

# No Alterations in the Frequency of FOXP3<sup>+</sup> Regulatory T-Cells in Type 1 Diabetes

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**Regulatory T-cells (Tregs) play a critical role in maintaining dominant peripheral tolerance. Previous characterizations of Tregs in type 1 diabetes have used antibodies against CD4 and  $\alpha$ -chain of the interleukin-2 receptor complex (CD25). This report extends those investigations by the addition of a more lineage-specific marker for Tregs, transcription factor forkhead box P3 (FOXP3), in subjects with type 1 diabetes, their first-degree relatives, and healthy control subjects. With inclusion of this marker, two predominant populations of CD4<sup>+</sup>CD25<sup>+</sup> T-cells were identified: CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> as well as CD4<sup>+</sup>FOXP3<sup>-</sup> T-cells expressing low levels of CD25 (CD4<sup>+</sup>CD25<sup>LOW</sup>FOXP3<sup>-</sup>). In all study groups, the frequency of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> cells was age independent, whereas CD4<sup>+</sup>CD25<sup>LOW</sup>FOXP3<sup>-</sup> cell frequencies strongly associated with age. In terms of additional markers for delineating cells of Treg lineage, FOXP3<sup>+</sup> cells were CD127<sup>-</sup> to CD127<sup>LOW</sup> whereas CD25<sup>+</sup> cells were less restricted in their expression of this marker, with CD127 expressed across a continuum of levels. Importantly, no differences were observed in the frequency of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> T-cells in individuals with or at varying degrees of risk for type 1 diabetes. These investigations suggest that altered peripheral blood frequencies of Tregs, as defined by the expression of FOXP3, are not specifically associated with type 1 diabetes and continue to highlight age as an important variable in analysis of immune regulation. *Diabetes* 56:604–612, 2007**

**A**lthough it is widely believed that type 1 diabetes results from the autoimmune destruction of insulin-producing pancreatic  $\beta$ -cells (1), a major void exists in our understanding of the defects in immune regulation that allow for this uncontrolled self-reactivity to occur. It has become increasingly

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APC, allophycocyanin; CBC, complete blood count; CD25,  $\alpha$ -chain of the interleukin-2 receptor complex; FITC, fluorescein isothiocyanate; FOXP3, transcription factor forkhead box P3; GADA, GAD autoantibody; IL, interleukin; MHC, major histocompatibility complex; PE, phycoerythrin; Tef, CD4<sup>+</sup>CD25<sup>-</sup> effector T-cell; Treg, CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> regulatory T-cell.

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accepted that autoreactive T-cells are present in healthy individuals, and their mere presence does not necessitate the development of autoimmune disease (2). The generation of autoimmune disease appears to require the presence of autoreactive T-cells concomitant with a breakdown in the mechanisms maintaining peripheral tolerance (3). Although multiple cells of the immune system have been identified as contributing to the pathogenesis of type 1 diabetes, a growing interest has recently been directed toward a population of so-called regulatory T-cells (Tregs), defined by their co-expression of CD4 and  $\alpha$ -chain of the interleukin (IL)-2 receptor complex (CD25) (4).

Multiple reports now exist, both in humans with type 1 diabetes and NOD mice, proposing a deficiency in either the frequency or function of Tregs (5,6). The first of these reports in humans indicated a reduced CD4<sup>+</sup>CD25<sup>+</sup> T-cell frequency in type 1 diabetic patients compared with healthy control subjects (7). Subsequent investigations uniformly failed to replicate these findings (8–10), with one study suggesting the discrepancy involving Treg frequency could, in part, be explained by the need for age-adjusted analysis (10).

However, a major limitation for previous studies of Tregs included their dependence on the measurement of CD25 (the  $\alpha$ -chain of the IL-2 receptor). Specifically, CD25 can also be expressed on T-cells of alternate lineages or activation states, allowing for a potential overestimation of the true Treg pool (11). Recently, reagents have been developed that are capable of identifying a lineage-specific transcription factor more intimately associated with Tregs, transcription factor forkhead box P3 (FOXP3). Although FOXP3 expression appears less restricted in humans compared with mice (12), its expression (whether naturally occurring from thymic origin or induced in the periphery after tolerogenic conditioning) does nonetheless suggest a demarcation of functional Tregs (13). In this report, we extend previous investigations addressing the important question of Treg frequencies in type 1 diabetes by analyzing FOXP3<sup>+</sup> T-cells in subjects with or at varying degrees of risk for the disease. Because age has previously been identified as a critical factor in studies of CD4<sup>+</sup>CD25<sup>+</sup> T-cells, this parameter was also included when considering its impact on FOXP3<sup>+</sup> T-cell development.

## RESEARCH DESIGN AND METHODS

Peripheral blood samples were obtained from 110 individuals, including those with recent-onset (<12 weeks from diagnosis) or established type 1 diabetes, their first-degree relatives, and healthy control subjects (patient demographics outlined in Table 1). Type 1 diabetes was diagnosed after physician examination according to American Diabetes Association criteria (14). Autoantibody

TABLE 1  
Patient demographics

Group	<i>n</i>	Sex (male/female)	Age (years)	GADA	Anti-IA2	Duration (years)
New-onset type 1 diabetes*	14	8/6	14.1 ± 4.1	12/14	9/14	0.11 ± 0.07
Median (range)			13.5 (8.4–23.5)			0.11 (0.01–0.24)
Established type 1 diabetes†	17	10/7	18.1 ± 10.2	15/17	12/17	7.0 ± 9.8
Median (range)			13.5 (8.4–23.5)			3.6 (1.0–38.2)
First-degree relatives	46	22/24	27.7 ± 17.5	4/46	5/46	
Median (range)			33.9 (3.5–62.6)			
Healthy control subjects	33	16/17	23.4 ± 10.1	0/32‡	0/32‡	
Median (range)			21.9 (6.3–44.9)			

Data are means ± SD and *n* unless otherwise indicated. \*New-onset type 1 diabetic patients were analyzed within 3 months of the date of diagnosis. †The established type 1 diabetic group contained all subjects with disease duration >3 months. ‡Serum was not available for one control subject.

