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Increased expression of regulatory T cell-associated markers in recent-onset diabetic children

Mikael Pihl[1*], Mikael Chéramy[1], Jenny Mjösberg[2], Johnny Ludvigsson[1], Rosaura Casas[1]

[1]Division of Pediatrics and Diabetes Research Center, Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden; [*]Corresponding Author: mikael.pihl@liu.se
[2]Division of Clinical Immunology, Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden.

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ABSTRACT

$CD4^+CD25^{hi}$ T cells are thought to be crucial for the maintenance of immunological tolerance to self antigens. In this study, we investigated the frequencies of these cells in the early stage of type 1 diabetes, as well as in a setting of possible pre-diabetic autoimmunity. Hence, the expression of FOXP3, CTLA-4, and CD27 in $CD4^+$ $CD25^{hi}$ T cells was analyzed using flow cytometry in 14 patients with recent onset type 1 diabetes, in 9 at-risk individuals, and 9 healthy individuals with no known risk for type 1 diabetes. Our results show there were no differences in the frequency of $CD4^+CD25^{hi}$ cells between groups. However, compared to controls, recent-onset type 1 diabetic patients had higher expression of FOXP3, CTLA-4, and CD27 in $CD4^+$ $CD25^{hi}$ cells from peripheral blood. The median fluorescence intensity of FOXP3 was significantly higher in $CD4^+CD25^{hi}$ cells from patients with type 1 diabetes than from controls. Furthermore, a positive correlation between the frequency of $FOXP3^+$ cells and the median fluorescence intensity of FOXP3 was observed among patients with type 1 diabetes. These data suggest that the frequency of $CD4^+CD25^{hi}$ $FOXP3^+$ T cells in the periphery is not decreased but rather increased at onset of type 1 diabetes. Thus, functional deficiencies rather than reduced numbers of $CD4^+CD25^{hi}$ cells could contribute to the development of type 1 diabetes.

Keywords: Regulatory T cells; Type 1 Diabetes; Autoantibodies

1. INTRODUCTION

There is little doubt today that a regulatory subset of T cells necessary for peripheral tolerance exists, and that absence of these cells causes autoimmunity in a variety of experimental settings [1]. Naturally occurring regulatory T cells (Treg) are $CD4^+$ T cells which constitutively express the Interleukin (IL)-2 receptor α chain (CD25), the transcription factor FOXP3 and Cytotoxic T lymphocyte associated antigen 4 (CTLA-4, CD152), and are normally produced in the thymus [2]. Expression of the transcription factor FOXP3 is vital to the development and function of Treg [3-4] and has therefore been used to delineate regulatory T cell populations. However, activation of human T cells induces transient expression of FOXP3 in non-regulatory T cells without conferring a regulatory phenotype to the affected cells [5-9]. Some report this phenomenon to be a consequence of T cell receptor (TCR) stimulation [7], while others have postulated that TCR stimulation does not produce FOXP3 expression at either gene or protein level [10]. Tran *et al* recently found that high levels of FOXP3 could be induced in $CD4^+CD25^-$ T cells by TCR stimulation, only in the presence of transforming growth factor (TGF)-β. FOXP3 expression induced in this way was maintained for weeks in the presence of IL-2 [9]. CTLA-4 is a co-stimulatory molecule with potent suppressive function constitutively expressed on Treg but also on activated effector T cells [11,12]. In addition to its negative co-stimulatory effect, CTLA-4 up-regulates Indolamine 2,3-dioxygenase in dendritic cells [13], resulting in catabolism of tryptophan to kynurine, which has potent local immunosuppressive effects [14]. Expression of the transmembrane costimulatory receptor CD27 was used to define Treg in inflamed synovia in conjunction with CD25 [15]. Other evidence suggests that Treg expressing CD27 are more suppressive than CD27-negative counterparts [16].

