

Altered Suppressor Function of Regulatory T Cells in Type 1 Diabetes

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ABSTRACT

Background: Type 1 diabetes (T1D) is a T cell mediated autoimmune disease targeting the insulin-producing β cells within pancreatic islets. Autoimmune diseases may develop as a consequence of altered balance between regulatory (Tregs) and autoreactive T cells. **Objectives:** To evaluate Treg cells frequency and suppressive function in the peripheral blood of newly diagnosed T1D patients in comparison with healthy controls. **Methods:** Fifteen new cases of T1D patients and 15 age- and sex-matched healthy controls were recruited to this study. Their peripheral blood mononuclear cells (PBMCs) were isolated and $CD4^+CD25^+FoxP3^+CD127^{low}$ Treg cells were studied by flowcytometry technique. Thereafter, Tregs were isolated by Magnetic-Activated Cell Separation (MACS) technology and by using CFSE (carboxyfluorescein succinimidyl ester) dilution assay, their suppressive activity was evaluated in the coculture of $CD4^+CD25^-$ T responder cells with Treg cells. **Results:** The percentage of $CD4^+CD25^+FoxP3^+CD127^{low}$ Tregs did not differ between T1D patients and healthy controls but the MFI (mean fluorescence intensity) of transcription factor FoxP3 (forkhead box protein P3) was significantly decreased in T1D patients (20.03 ± 1.4 vs. 31.33 ± 2.95 , $p=0.0017$). Moreover, the suppressive function of $CD4^+CD25^+CD127^{low}$ Treg cells was significantly diminished in T1D patients in comparison with control group ($35.16 \pm 4.93\%$ vs. $60.45 \pm 5.26\%$, respectively, $p=0.0015$). **Conclusion:** Present study indicates an impaired immune regulation among T1D patients, characterized by defects in suppressive function and expression of FoxP3 in Treg cells without any significant decrease in their frequency in peripheral blood.

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Keywords: Regulatory T Cells (Tregs), Suppressive Function, Type 1 Diabetes (T1D)

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INTRODUCTION

Type 1 diabetes (T1D) is a T cell-mediated autoimmune disease characterized by destruction of insulin-secreting pancreatic islet β cells that results in loss of insulin production, and consequently an inability in controlling glucose metabolism which leads to the long-term medical complications and considerable end organ damages (1,2). The immune system must simultaneously mount effective immunity against foreign pathogens while maintaining tolerance to self antigens. Clearly, any insufficiency in the mechanisms that promote self-tolerance may result in autoimmune disorders including T1D (3,4). Immune tolerance to self antigens is initially achieved during thymic development by the clonal deletion of potentially autoreactive T cells. However, a fraction of autoreactive T cells survive negative selection. Autoreactive T cells specific for the islet autoantigens including insulin and glutamic acid decarboxylase 65 (GAD65) are shown to be involved in T1D pathogenesis (5-7). In fact, autoreactive T cells are kept in check by mechanisms of peripheral tolerance, most notably by a specialized subset of CD4⁺ T cells called regulatory T cells (Tregs). Therefore, functional deficiency of this Treg population leads to the development of a range of autoimmune diseases, including T1D (1,8-10). Moreover, Treg cells can control activity of autoreactive T cells stimulated *in vitro* (11). Much evidence has been collected suggesting a fundamental role for Tregs in the control of autoreactivity against islet cells in both mouse and human (12-14).

To date, studies evaluating the frequency and function of Tregs in the peripheral blood of T1D patients have reported conflicting results including reduction in Treg frequency, impaired regulatory function, or no defect (15-17). Furthermore, as FoxP3 is a nuclear protein, assessment of its expression in T cells requires fixation and permeabilization of these cells, resulting in an inability to obtain viable cells for functional analysis. In the past few years, it has been demonstrated that low or negative expression of the CD127 (IL-7R chain) combined with expression of CD25 (IL-2R α) assist in the distinction of Treg cells from effector T cells and enable better identification and isolation of pure Treg populations that have substantial suppressive activity (18,19). Therefore, we used expression of CD25 and low or negative expression of CD127 (CD127^{-/low}) to more precisely characterize the regulatory properties of 'true' Tregs in T1D patients. In this case-control study, we investigated frequency and suppressive function of Treg cells in new onset of T1D and healthy subjects.