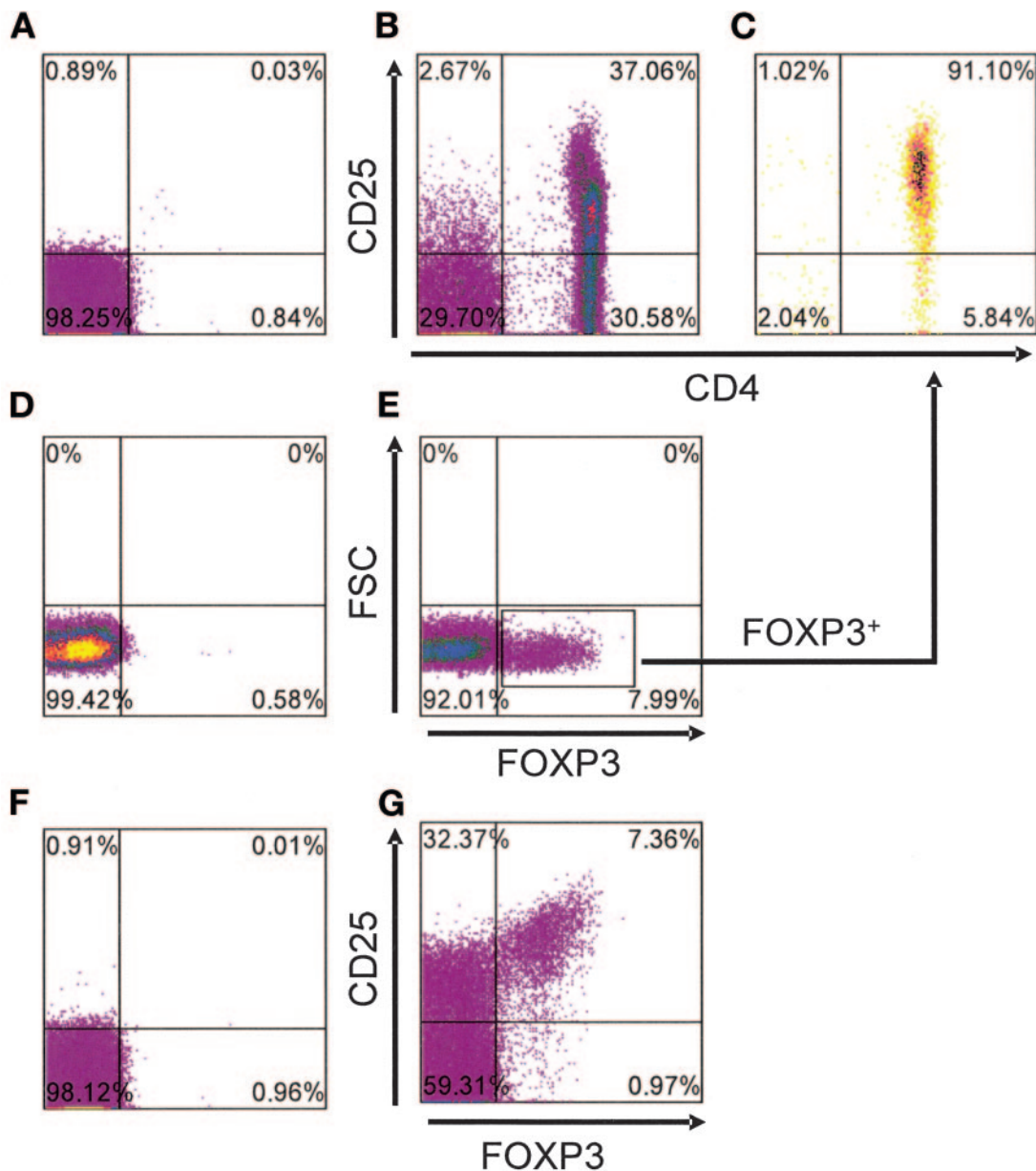
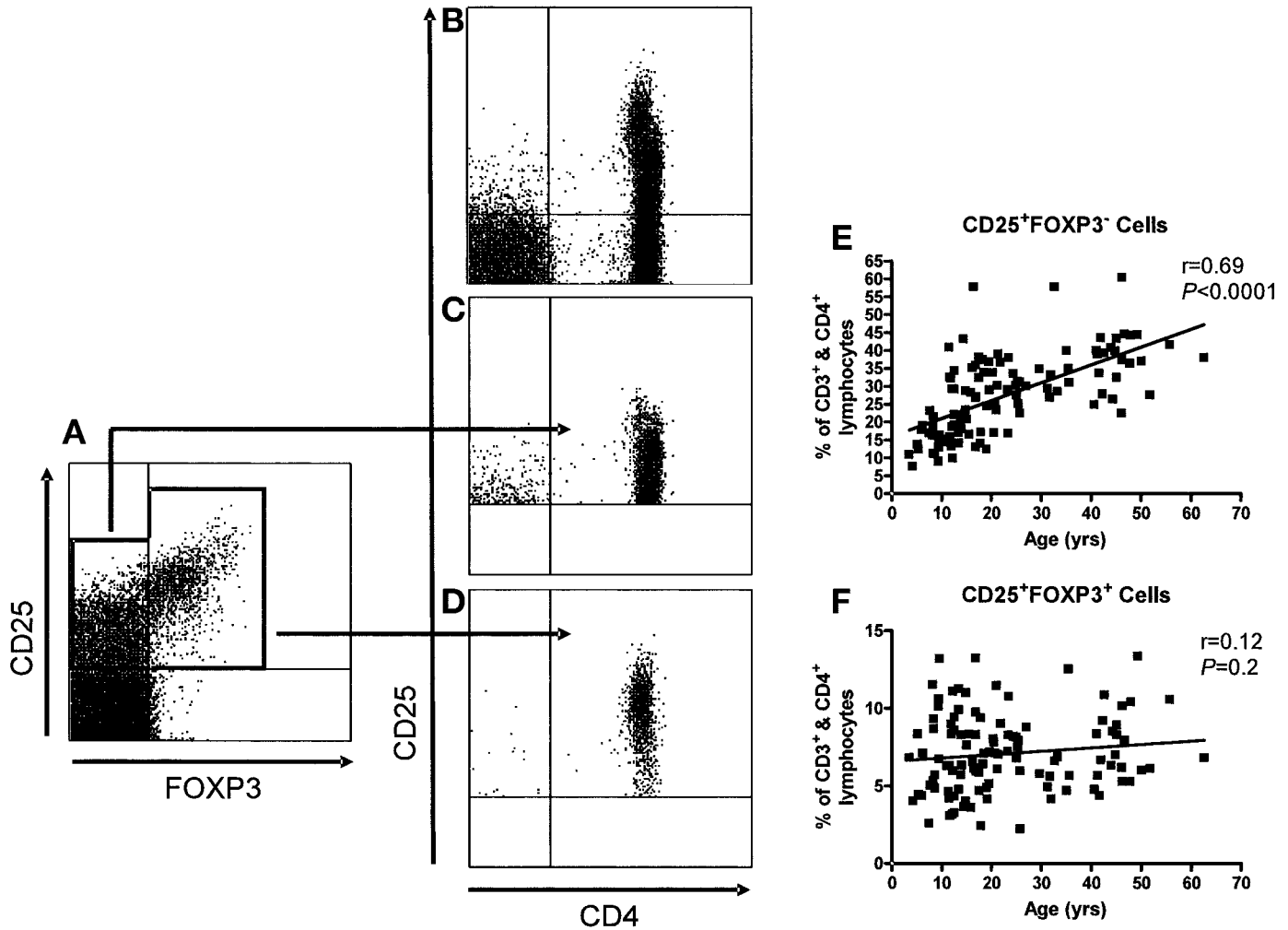