Defects in the function of Tregs have been hypothesized to be involved in the pathogenesis of numerous autoimmune diseases, including type 1 diabetes [17]. In mice, islet antigen specific FOXP3 transduced T cells were able to suppress recent onset type 1 diabetes [18].

However, studies of human Tregs in type 1 diabetes have produced contradictory results. Kukreja *et al.* reported reduced numbers of $CD4^+CD25^{hi}$ cells [19], while Lindley *et al.* and Brusko *et al.* could find no change in $CD4^+CD25^{hi}$ frequency between healthy and diabetic individuals [20,21]. A meta-analysis comparing these findings suggested that the lack of consensus between studies is a consequence of differently matched diabetic and control groups. The authors advocated further studies of phenotypical markers associated with Treg, including FOXP3 [22].

A conundrum of type 1 diabetes is the lack of good indicators before disease onset, since it is of interest to study individuals at risk of developing the disease. Thus, we are interested in describing T cell populations in at-risk individuals, with possible autoimmune activity prior to diagnosis of type 1 diabetes. Most people progressing to type 1 diabetes produce autoantibodies to one or more islet autoantigens, most commonly against insulin, glutamic acid decarboxylase (GAD), and the tyrosine phosphatase like protein IA-2 [23]. Therefore, a group of healthy children with autoantibodies was selected as a risk population in this study. To our knowledge, no previous studies have examined the expression of FOXP3 and CTLA-4 in autoantibody-positive children, nor in children with recent onset T1D with as closely age-matched healthy controls as in the present study. This is important as the immune system changes with age from childhood, through puberty until adulthood, and as the autoimmune process leading to type 1 diabetes is more rapid and aggressive in children than in higher age groups.

We hypothesized that patients with type 1 diabetes as well as healthy individuals expressing high levels of autoantibodies against islet antigens would have a decreased proportion of Treg compared to healthy individuals without autoantibodies. Therefore we analyzed the expression of proteins related to Treg function to determine their frequency in type 1 diabetes patients, healthy subjects and healthy subjects at risk of type 1 diabetes. Here, we report an increased expression of FOXP3 and CTLA-4 on $CD4^+CD25^+$ cells in patients with type 1 diabetes and subjects at risk of type 1 diabetes.

2. MATERIALS AND METHODS

2.1. Study Population

All the participants and their parents received written information on the study, and consent was obtained according the Declaration of Helsinki. Via proxy consent was obtained for participating children, and presumed consent was obtained from parents upon completing and submitting questionnaries on entering the study.

The study was approved by the Regional Ethics Committee for Human Research "Regionala etikprövningsnämnden i Linköping, Avdelningen för prövning av medicinsk forskning", Linköping University Hospital, Sweden (Dnr 03-092).

Venous blood samples were collected from 14 patients with recent onset type 1 diabetes with a median age of 10 years (range 3 - 17 years, SEM 1,123) and a diabetes duration of three months. Nine 8-year-old healthy children with autoantibodies against Insulin, GAD65, or IA-2 in the 95th percentile or higher at 5 and/or 2.5 years of age were included as a risk population. Some, but not all, at-risk children exhibited an antibody response to more than one autoantigen (**Table 1**). And some, but not all, were positive for autoantibodies on several occasions a few years apart prior to sampling. Nine healthy children, 8 years old, with no known type 1 diabetes-associated HLA-genotypes, allergy, or autoimmune disease were included as reference. All at-risk and control subjects were participating in the ABIS study (All Babies in Southeast Sweden).

Table 1. Presence of autoantibodies in at-risk children.