MATERIALS AND METHODS

Subjects. Peripheral blood samples were obtained from a total of 15 patients (8 males, 7 females) with clinically definite new onset T1D (17.00 ± 0.28 years old, range 15 to 18), and also from 15 healthy controls (8 males, 7 females, 17.67 ± 0.23 years old, range 16 to 19) without any family history of diabetes mellitus and past medical history of autoimmune disorders.

The clinical diagnosis of T1D was confirmed in patients after physical examination and paraclinical evaluations according to American Diabetes Association criteria (20). Patients were recruited from the T1D clinic at Children's Medical Center (Imam Khomeini Hospital Complex), and clinical information including age, sex, date of

diagnosis, complete blood count (CBC) was extracted from their medical records. The dermographic data of T1D patients and healthy controls are summarized in (Table 1). The protocol was approved by ethical committee at Tehran University of Medical Sciences and informed consent was obtained from all study subjects.

Table 1. Dermographic data of T1D patients and healthy controls.

	Normal	T1D
Number	15	15
Age in year (mean \pm SD)	17.67 \pm 0.23	17.00 \pm 0.28
Gender ratio (M/F)	8/7	8/7
Date of diagnosis (Week)	---	2.533 \pm 0.29

Collection of Peripheral Blood Mononuclear Cells (PBMCs). Blood samples (25mL of each patient blood) were drawn into heparinized tubes (BD Vacutainer, CA, USA), diluted 1:2 with sterile PBS at room temperature and centrifuged on Ficoll-Paque gradients (Sigma, CA, USA) at 2000 \times g for 15 minutes. PBMCs were collected from the interface and washed in PBS.

Antibodies and Cell Culture Reagents. Allophycocyanin (APC)-conjugated anti-human CD4, Phycoerythrin (PE)-conjugated anti-human Foxp3, Phycoerythrin-Cy-7 (PE-Cy7)-conjugated anti-human CD25, Fluorescein isothiocyanate (FITC)-conjugated anti-human CD127, and respective isotype controls were purchased from BD (CA, USA). RPMI 1640 media supplemented with 1% sodium pyruvate, 1% non-essential amino acids, 2 mM L-glutamine, 20 mM HEPES, 50 U/ml penicillin and 50 μ g/ml streptomycin (Sigma, CA, USA), 50 μ M 2-ME, 10% heat inactivated human AB serum (Invitrogen, CA, USA) were used as culture medium. Also, bovine serum albumin (BSA) and carboxyfluorescein succinimidyl ester (CFSE) were purchased from Sigma (CA, USA).

Immunofluorescent Staining of the Cells and Flowcytometry Analysis. Staining was performed according to the manufacturer's recommendations (BD, CA, USA) and modified as follows: 1-2 \times 10⁶ fresh PBMCs from Controls and T1D patients were suspended in 100 μ L staining buffer. Non-specific staining was blocked by 5% human AB serum for 30 min and cells were subsequently surface-stained with fluorescence-conjugated specific mAbs against CD4, CD25 and CD127. Cells were mixed and incubated for 30 min at 4°C. After washing by PBS containing 0.5% BSA, cells were fixed for 60 min using 1 mL Fix/Perm buffer. Afterwards, cells were permeabilized in Perm buffer in two washing steps. Non-specific intracellular staining was blocked by 5% human AB serum for 30 min and cells were subsequently stained by PE-conjugated anti-human FoxP3. After 30 min incubation at 4°C they were washed twice by Perm buffer (PBS in 2 mM EDTA and 1% BSA) and then resuspended in 200 μ L fixation buffer (1% paraformaldehyde) before running on a Flow cytometer (BD FASCalibur). Isotype controls were used to determine the gating parameters. Data were analyzed using Flowjo software (version 7.6.1).

