively; Figs. 3 and 4). In contrast, the percentages of CD4$^+$CD25$^+$ cells coexpressing HLA-DR or CD45R0 that represent chronically activated and memory cells, respectively, were similar in the two groups (Figs. 3 and 4).

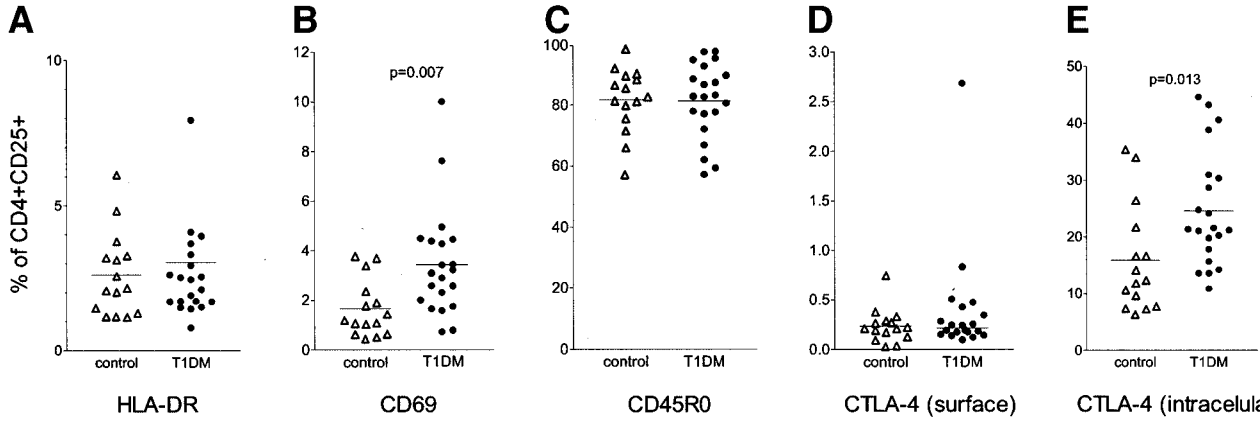

FIG. 3. Coexpression of activation and differentiation markers on CD4$^+$CD25$^+$ T-cells. Graphs show the coexpression of HLA-DR (*A*), CD69 (*B*), CD45R0 (*C*), surface expression of CTLA-4 (*D*), and intracellular expression of CTLA-4 by CD4$^+$CD25$^+$ T-cells (*E*). Results are expressed as percentage of CD4$^+$CD25$^+$ cells coexpressing the third marker. Mean (median for surface CTLA-4) values for each group are indicated by a horizontal line. T1DM, type 1 diabetes.