At-risk individuals	Aab at 1 year of age			Aab at 2.5 years of age			Aab at 5 years of age			Genotype where available				
	IAA	GAD	IA-2	IAA	GAD	IA-2	IAA	GAD	IA-2	DQB1	DQB2	DQA1	DQA2	DRB
3932							98th			02	0501	05		
8107							95th							
8772								95th		0302	0602			0401
11,124								98th		0302	0602			0405
14,903							98th			02	0301	0201	05	
17,450		95th	99th		99th					0602				
18,034					90th		95th	90th		0301	0302	03	05	0401
19,032		99th				99th		98th	>5.5 RA U	0301	0302	03	05	0401
23,735								98th	>10 RA U	02	0302	03	05	0401

2.2. Flow Cytometry

Peripheral Blood Mononuclear Cells (PBMC) were isolated from blood samples by Ficoll (Pharmacia Biotech, Sollentuna, Sweden) gradient centrifugation within 24 h of collection. Cells at interface were harvested and washed three times in RPMI 1640 (Gibco, Auckland, New Zealand).

PBMC were washed with Phosphate Buffered Saline (PBS)(Medicago AB, Uppsala, Sweden) containing 0.1% Bovine Serum Albumin (BSA)(Sigma-Aldrich, St Louis, MO, USA). Approximately 2×10^6 cells were used in each FACS tube for staining of Treg-like cells. In addition, ~10^5 cells were used to set compensation and as isotype and unstained controls. Cells were aliquoted (200 µl per tube) along with appropriate antibodies, peridin chlorophyll (PerCP) anti-CD4 (BD Biosciences, San Jose, CA, USA, clone SK3), fluorescein isothiocyanate (FITC) anti-CD27 (BD Pharmingen, M-T271), and Allophycocyanin (APC) anti-CD25 (BD Biosciences, 2A3). Cells were stained for 30 minutes at 4˚C, washed with PBS 0.1%BSA, and then fixed and permeabilized with the eBioscience intracellular staining kit (eBioscience, San Diego, CA, USA). Finally, cells were stained intracellularly with FITC- or Phycoerythrin (PE)- conjugated anti-FOXP3 (eBioscience, PCH101) and FITC- or PE-conjugated anti-CTLA-4 (R&D Systems, Minneapolis, MN, USA, clone 48815 and BD Biosciences, BNI3, respectively) as above. FITC- conjugated CTLA-4 antibody was combined with PE-con- jugated FOXP3 antibody, and FITC-conjugated FOXP3 with PE-conjugated CTLA-4. Though cells were stained with FITC- and PE-conjugated anti-FOXP3, only the samples stained with PE-conjugated antibody were used when analyzing FOXP3 expression alone. PCH101 has been shown to bind both isoforms of FOXP3 [8]. Stained cells were kept in the dark at 4˚C until analysis or were analyzed immediately. The following isotype control antibodies were used: FITC-conjugated mouse IgG1, PE-conjugated mouse IgG2a, PerCP-labeled mouse IgG1, and APC-labeled mouse IgG1. Unstained cells were used to estimate autofluorescence. Cells stained with single antibodies were used to compensate spectrally adjacent dyes.

Samples were acquired on a four-color BD FACSCalibur flow cytometer. The cytometer was calibrated daily using BD Calibrite 3 beads, with added APC beads (BD Biosciences). Compensation was set manually and gates were set subjectively (**Figures 1(a)-(c)**). Analysis was performed using Cellquest Pro software (BD Biosciences). All analyses were performed in a blinded manner, the evaluator did not know the identity of the sample. $CD4^+CD25^{hi}$ cells were defined by first gating on small lymphocytes by forward and side scatter, and then on CD4 and high CD25 expression [24]. The $CD25^{hi}$ gate was adjusted to contain $CD4^+$ cells that expressed higher levels of CD25 than the discrete population of $CD4^-$ cells [25]. This gate contained approximately 2% of small lymphocytes, and 2% - 6% of $CD4^+$ cells. Approximately 5×10^5 small lymphocytes were collected from each tube, while approximately 10^4 cells were acquired from tubes with unstained cells, cells stained with isotype controls, and tubes used to set compensation.