Isolation of CD4⁺CD25⁺CD127^{low/-} Treg Cells. CD4⁺CD25⁺CD127^{low/-} Treg cells were isolated from mononuclear cells using a CD4⁺CD25⁺CD127^{low/-}Regulatory T Cell Isolation Kit (Miltenyi Biotec, Germany) according to the manufacturer's instructions. Briefly, the isolation was performed in a two-step procedure. PBMCs were incubated with biotin-labeled cocktail antibodies, thus non-CD4⁺ and CD127^{high} cells were indirectly labeled with a cocktail of antibodies and beads. The labeled cells were subsequently depleted by separation over a Magnetic-Activated Cell Separation (MACS) column. CD4⁺CD127^{low/-} T cells were isolated using negative selection. Secondly, CD25⁻ T cells were removed using positive selection after incubation with streptavidin-labeled anti-CD25 and CD4⁺CD25⁺CD127^{low/-} cells were directly labeled by beads and isolated by positive selection from pre-enriched CD4⁺ fraction. To increase the purity, the cells were separated over a second (new) column. A purity of >90% for isolated CD4⁺CD25⁺CD127^{low/-} Tregs was confirmed by flow cytometry.

Isolation of CD4⁺CD25⁻ T responder Cells (Tresp). CD4⁺CD25⁻ T cells were isolated using a CD4⁺CD25⁺ Regulatory T Cell Isolation Kit (Miltenyi Biotec, Germany) according to the manufacturer's instructions. Briefly, PBMCs were first incubated with biotin-labeled cocktail antibodies, CD4⁺ T cells were isolated using negative selection, and then CD25⁻ T cells were isolated using positive selection after incubation with streptavidin-labeled anti-CD25. A purity of >95% for CD4⁺CD25⁻ T cells was confirmed by flowcytometry.

In Vitro Suppression Assay. CD4⁺CD25⁻ Tresp cells were re-suspended in PBS at 2×10^6 cells/ml and incubated with CFSE (2 μ M final concentration) for 10 min at 37°C. After addition of 5 volumes of ice-cold culture media, the cells were incubated on ice for 5 min. Then, they were washed and re-suspended in culture medium at the indicated cell concentrations. In separate experiments, CFSE labeled CD4⁺CD25⁻ Tresp cells from healthy controls activated by human Treg suppression inspector (Miltenyi Biotech, Germany) were cultured in duplicates in 96-well round bottom plates at 5×10^4 cells per well in 200 μ l medium in the absence or presence equal numbers of autologous CD4⁺CD25⁺CD127^{low/-} Treg cells (Tresp/Treg ratio of 1:0 or 1:1). Similarly, CFSE labeled CD4⁺CD25⁻ Tresp cells from T1D patients were cultured with autologous CD4⁺CD25⁺CD127^{low/-} Treg cells. After 5 days, cells were harvested, stained for CD4 and the proliferation of cells based upon the dilution of CFSE in CD4⁺ lymphocytes was analyzed by flowcytometry. Suppression of Tresp proliferation by Tregs in co-culture (suppressive capacity) was expressed as the difference between the percentage of CD4⁺CD25⁻ Tresp cells' proliferation (CFSE^{low} cells) cultured in the presence and absence of Tregs.

The percentage of suppression was calculated using the following formula:

(% Suppression = $(1 - [a/b]) \times 100$, a = % proliferation in the presence of Treg, b = % proliferation in the absence of Treg)

Statistical Analysis. The data were analyzed by Student's *t-test* to assess statistical significance between control subjects and T1D patients, and to evaluate differences in the percent proliferation of T cell co-cultures, with or without Treg cells using a computer based software package (GraphPad Prism 6). Data are expressed as mean \pm SEM. For all tests, p values of less than 0.05 were considered significant.