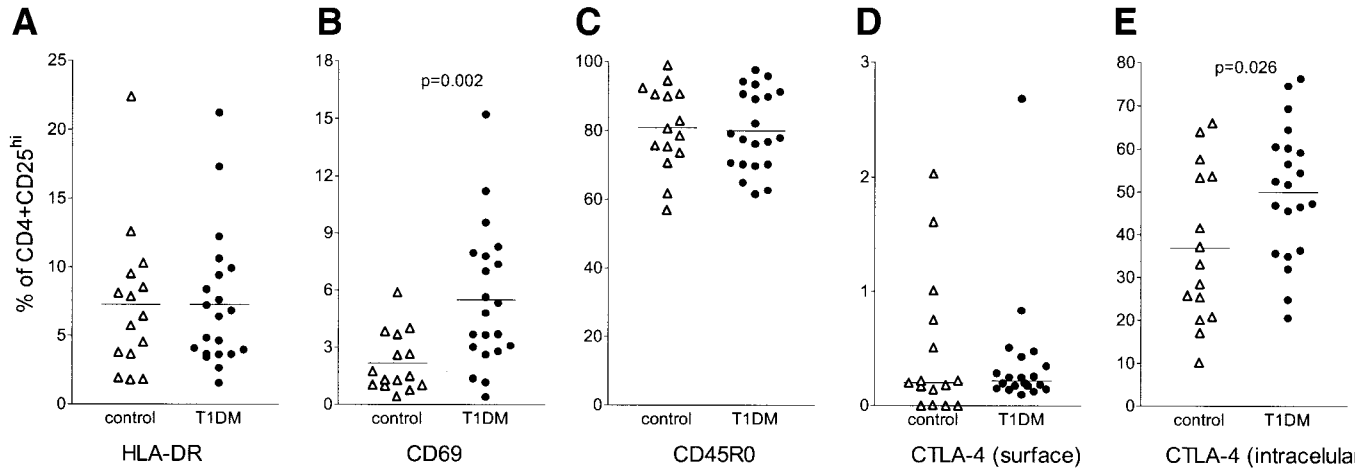

FIG. 4. Coexpression of activation and differentiation markers on $CD4^+CD25^{hi}$ T-cells. Graphs show the coexpression of HLA-DR (*A*), CD69 (*B*), CD45R0 (*C*), surface expression of CTLA-4 (*D*), and intracellular expression of CTLA-4 on the top 2% of $CD4^+CD25^+$ T-cells (*E*). Results are expressed as percentage of $CD4^+CD25^{hi}$ cells coexpressing the third marker. Mean (median for surface CTLA-4) values for each group are indicated by a horizontal line. T1DM, type 1 diabetes.

Because the T-cell inhibitory molecule CTLA-4 is stored intracellularly and expressed in its functional context only through transient surface expression, we examined both intracellular and surface CTLA-4 on $CD4^+CD25^+$ and $CD4^+CD25^{hi}$ T-cells. The percentage of $CD4^+CD25^+$ and $CD4^+CD25^{hi}$ cells expressing intracellular CTLA-4 was significantly elevated in patients with type 1 diabetes compared with control individuals ($P = 0.013$ and $P = 0.026$, respectively; Figs. 3 and 4). Furthermore, the amount of CTLA-4 per $CD4^+CD25^+$ cell, represented by the MFI of intracellular CTLA-4–associated immunofluorescence on $CD4^+CD25^+$ and $CD4^+CD25^{hi}$ cells, was significantly higher in type 1 diabetes ($P = 0.005$ and $P = 0.007$, respectively; Fig. 5). In contrast, surface CTLA-4 expression was similar in the two subject groups, in terms of both quantity and quality.

After synthesis, CTLA-4 is primarily located in intracellular vesicles and is translocated to the cell surface only after T-cell activation (27,28). Because we found elevated intracellular expression of CTLA-4 on $CD4^+CD25^+$ T-cells, we also carried out studies seeking a corresponding increase in the percentage of cells positive for CTLA-4 surface expression. Surface expression was detected only at extremely low levels, and because removal of CTLA-4 from the cell surface is known to be rapid, especially in activated T-cells, we also performed surface immunofluorescence staining at 37°C after 1, 3, and 6 h of in vitro culture in the continued presence of anti–CTLA-4 monoclonal antibody to examine CTLA-4 turnover. We observed little measurable increase in surface staining during culture and no difference in surface expression between patients with type 1 diabetes and control individuals at any time point (data not shown). One interpretation of these results is that the $CTLA\text{-}4^+$, $CD4^+CD25^+$ cells constitute a resting rather than recently activated population and therefore have not received stimulatory signals that would lead to surface translocation, but this will require confirmation in future studies.