2.3. Statistical Analysis

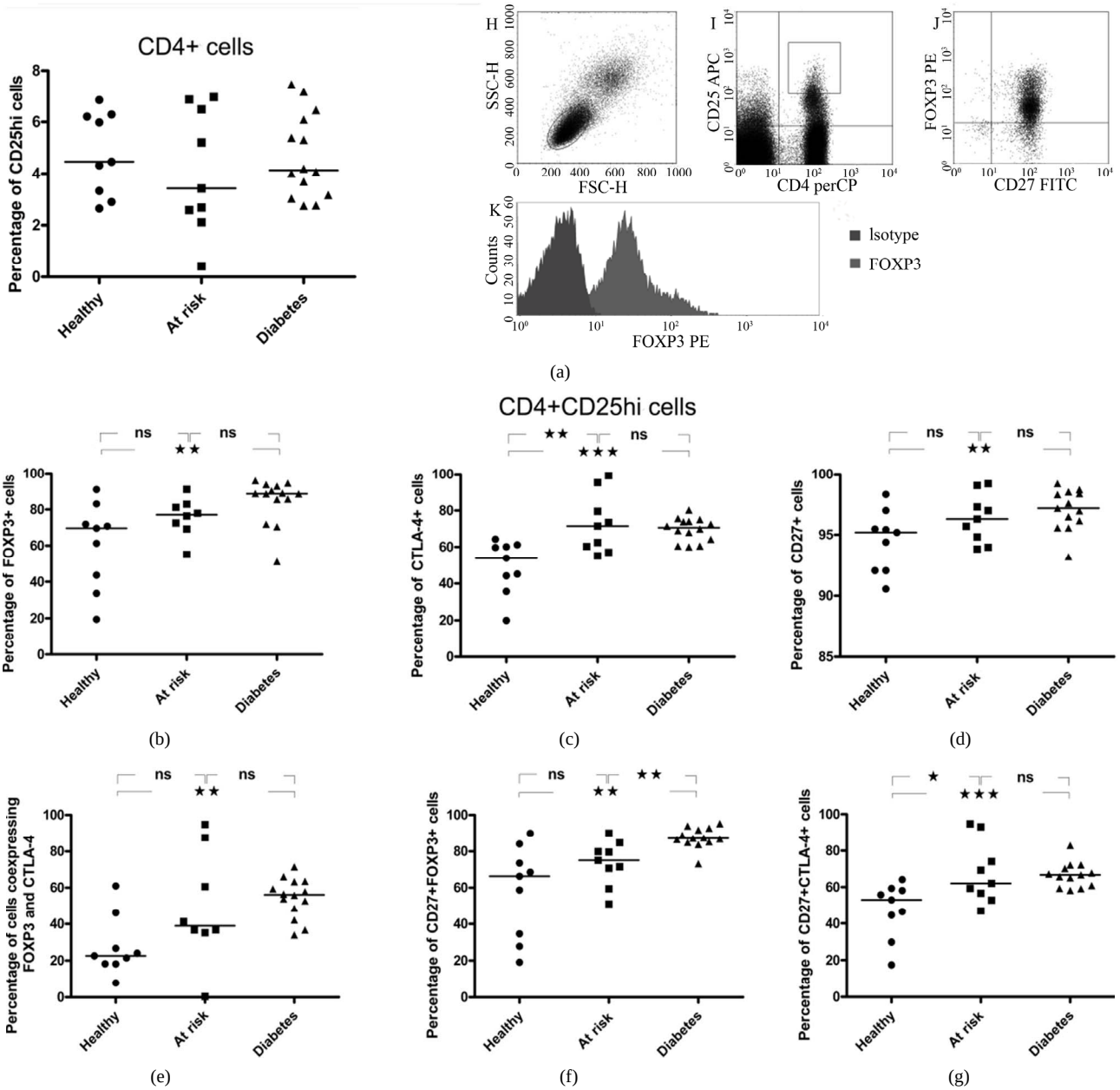
Some, but not all, of our material was normally distributed according to the D'Agostino & Pearson omnibus normality test. We decided to use non-parametric tests based partly on this and because of the small size of our population. Analysis of variance between groups was performed for each parameter using the Kruskal-Wallis test, followed by Dunn's multiple comparison test. Selected groups were compared using the Mann-Whitney U-test; two-tailed P-values were obtained throughout. Statistical testing was carried out with GraphPad Prism 5.01 and SPSS 14.0, both for Windows.