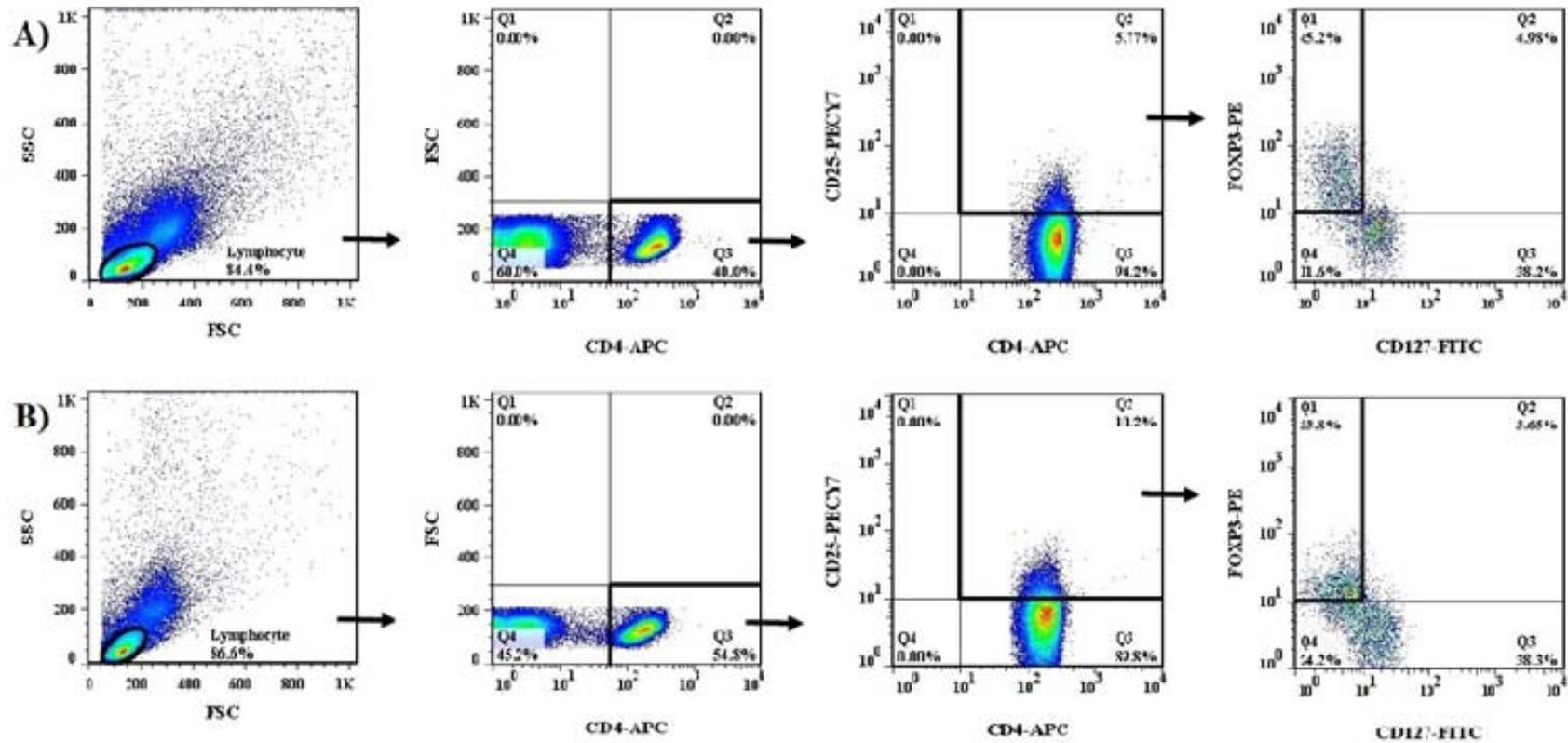


Figure 1. Flowcytometry analysis of Treg cells in representative A) control and B) patient. Freshly isolated peripheral blood mononuclear cells were first stained for cell surface markers CD4, CD25 and CD127. Then, the cells were fixed, permeabilized and stained for intracellular protein FoxP3. The cells in this analysis were gated for lymphocytes based on forward and side scatter properties. Then, the CD4⁺ cells and CD4⁺CD25⁺ cells were defined and FoxP3⁺CD127^{-/low} cells were determined within CD4⁺CD25⁺ cells.

