CD4+Foxp3+ regulatory T lymphocytes expressing CD62L in patients with long-standing diabetes type 1

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Abstract

Diabetes type 1 is a chronic autoimmune disease in which insulin-producing cells located in the pancreatic islets of Langerhans are gradually destroyed by autoreactive T cells. Quantitative or qualitative dysfunctions in naturally arising CD4+Foxp3+ T lymphocytes may result in ineffective suppression of autoreactive T cells. CD62L is a surface molecule that plays role in homing capabilities of Tregs in autoimmunity if they must be present in the target tissue in order to have the proper effect. Only cells with high expression of CD62L are able to protect against diabetes. We investigated CD4+Foxp3+ cells expressing CD62L after in vitro stimulation with anti-CD3 from children with long-standing diabetes type 1 and we found lower frequencies of these cells in diabetic group compared to their healthy counterparts.

Key words: *regulatory T cells, diabetes type 1, CD62L, Foxp3.*

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Introduction

Diabetes type 1 is a chronic autoimmune disease in which insulin-producing cells located in the pancreatic islets of Langerhans are gradually destroyed by autoreactive T cells. It is thought that the destruction of insulin producing cells is closely associated with T-lymphocytes [1]. It involves defects in central as well as peripheral tolerance [2]. Central tolerance involves a process of clonal deletion of autoreactive T cells during their development in thymus. Ineffective binding of self-antigen to HLA molecules may lead to incomplete deletion of autoreactive T cells thus allowing them to escape to pancreatic environment where they are able to destroy beta cells. Peripheral tolerance is maintained in part by natural regulatory CD4+ T lymphocytes, that constitutively express alfa chain for IL-2 receptor – CD25. Since CD25 is considered as a marker of activation and can be also expressed on T cells of alternate linages, it can't be used as a specific marker of Tregs. More closely related with Tregs seems to be a forkhead/winged helix (FKH) transcription factor, Foxp3 which is the key regulatory gene for the development and suppressive function of regulatory T lymphocytes [3, 4]. Foxp3+CD4+ T regulatory cells play critical roles in regulation of the immune response and prevention of autoimmunity [5]. CD62L is a key cell adhesion molecule that has a major role in directing naive T lymphocytes to peripheral lymphoid organs. It mediates the tethering and rolling of lymphocytes in specialized high endothelial venules (HEVs) of peripheral LNs [6]. Recent data suggest that the CD62L+ subset of Tregs is much better than the CD62L- subset of Treg cells in treating both autoimmunity [7] and graft-versus-host disease [8, 9]. This subset was also able to effectively inhibit bone marrow graft rejection [9]. Interestingly, naturally occurring regulatory T cells are able to maintain the expression of CD62L [10]. This is in contrast to conventional T cells which down-regulate the expression of CD62L after activation.

Some studies indicate that only Treg cells with high expression of CD62L are able to protect against diabetes, which was shown in NOD mice [11], while CD62L- CD4+

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CD25+ T cells expressing CCR2, CCR4, and CXCR3 were ineffective [7]. This differential activity of regulatory T cell subsets gives weight to the importance of regulatory T-cell migration to secondary lymphoid tissues for suppression of autoimmune disease.

Thus our studies were aimed to analyze the regulatory T lymphocytes subsets in children with diabetes type 1 after in vitro stimulation with anti-CD3 antibody.

Material and Methods

Fifty subjects consisting of 35 patients aged 16.1 (± 3.02) years with long standing diabetes type 1 and 15 age matched healthy controls had fresh blood samples collected for analysis. The mean duration of the disease in diabetic group was 8 (± 3.5) years. The patients were recruited from The Diabetic Department, Clinic of Pediatrics, Hematology, Oncology and Endocrinology Medical University of Gdańsk. Type 1 diabetes was defined according to the criteria of the American Diabetes Association [19]. A blood glucose level was taken at the time of sampling along with biochemical measurement of renal function, lipid status, and glycosylated haemoglobin (HbA1c). The study followed the principles of the Declaration of Helsinki and was approved by The Ethics Committee of The Medical University of Gdańsk.

Heparinised venous blood samples were collected aseptically into the tubes and stored on ice for maximum 30 minutes before flow cytometric analysis (LSRII, Becton Dickinson, USA). Blood samples (100 µl) were then stained with anti-CD4 (IgG1, κ mouse Pe/Cy5, Clone RPA-T4, BioLegend, USA), anti-CD25 (IgG1, κ mouse FITC, Clone BC96, BioLegend, USA) and anti-CD62L (IgG1, κ mouse APC, Clone DREG-56, BioLegend, USA) human antibodies. For each set, appropriate isotypic control was done. After 30 minutes incubation in the dark at room temperature stained probes were fixed using Immuno-prep reagents (Immunotech, USA) with Q-prep Immunology Workstation (Coulter, USA).

The remainder blood (3-4 ml) was used to isolate peripheral blood mononuclear cells.