3. RESULTS

Expression of Treg associated markers in $CD4^+CD25^{hi}$ T cells is more frequent among at-risk and recent onset type 1 diabetic children than among controls.

To determine the frequency of cells with a Treg phenotype, we compared the percentages of $CD4^+CD25^{hi}$ cells expressing FOXP3, CTLA-4, and CD27 determined by FACS. Lymphocytes were thus gated based on forward and side scatter (**Figure 1(a)**), followed by CD4 and CD25 expression (**Figure 1(b)**). Quadrant lines demarcate the isotype controls. Gated cells were analyzed for expression of FOXP3, CTLA-4, and CD27 (**Figure 1(c)**). Results are expressed as percentages of cells positively stained for each molecule. For clarity, no-risk individuals will be termed controls.

Approximately 2% small lymphocytes and 2% - 6% $CD4^+$ lymphocytes were gated as $CD4^+CD25^{hi}$. The groups were not significantly different in their frequencies of $CD4^+CD25^{hi}$ cells (**Figure 1(a)**). The percentage of FOXP3-expressing $CD4^+CD25^{hi}$ cells was significantly higher in diabetic children compared to controls ($p = 0.0061$, **Figure 1(b)**), and tended to be higher in diabetic children compared to at-risk children ($p = 0.0518$, **Figure 1(b)**). $CD4^+CD25^{hi}$ cells from at-risk and diabetic children more frequently expressed CTLA-4 than cells from control children ($p = 0.0078$, $p = 0.0006$, **Figure 1(c)**). CD27 was also more frequently expressed in $CD4^+CD25^{hi}$ cells among diabetic children compared to controls ($p = 0.0092$, **Figure 1(d)**). In addition, we analyzed the frequencies of $FOXP3^+CTLA\text{-}4^+$ $CD4^+CD25^{hi}$



(a) shows the frequency of $CD25^{high}$ cells among $CD4^+$ cells. The percentages of $CD4^+CD25^{hi}$ cells expressing FOXP3, CTLA-4, and CD27 are depicted in (b), (c), and (d), respectively. The percentage of $CD4^+CD25^{hi}$ cells coexpressing both FOXP3 and CTLA-4 is given in (e). (f) and (g) show percentages of $CD4^+CD25^{hi}$ cells that are $CD27^+FOXP3^+$ and $CD27^+CTLA\text{-}4^+$, respectively. Ns = not significant. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Significance was determined by Kruskal-Wallis test followed by Dunn's post test. A clear trend of increasing Treg-associated molecules with increasing autoimmune activity is discernable throughout. The manual setting of the lymphocyte gate is shown in H, with 5% of all events shown in the plot. In I, the manually set $CD4^+CD25^{hi}$ gate is depicted. Thirty-three percent of lymphocyte-gated events are shown, and the gate is set according to where the expression of CD25 in the CD4-negative population becomes scarce. J shows a typical plot of CD27 FITC (x-axis) and FOXP3 PE (y-axis); all collected events in the $CD4^+CD25^{hi}$ gate are shown. All plots show permeabilized cells. Isotype staining is compared to FOXP3 stained cells in K.

Figure 1. Differential expression of FOXP3 and CTLA-4 among healthy, at risk and recent onset diabetic children.