RESULTS

Frequency of Tregs in T1D Patients and Healthy Controls. The gating strategy and the Treg cells ($CD4^+CD25^+FoxP3^+CD127^{-/low}$) population are shown in dot plot diagram in Figure 1. The frequency of Treg cells was determined in both T1D patient and control groups (Figure 2).

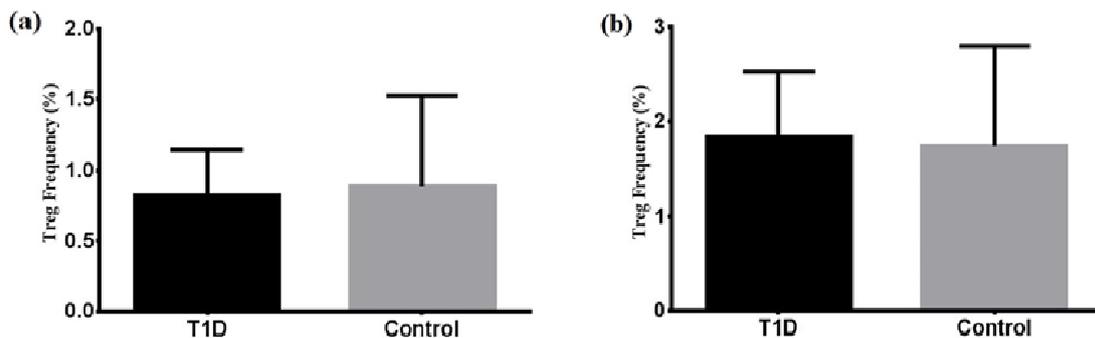


Figure 2. The frequency of ($CD4^+CD25^+FoxP3^+CD127^{-/low}$) Treg cells within a) Lymphocytes and b) $CD4^+$ T cells in T1D patients and healthy controls. We observed no difference in the percentage of Treg cells between patients with T1D and control subjects.

Table 2. T cell subset distribution in peripheral blood of T1D patients and healthy controls.

Subjects	P Value	Normal	T1D
% $CD4^+$ cells in lymphocyte	0.1261	45.58 ± 1.32	49.40 ± 2.03
($CD4^+CD25^+FoxP3^+CD127^{-/low}$) Treg/ $CD4$ ratio (%)	0.7674	1.83 ± 0.18	1.74 ± 0.27
($CD4^+CD25^+FoxP3^+CD127^{-/low}$) Treg/Lymphocyte ratio (%)	0.7263	0.83 ± 0.081	0.89 ± 0.16
Absolute ($CD4^+CD25^+FoxP3^+CD127^{-/low}$) Tregs count ($/\mu$ l)	0.6739	20.56 ± 2.63	19.02 ± 2.51
FoxP3 ⁺ cells within $CD4^+CD25^+CD127^{-/low}$ Tregs (%)	0.0001*	74.51 ± 1.18	58.76 ± 1.4
FoxP3 expression in ($CD4^+CD25^+FoxP3^+CD127^{-/low}$) Tregs (MFI)	0.0017*	31.33 ± 2.95	20.03 ± 1.4
Tregs suppressor capacity (% proliferation of Tresp)	0.0015*	60.45 ± 5.26	35.16 ± 4.93

* Statistically significant

We observed no difference in the percentage of (CD4⁺CD25⁺FoxP3⁺CD127^{-/low}) Treg cells within the peripheral CD4⁺ T cell pool between T1D patients and control group (Table 2).

Cellular Expression Levels of FoxP3 in T1D Patients and Healthy Controls. Tregs are characterized by the expression of FoxP3, which endows these cells with their suppressive function. Therefore, we analyzed intracellular FoxP3 expression within the population of (CD4⁺CD25⁺FoxP3⁺CD127^{-/low}) Treg cells. The expression of FoxP3 protein was evaluated by MFI (Figure 3). It was observed that the MFI of FoxP3 protein was significantly reduced in T1D patients compared to healthy controls (Table 2).