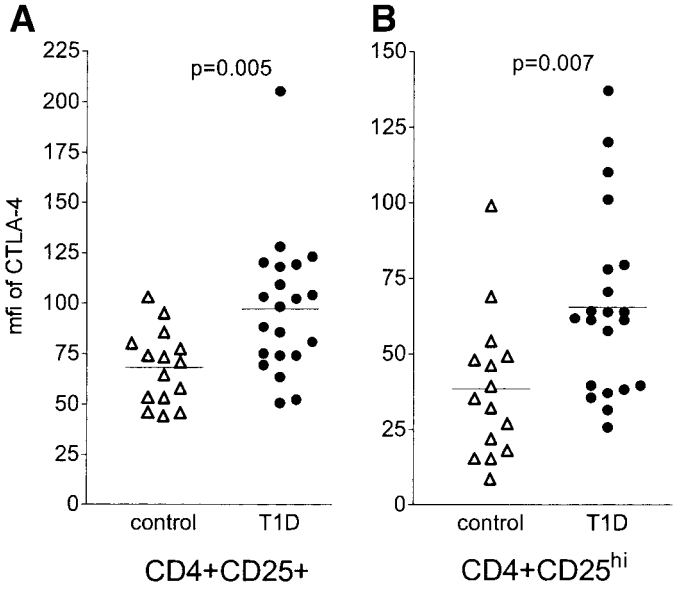

FIG. 5. Mean fluorescence intensities of intracellular CTLA-4 in $CTLA\text{-}4^+$, $CD4^+CD25^+$ (*A*), and $CD4^+CD25^{hi}$ (*B*) subsets. Mean values for each group are indicated by a horizontal line. T1D, type 1 diabetes.

Relationship between age, diabetes duration, and markers of activation and differentiation on $CD4^+CD25^+$ cells. There was no significant relationship among age, duration of diabetes, HLA genotype, and autoantibody status among patients with type 1 diabetes. In addition, there was no significant correlation between these variables and expression of CD25 or expression of activation markers on $CD4^+CD25^+$ T-cells.

Analysis of the CTLA-4 exon 1 + 49 A/G polymorphism. The human CTLA-4 gene contains an A/G dimorphism at position +49 that has been associated with the development of several autoimmune diseases (29) and is believed to affect cellular distribution of CTLA-4 (30). We therefore determined the alleles present at CTLA-4 +49 in the type 1 diabetic and control groups. Both the frequency of G allele–positive individuals and the frequency of genotypes were similar in control subjects and patients with type 1 diabetes (Table 1). Analysis of intracellular or surface expression of CTLA-4 stratified according to possession of A/G polymorphisms revealed no significant differences in either the level or percentages of cells expressing CTLA-4 (data not shown).

TABLE 1
Frequency of the CLTA-4 position +49 A/G polymorphism in patients with type 1 diabetes and control subjects

	Patients	Control subjects	*P* value
n	19	14	
Allele frequencies			0.45
G	14 (36.8%)	13 (46.4%)	
A	24 (63.2%)	15 (53.6%)	
Genotype frequencies			0.78
GG	3 (15.8%)	4 (28.5%)	
GA	8 (42.1%)	5 (35.7%)	
AA	8 (42.1%)	5 (35.7%)	

Data are *n* (%). Differences in the allele and genotype frequencies between the groups were assessed using Fisher's exact and χ^2 tests, respectively.

TABLE 2
Cytokine production

	IFN-γ (pg/ml)	Interleukin-10 (pg/ml)
T1DM $CD25^+$	0.58 (0.0–175.0)	1.6 (1.0–6.5)
T1DM $CD25^-$	893.7 (221.3–3303)	10.3 (6.6–50.0)
T1DM $CD25^+/CD25^-$	379.0 (74.9–3085)*	7.1 (2.2–27.8)
Control $CD25^+$	0.0 (0.0–15.0)	10.1 (0.0–149.0)
Control $CD25^-$	205.0 (0.0–1572)	8.4 (0.0–206)
Control $CD25^+/CD25^-$	41.1 (0.0–358)*	29.4 (0.0–81.2)

Data are median (range) Cytokine levels from each culture were determined by cytometric bead assay as described in RESEARCH DESIGN AND METHODS. *$P < 0.01$ comparing results from patients with type 1 diabetes (T1DM) and control subjects.