FIG. 1. Flow cytometric analysis of FOXP3 from fresh peripheral blood. Plots depict a representative healthy control sample gated on lymphocytes and CD3<sup>+</sup> T-cells showing isotype control (A) or CD4 and CD25 (B and C) staining, as well as isotype control (D) and intracellular (E) staining for FOXP3. In this sample, FOXP3<sup>+</sup> cells predominantly reside within the CD4<sup>+</sup>CD25<sup>+</sup> T-cell quadrant (91.1%; top right quadrant of plot C). In the 33 healthy control subjects used in this study, the FOXP3<sup>+</sup> population represented 1.05 ± 0.44 of all cells analyzed, or 4.31 ± 1.37 of CD3<sup>+</sup> lymphocytes. F: Isotype control staining. G: The correlation between CD25 expression and intracellular FOXP3 expression.



**FIG. 2.** Analysis of T-cell subpopulations using the markers CD25 and FOXP3 reveals an association between age and their frequency in peripheral blood. Representative plots (one healthy control shown) indicating expression of CD25 and FOXP3 (A) and CD4 and CD25 (B). C and D: The distribution of gated cell subsets (indicated boxed regions of A) are subsequently plotted to show their distribution within standard plots for CD4 and CD25. C: Analysis of gated CD25<sup>+</sup>FOXP3<sup>-</sup> cells (top left quadrant of plot A) indicates this cell subset co-expresses CD4 and low levels of CD25. D: Conversely, CD25<sup>+</sup>FOXP3<sup>+</sup> T-cells (top right quadrant of plot A) express CD4 and high levels of CD25. The frequency of CD25<sup>+</sup>FOXP3<sup>-</sup> T-cells from all subjects analyzed (*n* = 110) increases with age (E) whereas CD25<sup>+</sup>FOXP3<sup>+</sup> cells remain relatively stable (F).

assays for the type 1 diabetes-associated autoantibodies anti-GAD antibody (GADA) and anti-IA2 were performed on all study participants, using commercially available immunoassay kits (Kronus, Boise, ID). Healthy control criteria included lack of an autoimmune disorder or relative with type 1 diabetes. Institutional review board-approved informed consents and assents were obtained from all study participants.

**Flow cytometric analyses.** Whole blood was collected in K-EDTA S-Monovette tubes (Sarstedt, Newton, NC) and immediately subjected to cellular staining. Whole blood (100  $\mu$ l) was aliquoted (per tube) along with 20  $\mu$ l each appropriate test antibody or respective isotype control in four-color tests, fluorescein isothiocyanate (FITC) anti-CD3 (clone HIT3a), phycoerythrin (PE) anti-CD25 (M-A251), PE anti-CD127 (hIL-7R-M21), peridinin chlorophyll protein (PerCP) anti-CD4 (SK3), allophycocyanin (APC)-FOXP3 (clone PCH101), APC-CD45RA (HI100), APC-CD45RO (UCLH1), allophycocyanin (APC)-CD62L (DREG 56), and APC anti-HLA II DR (G46-6 [L243]). The following isotype control antibodies were used: FITC mouse IgG1 (X40), PerCP mouse IgG1 (MOPC-21), PE mouse IgG1 (MOPC-21), APC rat IgG2A (eBR2a), APC mouse IgG2b (27-35), and APC mouse IgG2a (G155-178). All antibodies for cytometric analyses were purchased from BD Biosciences (San Jose, CA), with the exception of FOXP3 (eBioscience, San Diego, CA). After surface staining for 30 min (4°C), erythrocytes were lysed and cells were fixed for 10 min at room temperature (BD FACS Lysing solution) followed by two washes with stain buffer containing 0.2% BSA. Surface-stained cells then underwent methods for intracellular FOXP3 staining using the anti-human FOXP3 staining kit according to manufacturer recommendations. Stained cells were then subjected to flow cytometric analysis using a BD FACSCalibur flow cytometer with  $1.5 \times 10^5$  cells acquired per test. FCS Express (version

2.200.0023; De Novo Software, Thornhill, ON, Canada) was used for analysis of cytometric data. To determine absolute cell counts, a small volume of blood was collected in potassium EDTA-containing tubes and analyzed for complete blood count (CBC) with differential when available on a Coulter Ac-T diff analyzer (Beckman Coulter, Fullerton, CA). Absolute counts were determined by multiplying the frequency of positive cells determined by cytometric analysis by the number of lymphocytes  $\times 10^9/\mu$ l as determined by CBC.

**Statistics and methods of analysis.** Statistical analysis used GraphPad Prism 4.00 software (GraphPad, San Diego, CA), with Mann-Whitney and Spearman's correlation analyses. Student's paired *t* test was used when assessing the stability of cellular phenotypes. *P* < 0.05 was deemed significant.

**RESULTS**

To determine the frequency of Tregs, lymphocytes and CD3<sup>+</sup> gated cells were analyzed for their expression of CD4, CD25, and FOXP3 (representative plots in Fig. 1A–G). The majority of FOXP3<sup>+</sup> T-cells co-expressed CD4 and CD25 (Fig. 1C), supporting specificity of the staining procedure. In addition, a strong correlation was observed between the levels of surface CD25 expression and the degree of intracellular FOXP3 observed (Fig. 1G).