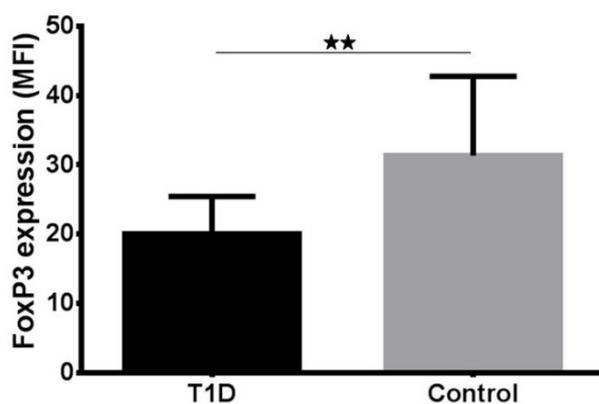


Figure 3. The expression of FoxP3 (MFI) in (CD4⁺CD25⁺FoxP3⁺CD127^{-/low}) Treg cells in T1D patients and healthy controls. It was shown that the MFI of FoxP3 protein was significantly reduced in T1D patients compared with healthy controls.

Percentage of FoxP3⁺ cells within CD4⁺CD25⁺CD127^{-/low} Cells in T1D Patients and Healthy Controls. The percentage of FoxP3⁺ cells within CD4⁺CD25⁺CD127^{-/low} cell population was determined in both T1D patient and control groups. It was shown that the percentage of FoxP3⁺ cells was significantly reduced in T1D patients compared to healthy controls (Table 2).

Treg Function in T1D Patients and Healthy Controls. We used isolated CD4⁺CD25⁺CD127^{-/low} Treg cells in a T cell suppression assays as described in the materials and methods section. The suppressive function of Tregs was evaluated by the ability of Tregs to suppress the proliferation of autologous Tresp cells (CD4⁺CD25⁻) in response to human Treg suppression inspector activation over 5 days in Treg-Tresp co-culture (Figure 4).

As expected, isolated CD4⁺CD25⁻ Tresp cells proliferated well in response to stimulation with human Treg suppression inspector, in T1D patients and controls. However, when CD4⁺CD25⁻ Tresp cells and autologous CD4⁺CD25⁺CD127^{low/-} Treg cells were co-cultured at a 1:1 ratio a significant reduction in the proliferation of Tresp was observed in T1D patients in comparison with control group (Figure 5) (Table 2).