Suppressive function of $CD4^+CD25^+$ T-cells. To test the suppressive function of Treg cells, we isolated populations of $CD4^+CD25^+$ and $CD4^+CD25^-$ T-cells by magnetic bead technology. Particular attention was paid to optimizing the conditions for the preferential isolation of $CD4^+$ T-cells expressing high levels of CD25. This was achieved through reducing the ratio of beads to cells, shortening incubation times of cells with the isolation beads, and performing the separation on ice. An example of the populations isolated is shown in Fig. 1*D* and *E*. No difference was observed between patients and control subjects in either the purity or the level of CD25 expressed on either population (data not shown). As previous studies have demonstrated that the strength of stimulus used in suppression assays can have a profound effect (25,26), we performed a series of pilot experiments in which the amount of stimulating anti-CD3 antibody was titrated. For the analyses presented, 5–10 μg/ml anti-CD3 antibody offered optimal levels of stimulation of responder T-cells and suppression by $CD4^+CD25^+$ T-cells in most individuals tested. All data presented here are from cultures that were stimulated with the 5-μg/ml dose.

As previously reported, $CD4^+CD25^+$ T-cells from control individuals were relatively anergic to polyclonal stimulation and able to suppress the proliferation of $CD4^+CD25^-$ cells in a dose-dependent manner (Fig. 6*A*). We observed no difference in the level of proliferation (Fig. 6*B* and *C*) or cytokine production (Table 2) of the individual $CD4^+CD25^+$ or $CD4^+CD25^-$ populations between patients with type 1 diabetes and control subjects. However, analysis of proliferation in cocultures of anti-CD3–stimulated $CD4^+CD25^+/CD4^+CD25^-$ cells at a ratio of 1:1 indicated that $CD4^+CD25^+$ T-cells from patients with type 1 diabetes displayed significantly impaired suppressor function when compared with control individuals (mean ± SD: 25.9 ± 17.9 vs. 57.3 ± 23.5%; $P = 0.007$; Fig. 6*D*). Furthermore, analysis of secreted cytokines revealed that in comparison with control subjects, cocultures from patients with type 1 diabetes contained significantly more interferon-γ (IFN-γ) (816.0 ± 991.9 vs. 98.2 ± 136.8 pg/ml; $P = 0.005$) and significantly less interleukin-10 (10.1 ± 8.5 vs. 36.2 pg/ml ± 27.4; $P = 0.03$; Fig. 7*A*). The balance of cytokines produced by $CD4^+CD25^-$ cells and cocultures is also expressed as a ratio of IFN-γ (pg/ml) to interleukin-10 (pg/ml) (Fig. 7*B* and *C*).

Suppression assays were also performed using a stronger stimulus (10 μg/ml anti-CD3 antibody). Consistent with other studies (22,25,26), increasing the strength of stimulus decreased the level of suppression in the control group, and no differences were seen between patients with type 1 diabetes and control subjects (mean suppression ± SD, 35.2 ± 21.6% and 35.6 ± 22.9%, respectively; data not shown).