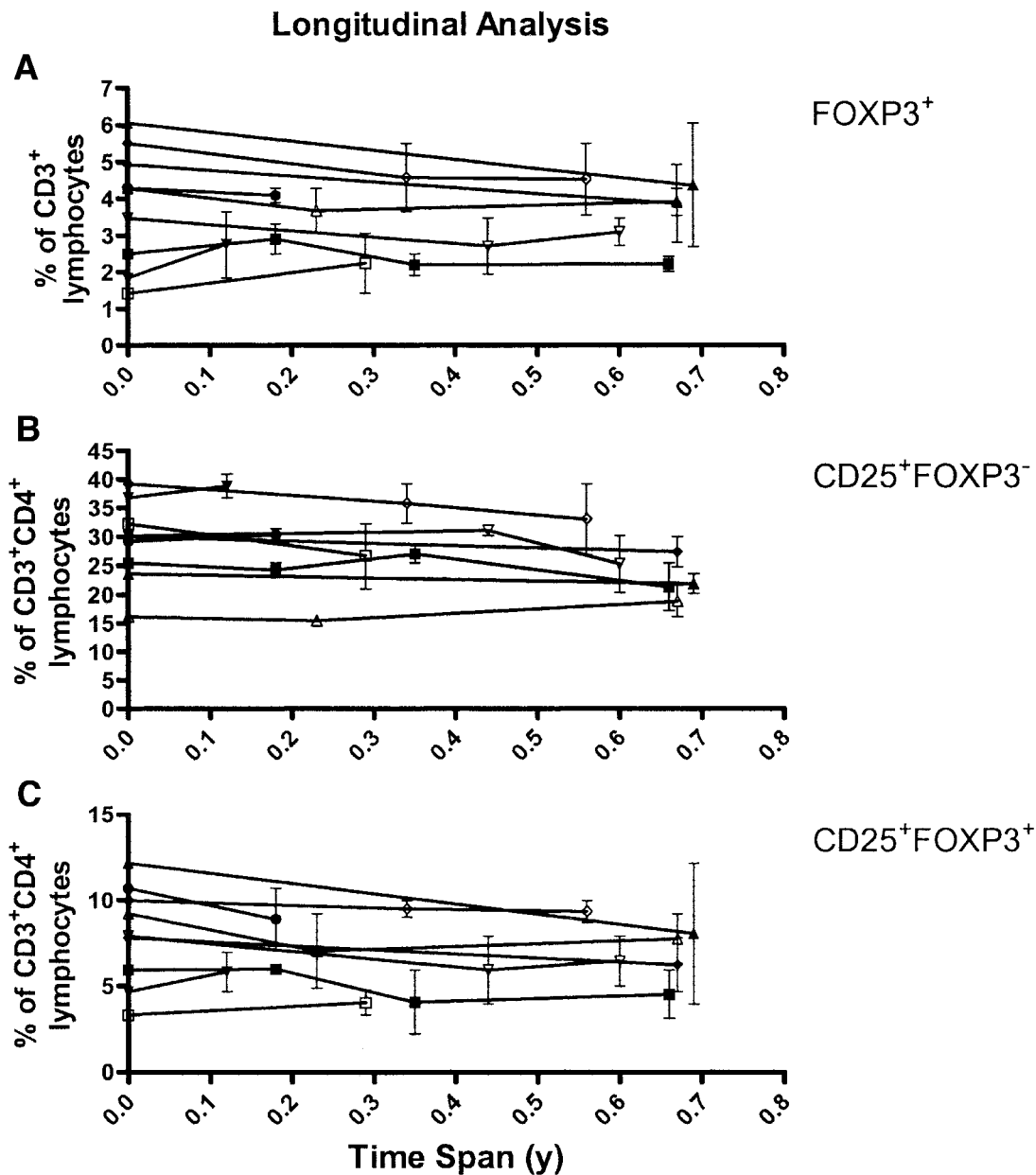
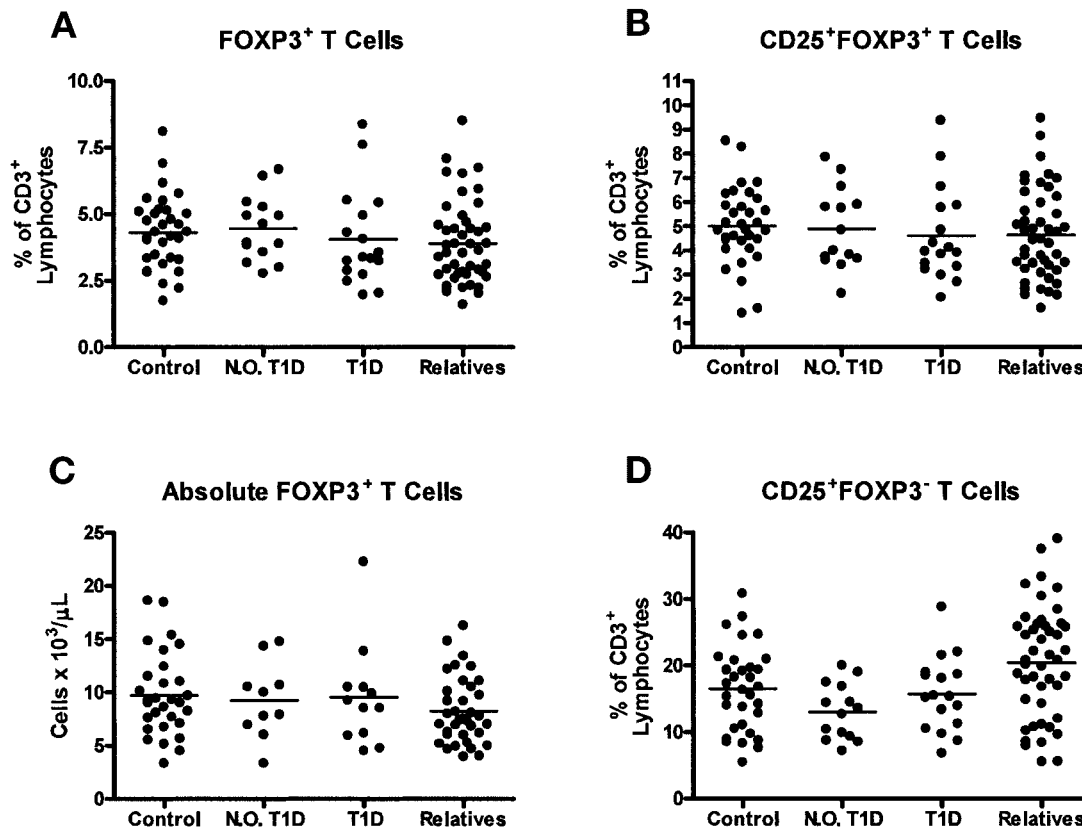


FIG. 3. The longitudinal stability in the frequency of FOXP3<sup>+</sup> (A), CD25<sup>+</sup>FOXP3<sup>-</sup> (B), and CD25<sup>+</sup>FOXP3<sup>+</sup> (C) T-cells over a period of time up to 8 months ( $n = 9$ ). Bars shown represent mean and SE from the initial analysis to each subsequent reanalysis.