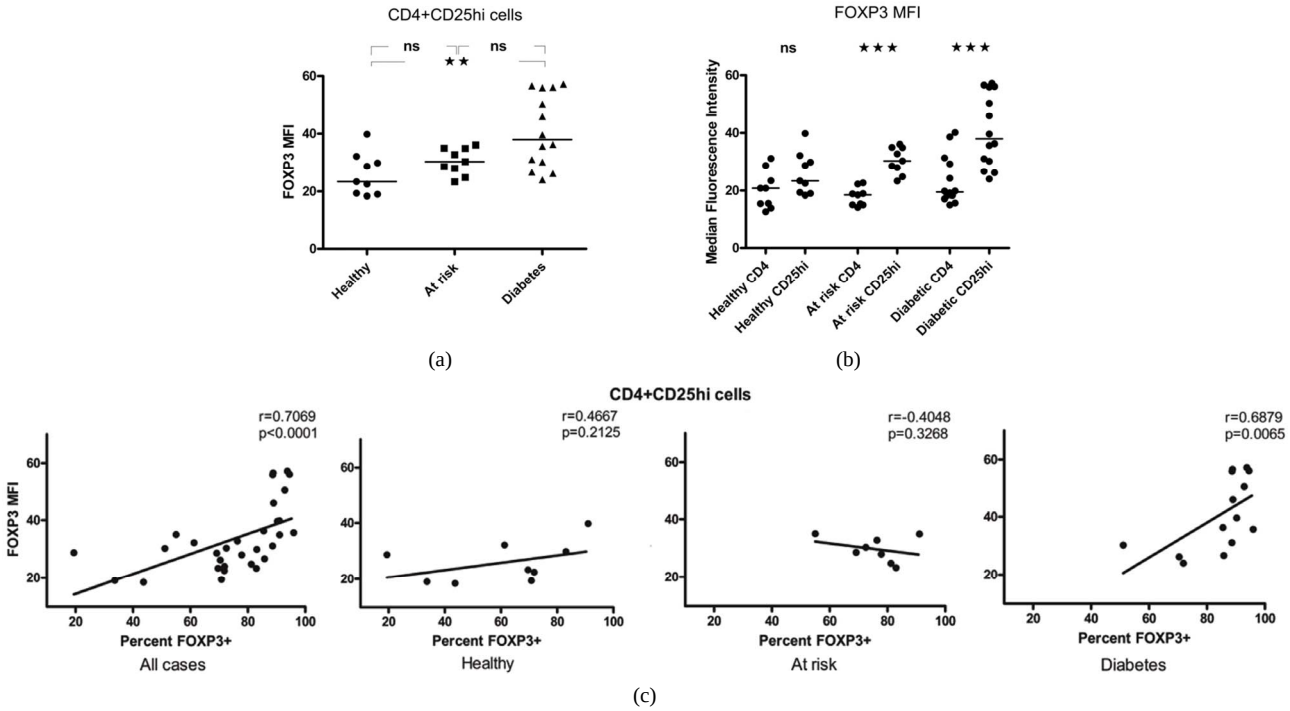
cells. In concordance with separately determined FOXP3 and CTLA-4 levels, co-expression on single cells was higher among children with type 1 diabetes than controls ($p = 0.0018$, **Figure 1(e)**).

Single cells expressing both FOXP3 and CD27 were common among $CD4^+CD25^{hi}$ cells regardless of group, as were $CTLA\text{-}4^+CD27^+$ cells. As the majority of $CD4^+CD25^{hi}$ cells expressed CD27, the frequencies of $FOXP3^+CD27^+$ cells are essentially the same as those of FOXP3 single positive cells. Hence, both $FOXP3^+CD27^+$ and $CTLA\text{-}4^+CD27^+$ co-expression was significantly higher in $CD4^+CD25^{hi}$ cells from diabetic chil-

dren compared to controls (p = 0.0021, **Figure 1(f)**, p = 0.0006, **Figure 1(g)**). Recent onset diabetic children had a higher percentage of $FOXP3^+CD27^+$ coexpressing $CD4^+CD25^{hi}$ cells than at-risk children (p = 0.0033), whereas at-risk children more frequently co-expressed $CTLA\text{-}4^+CD27^+$ than no-risk controls (p = 0.0315). While the at-risk population did not always differ significantly from the control and diabetic groups, a clear trend is discernable from the graphical presentation of our findings, where the at-risk group has higher expression of most analyzed markers than controls, and lower expression than recent onset type 1 diabetic patients. Even though the most distinct variations in FOXP3 and CTLA-4 expression were present within the $CD4^+$ $CD25^{hi}$ subset, the groups remain significantly different when analyzing $CD4^+$ cells in general (data not shown).