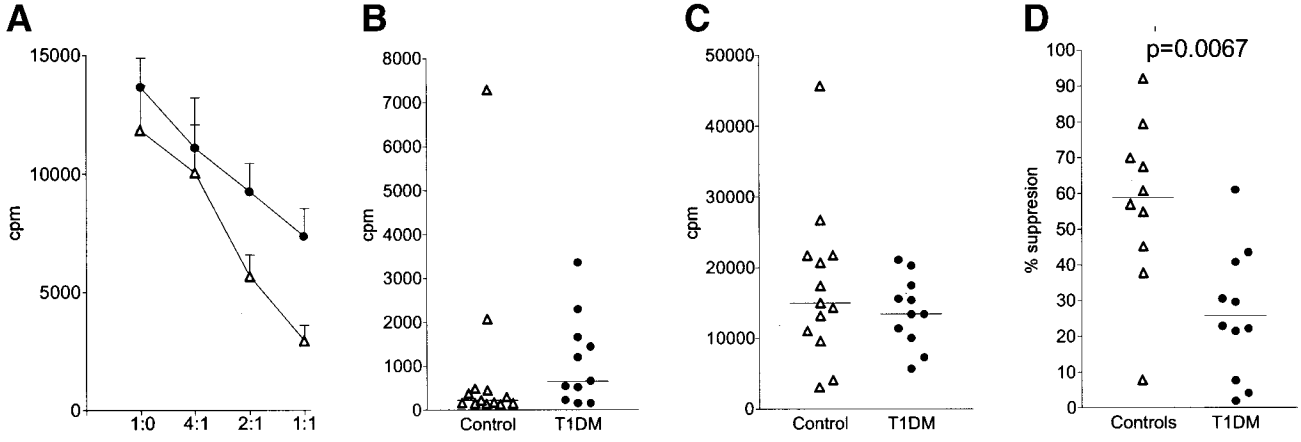

FIG. 6. Proliferation and percentage suppression in separated cell populations and cocultures. *A*: Representative proliferation of 5,000 $CD4^+CD25^-$ cells from a patient with type 1 diabetes (T1DM) (●) and from a control nondiabetic subject (△) in the presence of different ratios of $CD4^+CD25^+$ cells. Proliferation of $CD4^+CD25^+$ (*B*), $CD4^+CD25^-$ (*C*), and percentage suppression of $CD4^+CD25^-$ proliferation by $CD4^+CD25^+$ cells in cocultures (*D*). Median values are indicated by a horizontal line.

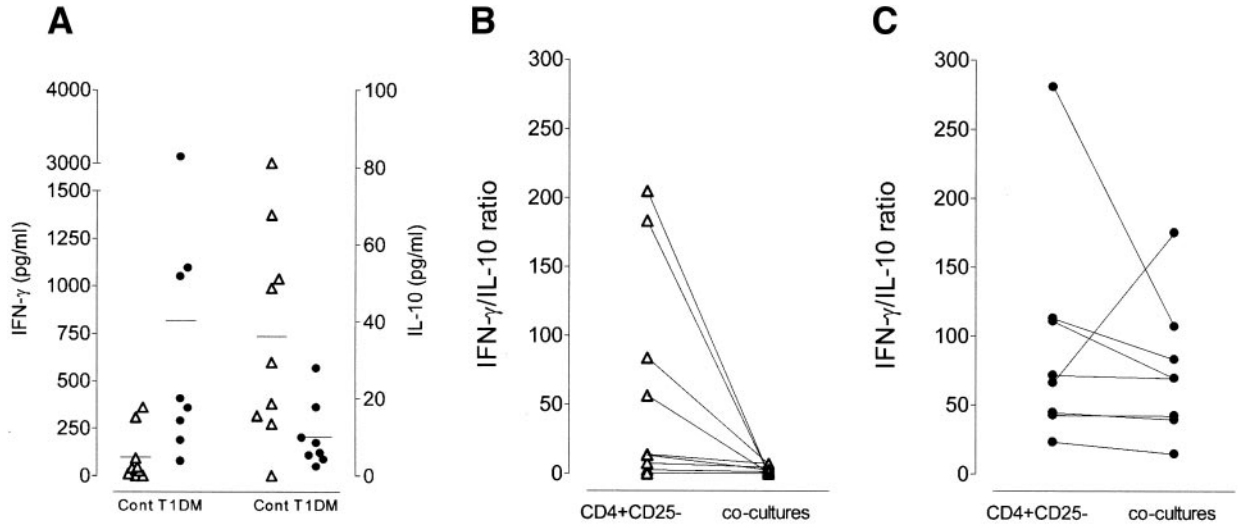

FIG. 7. Cytokine production. *A*: Levels of IFN-γ and interleukin-10 secreted in cocultures from patients with type 1 diabetes (T1DM) and control subjects. Median values are indicated by a horizontal line. Cytokine data are also represented as a ratio of IFN-γ to interleukin-10 produced by $CD4^+CD25^-$ cells and cocultures for control subjects (*B*) and patients with type 1 diabetes (*C*). Results from individual subjects are joined by a horizontal line.