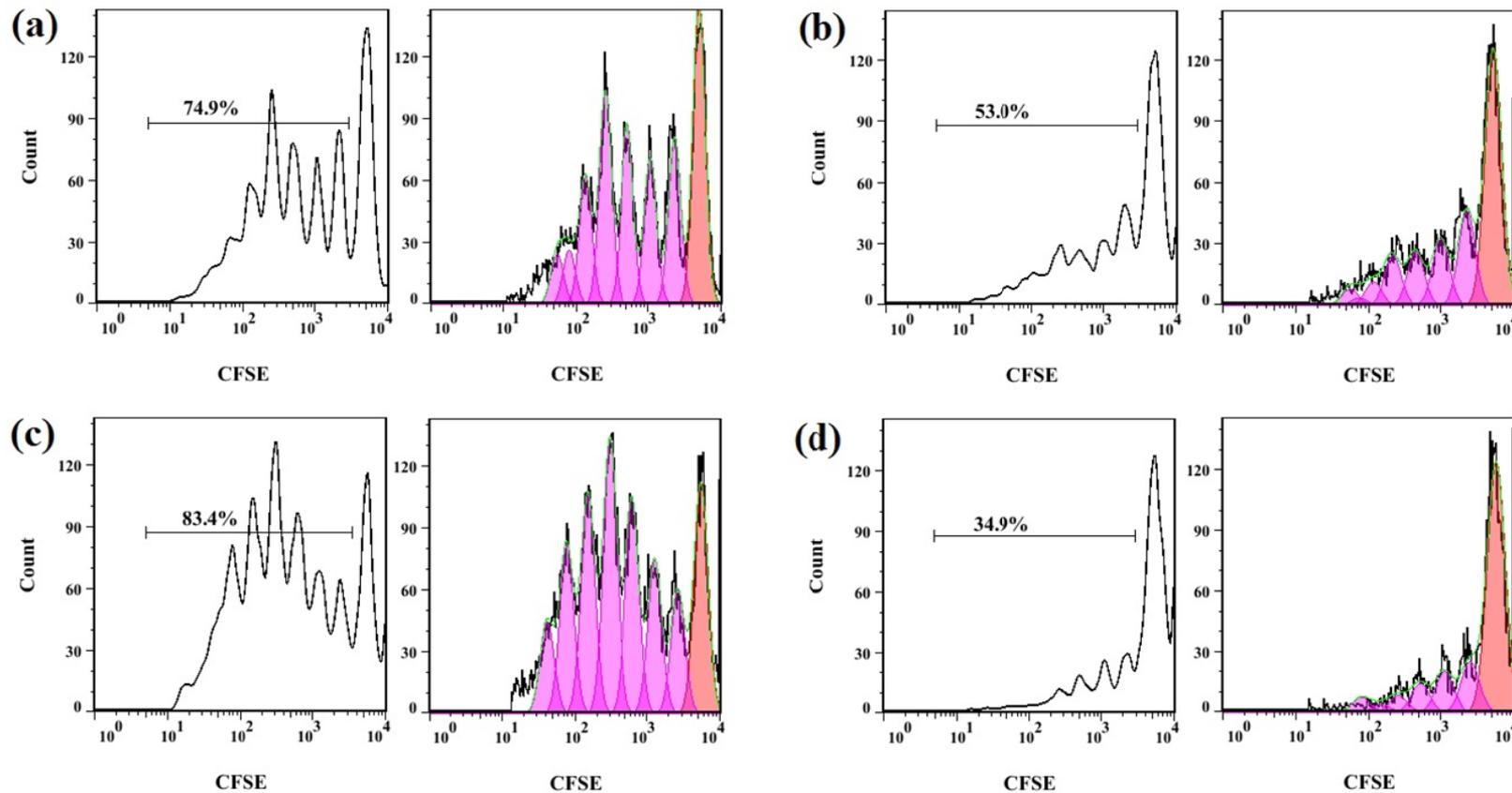


Figure 4. In vitro suppressive function of Treg cells. Treg and Tresp cells were isolated from PBMCs of subjects by using of MACS technology. To examine in vitro suppressive function of isolated Tregs, equal ratio of Treg:Tresp (1:1) were co-cultured in presence of anti-CD2/CD3/CD28 mabsibeads as stimulators of proliferation. As shown, isolated Treg cells suppressed proliferation of CFSE-labeled Tresp cells. Suppressive capacity was expressed as the difference between the percentage of proliferation of CD4⁺ CD25⁻ Tresp cells cultured in the presence or absence of Treg cells. a) %proliferation Tresp cells alone and b) co-culture with Treg cells in T1D patient, c) %proliferation Tresp cells alone and d) co-culture with Treg cells in healthy control demonstrating a robust Treg suppressive effect in the healthy control sample and an attenuation of this suppressive effect in the sample from the T1D patient.

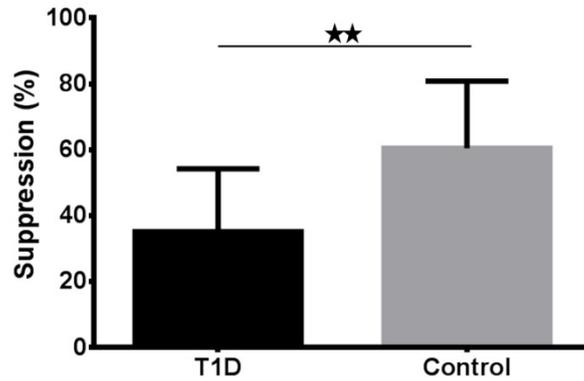


Figure 5. The suppressive function of (CD4⁺CD25⁺CD127^{-/low}) Treg cells based on difference between the percentage of proliferation of CD4⁺ CD25⁻ Tresp cells cultured in the presence or absence of Treg in T1D patients and healthy controls. when CD4⁺CD25⁻ Tresp cells and autologous CD4⁺CD25⁺CD127^{-/low} Treg cells were co-cultured at a 1:1 ratio a significant reduction in the level of suppression of proliferation was observed in the co-cultures of T1D patients in comparison with control group.