**Age influences CD25<sup>+</sup>FOXP3<sup>-</sup> but not CD25<sup>+</sup>FOXP3<sup>+</sup> T-cell frequencies.** We previously reported the frequency of CD4<sup>+</sup> T-cells expressing low to intermediate levels of CD25 (CD4<sup>+</sup>CD25<sup>LOW</sup>; defined by CD25 staining above isotype control and <100 fluorescence intensity units) increases with age, and the frequency of this cell type is independent of type 1 diabetes (10). One question that was difficult to address previously was whether increases in CD25<sup>+</sup> T-cells were accompanied by increasing frequencies of FOXP3<sup>+</sup> T-cells. Using the previous commonly used markers of CD3, CD4, and CD25, and now including FOXP3 as a more specific indicator of Tregs (Fig. 2), we have now identified a significant correlation (using all study subjects,  $n = 110$ ) between the frequency of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>-</sup> T-cells (Fig. 2A, top left quadrant) and age ( $r = 0.69$ ,  $P < 0.0001$ ). This population corresponds to the CD4<sup>+</sup>CD25<sup>LOW</sup> T-cell population previously noted to increase with subject age (Fig. 2C). In contrast,

the frequency of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> T-cells (Fig. 2A, top right quadrant) appears independent of subject age (Fig. 2F;  $r = 0.12$ ,  $P = 0.2$ ). As previously observed with the frequency of CD4<sup>+</sup>CD25<sup>+</sup> T-cells, the influence of age was independent of disease state (data not shown).

**The frequencies of FOXP3<sup>+</sup>, CD25<sup>+</sup>FOXP3<sup>-</sup>, and CD25<sup>+</sup>FOXP3<sup>+</sup> T-cells are stable over short periods of time.** To establish the stability of these cellular phenotypes and determine the reproducibility of our testing procedures, we performed a longitudinal analysis on a subset of individuals participating in this study. No significant variations were observed in a period up to 8 months, because the frequency of CD25<sup>+</sup> and FOXP3<sup>+</sup> cells (alone or in combination) remained relatively stable (Fig. 3, A–C, Student's paired  $t$  test, NS). These findings, taken together with the relative stability of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>-</sup> T-cells over time (Fig. 3B; mean coefficient of variation  $\pm$  SD  $16.6 \pm 7.9\%$ ), suggest that long-term alterations in T-cell



**FIG. 4.** Frequencies of FOXP3<sup>+</sup> Tregs do not differ in subjects with type 1 diabetes. For all populations analyzed, the frequency of total FOXP3<sup>+</sup> cells (A), CD25<sup>+</sup>FOXP3<sup>+</sup> T-cells (B), and the absolute number of FOXP3<sup>+</sup> cells (C), no significant differences were identified as a function of the study groups (all NS). D: The frequency of CD25<sup>+</sup>FOXP3<sup>-</sup> T-cells was elevated in first-degree relatives compared with the other three patient groups: relatives of type 1 diabetic patients (20.5% ± 8.4) versus control subjects (16.5 ± 6.3, *P* = 0.02), new onset type 1 diabetic subjects (13.0 ± 4.2; *P* = 0.002), and patients with established type 1 diabetes (15.7 ± 6.6; *P* = 0.04).

phenotype rather than a brief increase in acutely activated cells underlie the observed age-associated increases in the frequency of CD4<sup>+</sup>CD25<sup>+</sup> cells and CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>-</sup> T-cells.

**The frequency and absolute number of FOXP3<sup>+</sup> T-cells does not differ in normal healthy control subjects and patients with type 1 diabetes.** Because lymphopenia has been reported in association with type 1 diabetes of NOD mice (15), we assessed both the frequency and absolute number of FOXP3<sup>+</sup> T-cells in our study populations. The frequency of Tregs defined by the frequency of total FOXP3<sup>+</sup> T-cells, CD25<sup>+</sup>FOXP3<sup>+</sup> T-cells, or the absolute number of FOXP3<sup>+</sup> T-cells did not significantly differ as a function of type 1 diabetes state or disease risk (Fig. 4A–C). Significant differences were, however, observed in the frequency of CD25<sup>+</sup>FOXP3<sup>-</sup> T-cells for first-degree relatives (Fig. 4D; 20.5 ± 8.4%) compared with control subjects, new-onset type 1 diabetes, and established type 1 diabetic subjects (16.5 ± 6.3%, *P* = 0.02; 13.02 ± 4.2%, *P* = 0.002; and 15.7 ± 5.6%, *P* = 0.04, respectively). No other groups were significantly different in CD25<sup>+</sup>FOXP3<sup>-</sup> T-cell frequency (all NS). This facet was most likely related to the influence of subject age on CD25<sup>+</sup>FOXP3<sup>-</sup> T-cell frequency (Fig. 2E), as opposed to disease state. This patient population contained a large number of parents of affected offspring (see Table 1) and thus contained a larger proportion of older subjects than the other three subject groups. Likewise, neither the duration of type 1 diabetes nor the autoantibody titer associated with the frequency of CD25<sup>+</sup>FOXP3<sup>+</sup>

T-cells (NS). For reasons of limited subject availability, we elected to study a random sampling of first-degree relatives of type 1 diabetic patients independent of their autoantibody status in order to identify potential genetic associations between Treg frequency and disease risk. Although none were identified, future efforts could benefit from analysis of individuals at high (i.e., autoantibody-positive) versus low (i.e., autoantibody-negative) risk for the disease.

**CD4<sup>+</sup>CD25<sup>+</sup> T-cells predominantly express the memory T-cell marker CD45RO.** In an effort to understand what factors influence the development of CD25<sup>+</sup>FOXP3<sup>-</sup> T-cells, we sought to monitor additional markers associated with T-cell developmental state and antigen exposure and lymphocyte homing capacity. Therefore, markers of naive (CD45RA) and memory (CD45RO) T-cells and the homing marker CD62L were analyzed in association with CD4 and CD25 expression levels (Fig. 5). Analysis of these markers indicated that CD4<sup>+</sup>CD25<sup>+</sup>-gated T-cells (Fig. 5A, *top right quadrant*) predominantly express the memory T-cell marker CD45RO (Fig. 5B; 82.3 ± 9.8% for CD45RO for the control group vs. 40.4 ± 14.8% for CD45RA). Conversely, CD4<sup>+</sup>CD25<sup>-</sup>-gated T-cells (Fig. 5A, *bottom right quadrant*) predominantly express the naive T-cell marker CD45RA (Fig. 5C; 76.3 ± 11.0% for CD45RA vs. 41.3 ± 17.6% for CD45RO). Although some significant differences were observed for the subject groups in CD45RA and CD45RO expression levels (Fig. 5B and C), once again, these results most likely represent differences in cohort age as opposed to disease-associated differences.