The FOXP3 Median Fluorescence Intensity (MFI) of $CD4^+CD25^{hi}FOXP3^+$ cells is higher among recent onset Type 1 diabetic children than among controls.

The median fluorescence intensity for FOXP3 PE in $CD4^+CD25^{hi}FOXP3^+$ cells was higher in diabetic children than in controls (p = 0.0061, **Figure 2(a)**). Intriguingly, $CD4^+CD25^{hi}FOXP3^+$ cells had higher FOXP3 MFI than $CD4^+$ cells among children with type 1 diabetes (p = 0.0007, **Figure 2(b)**). The same pattern was evident in children with risk for type 1 diabetes (p < 0.0001). In contrast, no difference in the FOXP3 MFI was detected between $CD4^+$ cells and $CD4^+CD25^{hi-}FOXP3^+$ cells in the control group. Furthermore, there is a distinct correlation between the frequency of $CD4^+CD25^{hi}FOXP3^+$ cells and the MFI of FOXP3 among $CD4^+CD25^{hi}FOX\ P3^+$ cells (**Figure 2(c)**). This correlation is found exclusively in the diabetic population when the groups are examined independently.



(a) illustrates the FOXP3 MFI of $CD4^+CD25^{hi}$ cells, whereas (b) shows FOXP3 MFI between the $CD4^+$ and $CD4^+CD25^{hi}$ populations in each group. (c) shows the correlation of $CD4^+CD25^{hi}FOXP3^+$ cell frequency and FOXP3 MFI in $CD4^+CD25^{hi}$ cells in a mixed population and among healthy, at risk and diabetic children separately. r and p values were calculated using Spearman's correlation test.

Figure 2. Analysis of FOXP3 MFI in $CD4^+CD25^{hi}$ cells.

CD27 cannot define $CD4^+CD25^{hi}$ cells in peripheral blood.

To explore whether CD27 could define $CD4^+CD25^{hi}$ cells in peripheral blood, the expression of this receptor was analyzed. CD27 was commonly expressed on more than 95% of $CD4^+$ cells. Further, CD27 expression did not differ between the $CD4^+CD25^{hi}$ and the $CD4^+$ population, regardless of the autoimmune state of the individual, defined as diagnosed type 1 diabetes or the presence of autoantibodies.

4. DISCUSSION

In this study we found that the percentages of $CD4^+$ $CD25^{hi}$ cells expressing FOXP3 and CTLA-4 was higher in the peripheral blood of diabetic and at-risk children compared to healthy individuals. It has been argued that the age of the control population relative to the studied

population may affect the outcome of such comparisons [21]. In the present study, both at-risk and control subjects were 8-year-old children, and the recent-onset type 1 diabetic patients were only slightly older (median 10 years). Thus, the differences between at risk and control groups cannot be explained by differences in age.

In agreement with most of the studies including adults [20,21,26], we could not detect a difference in the frequency of $CD4^+CD25^{hi}$ cells. One previous publication on the subject has reported reduced frequencies of $CD4^+CD25^+$ cells in children with type 1 diabetes, but the control population was considerably older than the diabetic population [19]. Brusko *et al.* found no changes in the frequency of $CD4^+CD25^+$ FOXP3 regulatory cells in type 1 diabetics [27]. However, the samples from first degree relatives and healthy controls were from individuals considerably older than the diabetic children. Lawson *et al.* has also reported no difference in the percentages of CD4+$CD25^{hi}$ cells co-expressing FOXP3, but in contrast to our study the patients had long-standing diabetes, and both patients and controls were adult subjects [28].