DISCUSSION

Previous studies investigating Tregs in human T1D reported contradictory results, partly due to considering CD25⁺ or CD4⁺CD25⁺ cells for isolating Treg cells and assessing function. In this regard the findings were likely obscured by the presence of contaminating non-Treg cells expressing CD25. Recently, it has been shown that activated/memory cells expressing high CD127 are also present within the CD4⁺CD25⁺ T cell population, potentially confusing the classical functional assays for measuring CD4⁺CD25⁺ Treg suppressive function (21-23). Therefore, the present study utilized CD127 expression (CD127^{-/low}) to identify relatively more pure Treg and eliminate the effector cells. We examined the expression of FoxP3 across the full range of CD25 expression levels. However, in agreement with the majority of published studies (13,15,24), we could not find any significant difference in circulating populations of Treg in patients with T1D but MFI of FoxP3 was significantly decreased in them. These results suggest that the frequency of Treg cells in T1D patients is not abnormal and alteration of balance between regulatory T cells and activated T cells could not be responsible for defective suppression observed in T1D patients. However, until the discovery of definitive phenotypic markers of Treg cells this will remain an unresolved question. Moreover, the number of Treg cells found in the peripheral blood of patients with autoimmune disease is influenced by Treg development and proliferation in the periphery, which are likely affected by disease status.

In our study, circulating CD4⁺CD25⁺CD127^{-/low} Treg cells from healthy controls effectively suppressed the proliferation of Tresp cells. In contrast, Tregs from patients with T1D showed an impaired ability to suppress the polyclonal proliferation of co-cultured autologous Tresp cells. Indeed, our study, inconsistent with previous studies (13,25,26) suggests that defective suppressor function of Treg cells is a feature of T1D. However, conflicting results could be due to differences in isolation technique and the T cell stimulation conditions employed during the suppression assay. In this study we

isolated T cell populations using magnetic bead technology, whereas some studies analyzed isolated populations by Fluorescent Activated Cell Sorter (FACS). A more attractive possibility is the *in vitro* stimulation conditions (The level of TCR stimulation) that is known to affect both the Tregs ability to suppress and the effector T cell susceptibility to regulation (27).

Our results suggest that this defect primarily resides within the population of Treg cells, and may potentially be due to instability of FoxP3 expression in these cells and conversion of Treg cells to Th1, Th2 or Th17 effectors, a hypothesis that will require further study. While it is not yet clear whether Treg dysfunction plays a direct causal role in T1D, it may provide a general explanation for why tolerance against auto-antigens becomes imbalanced, leading to enhancement of an individual's susceptibility to autoimmunity. Further studies are needed to define the nature of the Treg dysfunction in T1D.

Foxp3 is predominantly expressed in Treg cells and is the master regulator for the development and function of these cells (8). Mutations of the FoxP3 gene in humans results in an X-linked clinical syndrome characterized by immune dysregulation, polyendocrinopathy, and enteropathy (IPEX) (28). It has been generally thought that FoxP3 expression serves as an on-off switch endowing T lymphocytes with suppressive ability. However, emerging evidence, including our own work showing an association between attenuated FoxP3 expression in Tregs and human autoimmune disease, proposes a paradigm in which alterations in its expression in Treg cells may lead to loss of immune tolerance (29). Recently, some studies have shown that reduced or aberrant FoxP3 expression is correlated with Treg dysfunction (30,31). Consistent with previous studies (32), we demonstrate herein that FoxP3 expression is significantly reduced in peripheral Tregs in T1D patients at the protein levels.

In summary, we have shown a decrease in Treg cell suppressive capacity and MFI of FoxP3 but no alteration in Treg cell frequency. These results suggest that a decrease in MFI of FoxP3 may be important in defective suppression observed in T1D patients. We suggest that defective suppression may contribute to pathogenesis of disease and assessment of Treg cell function may have clinical value. More studies evaluating the immune regulation of T1D patients are of great importance in this regard.

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