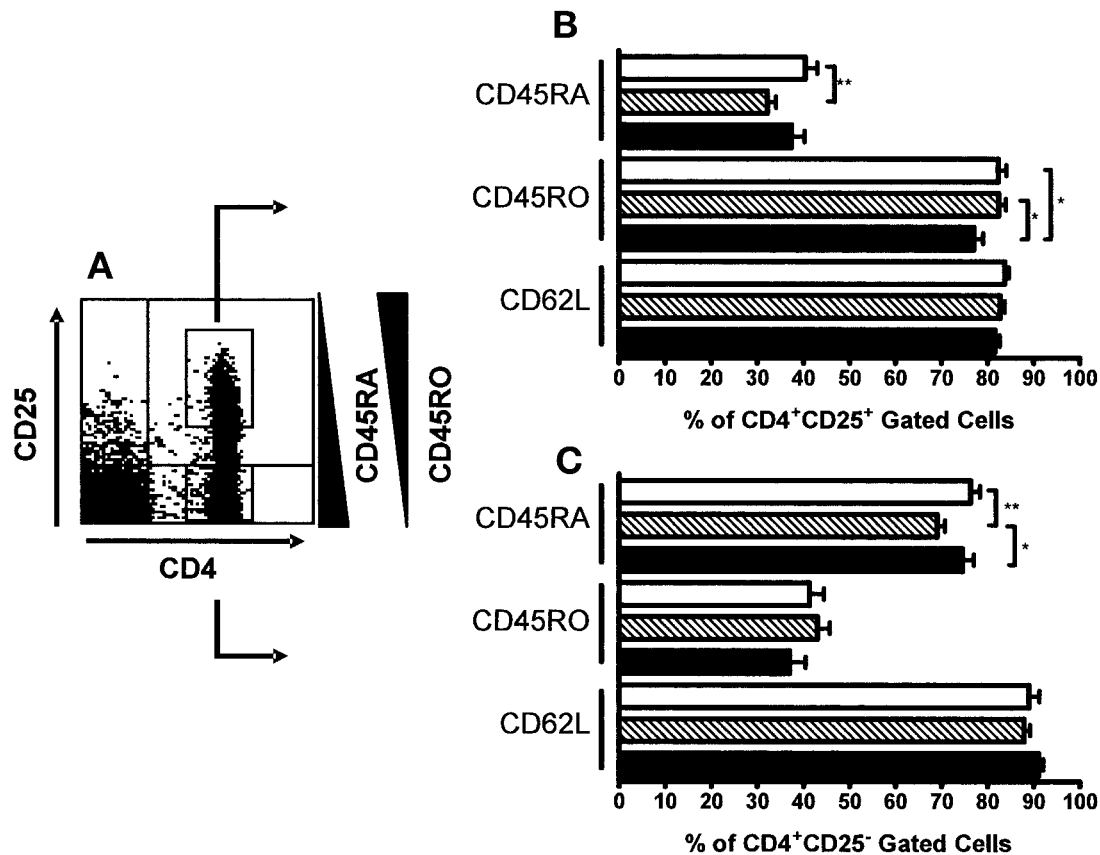


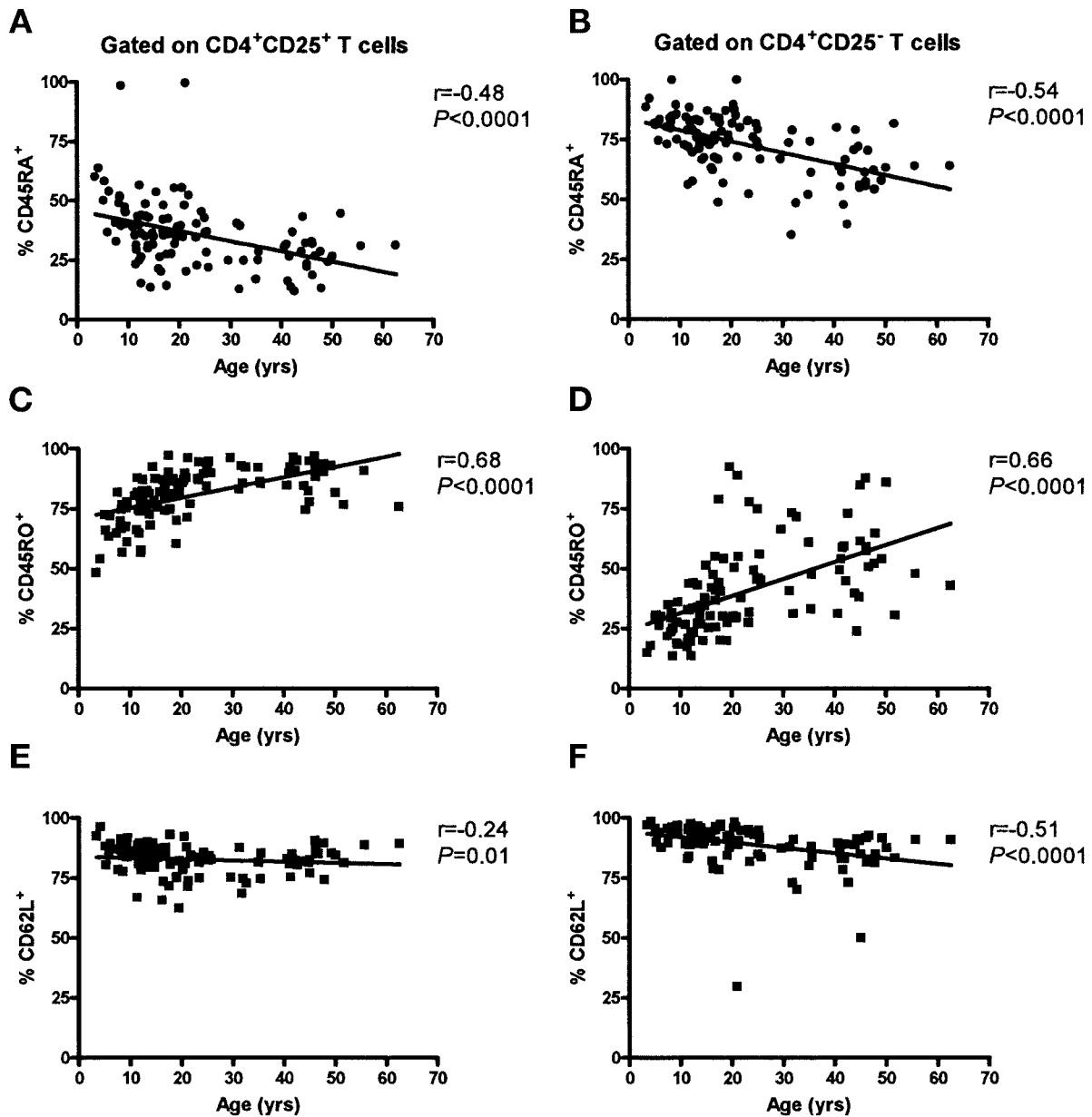
FIG. 5. CD4<sup>+</sup>CD25<sup>+</sup> T-cells and CD4<sup>+</sup>CD25<sup>-</sup> T-cells differ in their composition of memory and naive T-cells. *A*: A representative plot shows the analysis of CD4 and CD25 expression, and gates (indicated with boxed regions) for the analysis of phenotypic markers of memory (CD45RO) and naive (CD45RA) T-cells, along with the lymphocyte homing marker CD62L on control subjects (open bars), first-degree relatives (hatched bars), and patients with type 1 diabetes (closed bars). Analysis of CD45RA and CD45RO on CD4<sup>+</sup>CD25<sup>+</sup> T-cells (top right boxed region of *A*) or CD4<sup>+</sup>CD25<sup>-</sup> T-cells (bottom right boxed region) indicate that CD4<sup>+</sup>CD25<sup>+</sup> T-cells predominantly express the memory T-cell marker CD45RO. In contrast, CD4<sup>+</sup>CD25<sup>-</sup> T-cells predominantly express the naive T-cell marker CD45RA (depicted in sliding scale to the right of *A*). Situations identifying significance between subject groups are indicated. \* $P < 0.05$ , \*\* $P < 0.01$ .