DISCUSSION

The present study is the first in patients with type 1 diabetes to examine the suppressor function of regulatory cells contained within the $CD4^+CD25^+$ T-cell population. Analysis of the functional regulatory capacity of cells defined by these markers demonstrates a marked deficiency in their ability to suppress proliferation of cocultured $CD4^+CD25^-$ T-cells in patients with type 1 diabetes. In addition, the cytokine milieu generated in $CD4^+CD25^+$/$CD4^+CD25^-$ cocultures differs markedly and significantly between patients with type 1 diabetes and nondiabetic subjects, with a failure to suppress effector T-cell production of a proinflammatory cytokine and failure to promote production of an anti-inflammatory cytokine observed among the patients. Taken together, these novel data support the proposal that the development of type 1 diabetes is accompanied by defective immune regulation by at least one of the major suppressor T-cell populations defined to date.

By its nature, a cell type that negatively regulates other immune effectors is difficult to identify with ease and precision at the single-cell level. For this reason, the identification of a phenotype, CD25 positivity, which defines a population of cells with regulatory properties both in vitro and in vivo, has been heralded as an important advance (14,15). However, it is apparent that although CD25 defines a population with potent regulatory activity, it encompasses a heterogeneous group of cells (25), including recently activated effector cells (31). Before the advent of knowledge that $CD4^+CD25^+$ T-cells regulate, studies demonstrating increases in these cells in patients with type 1 diabetes were interpreted as consistent with the presence of an increased percentage of circulating autoreactive effector cells (32). In contrast, more recently, changes in this population of cells in patients with type 1 diabetes have been interpreted in the context of their regulatory function (33). Importantly, to date, no studies have attempted to examine this controversy by analyzing the regulatory capacity of $CD4^+CD25^+$ T-cells in patients with type 1 diabetes.

We used a conventional approach to examining the functional regulatory capacity of $CD4^+CD25^+$ T-cells by assessing their ability to suppress mitogen-induced proliferation of $CD4^+CD25^-$ T-cell populations and modulate the accompanying cytokine production. As a source of regulatory cells, we purified $CD4^+CD25^+$ T-cells using an immunomagnetic approach designed to preferentially recruit $CD25^{hi}$ cells, which are reported to harbor the most potent regulatory capacity. In accordance with other reports (17–20,25,34), we found that upon polyclonal activation, $CD4^+CD25^+$ T-cells that were purified in this way were relatively anergic in comparison with the $CD4^+CD25^-$ population and were able to suppress proliferation and cytokine production of the $CD4^+CD25^-$ cells in a dose-dependent manner. However, the regulatory capacity of $CD4^+CD25^+$ T-cells from patients with type 1 diabetes was markedly diminished in comparison with control subjects in terms of both suppression of proliferation and control of secretion of the proinflammatory cytokine IFN-γ. Conversely, the anti-inflammatory cytokine interleukin-10 was found at higher concentrations in cocultures from control subjects. IFN-γ is believed to have a central role in T-cell–mediated destruction of pancreatic β-cells, and it is known that islet-autoreactive T-cells from patients with type 1 diabetes are characterized by secretion of this cytokine (35–37), whereas cells from control individuals are characterized by secretion of interleukin-10 (35). Although it has been demonstrated that, in vitro, suppression of $CD4^+CD25^+$ T-cells seems to be independent of interleukin-10, secretion of this cytokine may represent a useful marker to identify effective regulation by cells in the $CD4^+CD25^+$ population.

In the present study, we found no significant abnormality in either the number of $CD4^+CD25^+$ T-cells or the level of CD25 expression in type 1 diabetes. This finding contrasts with an earlier published report suggesting that expression of CD25 is elevated in patients with type 1 diabetes (32) and a more recent study demonstrating a decrease in CD25 expression on CD4 T-cells (33) but agrees with observations published by Gessl and Waldhausl (38).

Factors that could account for the differences between these studies include the definition of CD25$^+$ cells, disease duration, and, most important, appropriate matching for age and HLA type in the control population.