It has been shown recently that the effector cells of diabetic subjects are resistant to regulation via Treg, and that this resistance is intrinsic to the effector population [29]. Thus, the increased level of FOXP3 expression we detect might be due to a resistance to Treg-mediated suppression in effector T cells. Marwaha *et al.* recently demonstrated that recent-onset type 1 diabetes patients have a higher percentage of non-suppressive CD45RA-$CD25^{int}FOXP3^{low}$ cells that secrete IL-17 [30]. Since CD 45RA was not included as a marker in the present study, it cannot be excluded that the increased percentage of FOXP3-expressing cells presented here may represent a population of non-suppressive cells.

Our study also showed higher frequencies of $CD4^+$ $CD25^{hi}$ cells expressing intracellular CTLA-4 among recent-onset type 1 diabetes children. This is in agreement with a previous result from a study in type 1 diabetic adults [20]. In addition we observed that children with risk for type 1 diabetes also had increased percentages of $CD4^+CD25^{hi}$ expressing CTLA-4. CTLA-4 is constitutively expressed by Treg and has been linked to Treg function in vitro [12,31,32]. However, sharply contrasting results indicate that CTLA-4 blockade does not alter the ability of Treg to suppress proliferation of responder T cells [24]. $FOXP3^+$ Treg capable of in vitro suppression are present in CTLA-4 deficient mice, which further questions the role of CTLA-4 in the mechanism of Treg suppression [33]. Our results indicate that it is unlikely that a lack of CTLA-4 is a causative factor in type 1 diabetes development in children.

CD27 has previously been suggested to define Treg in combination with CD25 [15]. It has also been reported that CD27 expression correlates with FOXP3 expression in peripheral blood of patients with relapsing-remitting multiple sclerosis [34]. In the present study, expression of CD27 did not vary noticeably between $CD4^+$ and $CD4^+CD25^{hi}$ cells. It has been shown that 80% of $CD4^+CD25^{int}$ cells from synovial fluid expressed CD27, arguing against the use of CD27 as a marker to define Treg, since it is unlikely that as many as 80% of $CD4^+CD25^{int}$ cells are Treg [35]. Thus, our results indicate that CD27 is not suitable to define Treg in peripheral blood from children with type 1 diabetes.

The median fluorescence intensity of FOXP3 has been shown to be higher in Treg than in effector T cells [8]. Thus, increased FOXP3 expression in children with type 1 diabetes might represent Treg and not be due to activation of effector T cells. Both diabetic and at-risk children exhibited higher FOXP3 MFI in $CD4^+CD25^{hi}$ cells compared to $CD4^+$, whereas controls did not. This could represent a higher fraction of $CD4^+CD25^{hi}$ cells with a regulatory phenotype in the at-risk and diabetic groups compared to controls. Finally, we found a correlation between the frequency of $FOXP3^+$ cells and FOXP3 MFI among patients with type 1 diabetes but not in at-risk or healthy children. This is in agreement with a previous study where the authors observed a correlation between $FOXP3^+$ cell frequency and FOXP3 MFI in patients with multiple sclerosis [34]. They further demonstrated that the suppressive capacity of $CD4^+CD25^{hi}FOXP3^+$ cells correlates with the MFI of FOXP3 in-vitro. Thus, it cannot be excluded that an abundant population of functional Treg exists in the peripheral blood of children with type 1 diabetes.

In conclusion, the result of our study provide evidence of an altered frequency of cells with a regulatory T cell phenotype in peripheral blood of children with type 1 diabetes and in children with risk for developing the disease. It will be important to clarify whether the increased frequency of CD4CD25hiFOXP3 cells is a failed attempt at controlling autoimmunity.

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