**Age leads to long-term alterations in T-cell phenotype.** A common caution when interpreting studies involving patients with type 1 diabetes is whether age influences the factor(s) under assessment. To determine the influence of age on the frequency of CD45RA<sup>+</sup>, CD45RO<sup>+</sup>, and CD62L<sup>+</sup> T-cells, we correlated the frequency of these markers, determined by flow cytometry at the time of testing versus subject age. These analyses indicated long-term alterations in T-cell immune phenotype associated with subject age (Fig. 6;  $n = 110$ , all subjects analyzed). Specifically, whether assessed on CD4<sup>+</sup>CD25<sup>+</sup>-gated T-cells (Fig. 5A, top right quadrant gate) or CD4<sup>+</sup>CD25<sup>-</sup> T-cells (Fig. 5A, bottom right quadrant gate), the general trend indicates a reduction in CD45RA expression with age (Fig. 6A and B;  $r = -0.48$ ,  $P < 0.0001$ , and  $r = -0.54$ ,  $P < 0.0001$ , respectively). Assessment of the memory T-cell marker CD45RO exhibited the inverse relationship, with the frequency of CD45RO<sup>+</sup> T-cells increasing with subject age on both CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T-cells (Fig. 6C and D;  $r = 0.68$ ,  $P < 0.0001$ , and  $r = 0.66$ ,  $P < 0.0001$ , respectively). In terms of the lymphocyte homing marker CD62L, which has been reported to influence Treg tracking and function (16), a negative correlation was again observed with subject age (Fig. 6E and F;  $r = -0.24$ ,  $P = 0.01$ , and  $r = -0.51$ ,  $P < 0.0001$  for CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T-cells, respectively).

**Extended analysis of markers characterizing the lineage of Tregs.** To lend support to our abilities to provide staining procedures identifying Tregs, and to verify recent efforts defining markers of Tregs, the cellular distribution of CD127 (the  $\alpha$ -chain of the IL-7 receptor) was analyzed along with the markers CD25, FOXP3, and the major histocompatibility complex (MHC) class II molecule DR on CD3<sup>+</sup> lymphocytes (Fig. 7D–F). This analysis was consistent with the findings of Liu et al. (17) and Seddiki et al. (18), in that FOXP3<sup>+</sup> cells were CD127<sup>-</sup> to CD127<sup>LOW</sup>, whereas CD25<sup>+</sup> cells were less restricted in their expression of this marker, with CD127 expressed across a continuum of levels. On the other hand, MHC class II DR expression appears across the continuum of cells expressing CD127, indicating that class II DR expression is not unique to Tregs.

## DISCUSSION

The robust interest in Tregs over the past decade likely results from the prevailing paradigm that autoimmunity results from an eventual imbalance in CD4<sup>+</sup> T-cells, favoring pathogenic effector T-cell phenotypes over a regulated response (19). To these studies, we add our findings that there does not appear to be a simple deficiency in the peripheral blood frequency or absolute number of FOXP3<sup>+</sup> T-cells in patients with type 1 diabetes versus



**FIG. 6.** Subject age influences the frequency of cells expressing the phenotypic markers CD45RA, CD45RO, and CD62L. Expression of the naïve T-cell marker CD45RA decreases with age on both CD4<sup>+</sup>CD25<sup>+</sup> T-cells (A) and CD4<sup>+</sup>CD25<sup>-</sup> T-cells (B) ( $n = 110$ , all subjects analyzed). The frequency of the memory T-cell marker CD45RO increases with age on CD4<sup>+</sup>CD25<sup>+</sup> T-cells (C) and CD4<sup>+</sup>CD25<sup>-</sup> T-cells (D). A negative correlation was observed between subject age and the T-cell homing marker CD62L on CD4<sup>+</sup>CD25<sup>+</sup> T-cells (E) and CD4<sup>+</sup>CD25<sup>-</sup> T-cells (F).

healthy control subjects. Inclusion of the Treg lineage marker FOXP3 did, however, identify important age-related influences over CD4<sup>+</sup> T-cells, which likely confounded previous studies of Tregs that relied primarily on the markers CD4 and CD25. As part of these analyses, our studies identify several key phenotypic properties of CD4<sup>+</sup> T-cells influenced by subject age. Specifically, we note that CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>-</sup> but not CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> T-cells appear to increase with age. Furthermore, this increase in CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>-</sup> T-cells is accompanied by a concomitant increase in CD45RO<sup>+</sup> memory T-cells and a reduction in CD45RA<sup>+</sup> and CD62L<sup>+</sup> T-cells. We speculate that this represents a combination of reduced thymic output with age as well as an accumulation of antigen exposures over time. In line with these findings, a recent report tracking T-cell populations in vivo indicated relatively stable frequencies of CD4<sup>+</sup>CD25<sup>HI</sup> cells despite their rapid turnover

rate (20). From this observation, Vukmanovic-Stejic et al. (20) suggest that Treg populations are continuously replenished by pre-existing CD4<sup>+</sup>CD25<sup>HI</sup> cells themselves or replenished by conversion of CD4<sup>+</sup>CD25<sup>-</sup> cells into the CD4<sup>+</sup>CD25<sup>HI</sup> T-cell pool.