When our phenotypic study was broadened to analyze the CD4$^+$CD25$^+$ population in greater depth, we found significant differences within distinct cellular subsets between patients with type 1 diabetes and control subjects in CD69 and CTLA-4 expression. The relationship of these changes to the defective suppression observed in functional assays remains to be established. We observed a significantly higher percentage of CD4$^+$CD25$^+$ and CD25^{hi} (but not CD25$^-$; data not shown) cells coexpressing CD69. CD69 is a marker of very recent T-cell activation, detectable on T-cells within 4 h of activation and declining rapidly after 24 h (39). In the context of an inflammatory autoimmune disease such as type 1 diabetes, therefore, it is possible that CD69$^+$ cells represent very recently activated effector cells, although it cannot be excluded that they represent activated regulatory cells. Levels of HLA-DR and CD45R0 on CD4$^+$CD25$^+$ T-cells, which more reliably reflect chronicity of immune activation, were similar in patients and control subjects.

Our study also showed that in patients with type 1 diabetes, there is an increase in the percentage of CD4$^+$CD25$^+$ and CD25^{hi} cells positive for CTLA-4 intracellular expression and that the amount of CTLA-4 per cell is also increased. CTLA-4 is a negative regulator of T-cell activation pathways (40,41) expressed de novo after T-cell activation and is considered to constitute part of a physiological postactivation negative feedback pathway in T-cell homeostasis (42). More recently, it has been identified as a constitutive marker of CD4$^+$CD25$^+$ regulatory T-cells in mice (43), although it remains to be seen whether such a specific role can be ascribed in humans. At present, therefore, it remains unclear as to whether the elevation in CTLA-4 expression that we observe can be interpreted as representing an increased, stable regulatory capacity of CD4$^+$CD25$^+$ T-cells in type 1 diabetes or as further corroboration that CD4$^+$CD25$^+$ cells in patients contain more recently activated T-cells, as for CD69. Future studies in this context with polychromatic flow cytometry should be able to establish, for example, whether CD69$^+$CD4$^+$25$^+$ cells coexpress CTLA-4. These studies on CTLA-4 expression in the CD25$^+$ population will also be important given the added impetus of recent studies implicating genetically determined lower mRNA levels of the soluble splice form of CTLA-4 in susceptibility to autoimmune diseases, including type 1 diabetes (44).

Because CD25 is expressed by both activated effector and regulatory T-cells and that, at any given moment, the CD4$^+$CD25$^+$ population therefore contains a balance of these two different cell types, careful interpretation of our observations is required. One reading of our data is that the numeric balance of regulatory versus effector T-cells within this critical cell population is subtly weighted in favor of inflammation over regulation in patients with type 1 diabetes. However, the vast majority of cells within the CD4$^+$CD25$^+$ population do not show signs of activation, and, given that we observed no significant difference in the proliferation or cytokines secreted by the CD4$^+$CD25$^+$ populations from patients or controls, it is unlikely that the differences observed in regulatory capacity are entirely due to a "dilution" of Treg with activated T-cells. It therefore is tempting to speculate that the skewed cytokine secretion seen in cocultures (but not single cultures) indicates that CD4$^+$CD25$^+$ regulatory cells from patients with type 1 diabetes may be deficient at regulating the type of proinflammatory responses believed to be involved in islet destruction. Alternatively, regulatory cells in patients with type 1 diabetes could be susceptible to clonal exhaustion in an attempt to control the immune activation that precedes diagnosis. Studies on individuals who are at risk for developing type 1 diabetes and those with long-established disease will be useful in establishing whether the phenotypic alterations identified here are stable and contribute to disease susceptibility.

These findings corroborate similar studies in the autoimmune conditions multiple sclerosis and autoimmune polyglandular syndrome II, which also report a defect in CD4$^+$CD25$^+$ cell function (22,23). Taken together, these studies may point to a central role for this regulatory cell population in the development of a wide range of autoimmune conditions.

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