Despite our finding that the frequency of Tregs, defined by expression of the markers CD25 and FOXP3, does not appear to be deficient in type 1 diabetes, several caveats exist before we can definitely state that no differences in Tregs are associated with type 1 diabetes. First, these studies were conducted in peripheral blood and may not reflect the T-cell repertoire present at the site of inflammation (i.e., the pancreas and pancreatic draining lymph node). Furthermore, although the markers of CD25 and FOXP3 represent appropriate markers identifying a lineage of Tregs, additional phenotypic properties responsi-

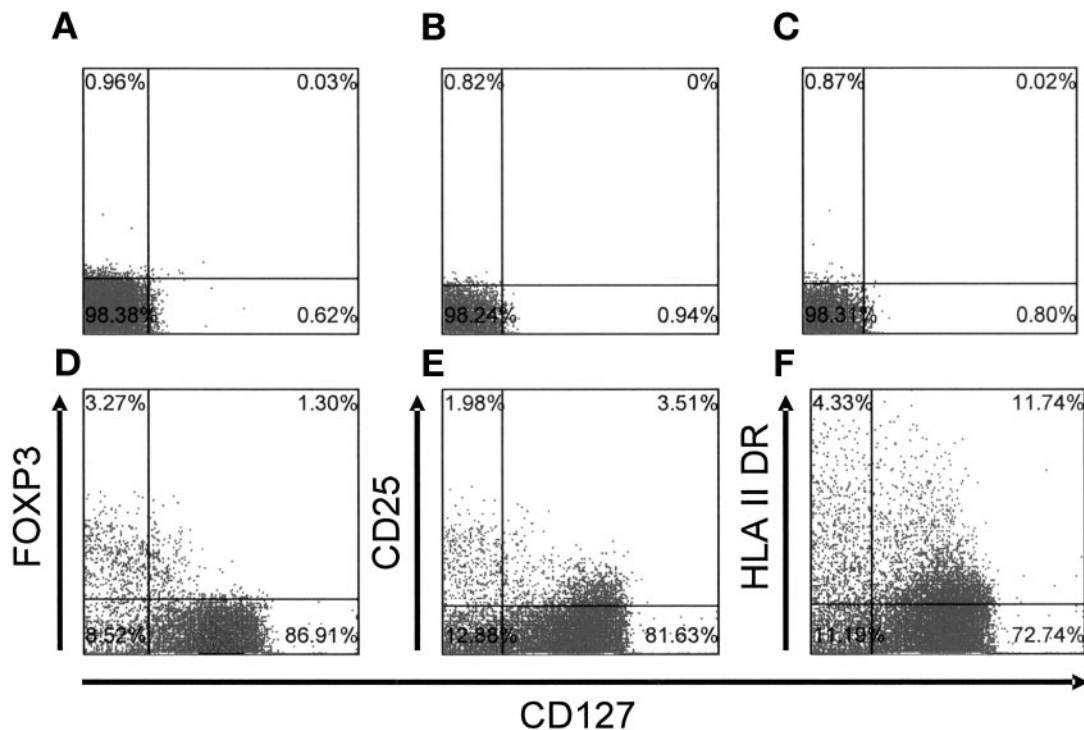


FIG. 7. Extended phenotypic analysis of markers that identify functional subsets of Tregs and Teffs. Shown are representative plots from a normal healthy control subject showing isotype control staining (A–C) or co-expression of the markers CD127 (IL-7 receptor  $\alpha$ -chain) and FOXP3 (D), CD25 (IL-2 receptor  $\alpha$ -chain) (E), or the MHC class II molecule HLA-DR (F).

ble for the functional capacity of Tregs may be deficient in the case of type 1 diabetes.

It has been reported that aging is associated with a progressive decline in immune function and speculated that increasing CD4<sup>+</sup>CD25<sup>+</sup> T-cells may contribute to these defects (21). In terms of alterations in the functional capacity of Tregs with age, Tsaknaridis et al. (22) report declining Treg function with age in humans. However, a report by Sakaguchi and colleagues (23) reported equivalent Treg function in aged mice but noted a relative hyporesponsiveness of CD4<sup>+</sup>CD25<sup>-</sup> T-cells. These events may not be mutually exclusive. One can envision a situation in which defects in Treg function may also be linked to improper activation of CD4<sup>+</sup>CD25<sup>-</sup> effector T-cells (Teffs) (24). Teffs represent the primary source of IL-2, and Tregs require IL-2 signaling for proper development and function (25). In the case of type 1 diabetes, we and others have previously reported a hyporesponsiveness of T-cells in NOD mice and type 1 diabetes, characterized by reduced T-cell proliferation and production of IL-2 (10,26–28). Genetic association studies have identified type 1 diabetes susceptibility loci related to *IL-2* in NOD mice and in the gene encoding the IL-2R $\alpha$  (CD25) in humans (29–31). Furthermore, downstream elements associated with the IL-2 signaling pathway, including that of STAT5, also appear to be defective in type 1 diabetes (32–35). Clearly, more research in this area is required.

In line with the notion of a need for additional lineage-specific identifiers, two additional markers, CD127 and HLA class II DR, have recently been reported to identify distinct functional populations of Tregs. The surface marker CD127 (IL-7 receptor  $\alpha$ -chain) appears to be expressed at the highest levels on effector T-cells, whereas functional Tregs are CD127<sup>LOW</sup> or negative (17,18). HLA II-DR has also been reported to be preferentially ex-

pressed on functional Tregs with the greatest suppressive capacity in vitro (36). Our own phenotypic analysis of these markers does in fact identify a population of CD127<sup>HI</sup>CD25<sup>LOW</sup> T-cells that might influence suppression when selecting solely based on CD25 expression (Fig. 7E, bottom right quadrant) and should be subject to further investigation. In addition, the influence of a CD25<sup>LOW</sup> population would be more apparent in people of older age (Fig. 2E). These recent advances in the capacity to segregate functional Tregs from Teffs should help to address the current limitations on isolation and analysis of functional human Tregs in studies of type 1 diabetes.

Although the exact mechanisms of suppression used by Tregs remains subject to debate, it appears clear that Tregs require IL-2 signaling and FOXP3 expression for normal development and function (37–39). Previous studies of Tregs in type 1 diabetes (8–10) lack agreement with respect to the question of whether functional differences exist between type 1 diabetic patients and healthy individuals in the capacity of their CD4<sup>+</sup>CD25<sup>+</sup> T-cells to suppress proliferation of autologous Teff cell proliferation after polyclonal stimulation. One key aspect in need of further investigation is to correlate the degree of suppression with the percentage of FOXP3<sup>+</sup> cells isolated for use in suppression assays. In a partial yet indirect step toward this question, we have observed that the degree of FOXP3<sup>+</sup> T-cells detected in peripheral blood does not dictate the degree of in vitro suppression (data not shown). Like efforts should also be addressed by testing for the degree of suppression in association with the aforementioned makers of HLA-DR and CD127.

In summary, these studies provide substantive evidence that altered Treg frequencies are not specifically associated with type 1 diabetes. This finding should not be viewed as eliminating the potential mechanistic impact



these cells might have on the pathogenesis of type 1 diabetes. Instead, we believe future efforts should be directed toward the identification of factors capable of influencing the functional activity of Tregs and Teffs in a setting of type 1 diabetes (e.g., CTLA-4 or TGF- $\beta$ ), to elucidate how these cells act at the site of pancreatic inflammation, and to determine how treatments aimed at augmenting the frequency and/or functionality of Tregs (40) modulate the pathogenesis of type 1 diabetes.

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