PBMC were separated from heparanized whole blood samples by density gradient preparation over Ficoll-Uropoline. Mononuclear cells at the interface were carefully transferred into a Pasteur Pipette, then treated with hemolysis buffer and washed twice in PBS. Cells were suspended at a density of 1×10^6 cells/ml and cultured in 1 ml RPMI 1640 supplemented with 5% heat-inactivated fetal calf serum (FCS). The cultured cells (10^6/ml) were stimulated with anti-CD3 antibody (clone UCHT1, eBioscience, USA) at the concentration 5 µg/ml for 48 h. After this time the cells were harvested and stained for the surface expression of CD4 and CD62L using previously described antibodies and for intracellular expression of Foxp3 (IgG1, κ mouse Alexa-Fluor 488, Clone 206D, BioLegend, USA) according to the manufacturer protocol. Flow cytometry gating was used to detect and separate leukocytes by their different light scattering properties and to analyze the T cells (i.e., CD4-positive cells) for expression of CD62 and Foxp3).
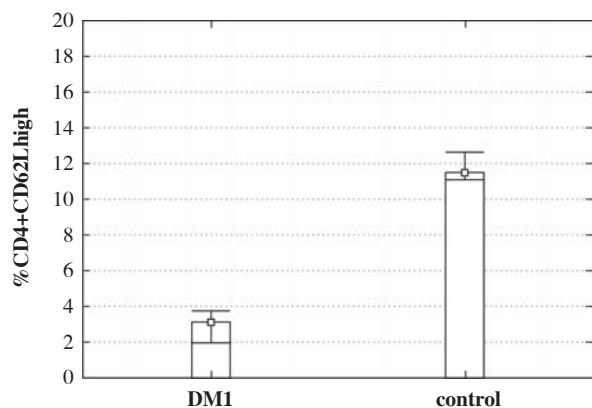
Results

No significant differences were found in frequencies of CD4+CD25high T cells isolated from fresh blood of DM1 and control subjects. Instead, we found decreased percentage of CD4+CD62Lhigh and CD4+CD25highCD62Lhigh T cells isolated from fresh blood of DM1 patients in comparison with their healthy counterparts (Figs. 1, 2).

After *in vitro* stimulation with anti-CD3 antibody the percentage of regulatory T cells CD4+Foxp3+ was higher in control group in comparison with DM1 group (Fig. 3). After *in vitro* stimulation with anti-CD3 in control group there were more regulatory CD4+Foxp3+ cells expressing CD62L than in DM1 group (Fig. 4).

Discussion

Our findings indicate that lower frequency of CD4+Foxp3+ regulatory T cells in patients with diabetes type 1 may impact the development of autoimmunity. Brusko et al. showed no differences in the frequency of Foxp3 expressing regulatory T cells between patients with diabetes type 1 and healthy controls [12]. The studies done by his group were conducted in peripheral blood and obtained results are in contrast with suggestion that in case of diabetes only subpopulation of Treg that carries CD62L is able to protect against the disease [11]. CD62L plays role in homing capabilities of lymphocytes. If CD62L makes CD4+Foxp3+ cells able to protect against diabetes, Tregs
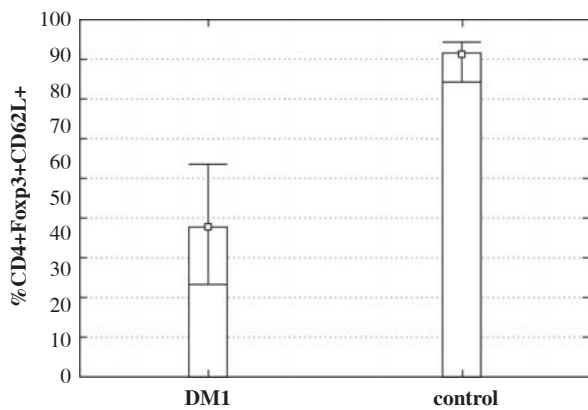

Figure 1. The percentage of CD4+CD62Lhigh cells in peripheral blood. Results from FACS analysis of 35 subjects with DM1 and 15 non-diabetic controls. The range (the 25th and 75th percentiles are indicated by the lower and upper border of the box) and the median value are indicated. Differences are significant with p < 0.001 (Mann-Whitney test)
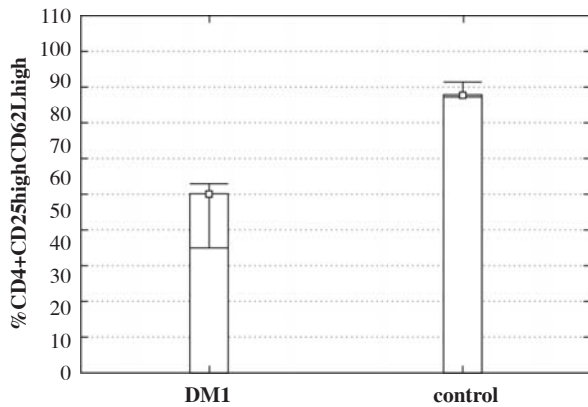
Figure 2. The percentage of CD4+CD25highCD62Lhigh cells in peripheral blood. Results from FACS analysis of 35 subjects with DM1 and 15 non-diabetic controls. The range (the 25th and 75th percentiles are indicated by the lower and upper border of the box) and the median value are indicated. Differences are significant with p < 0.001 (Mann-Whitney test)
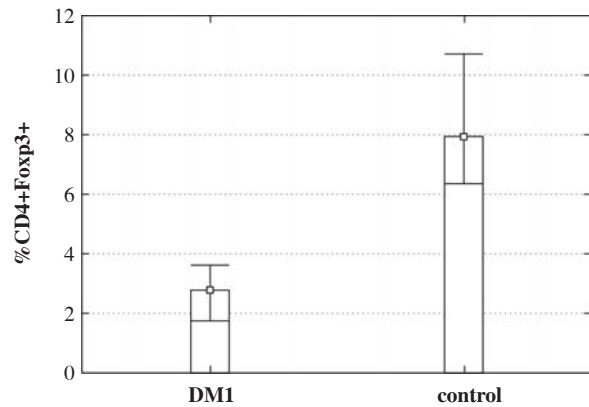
Figure 3. The percentage of CD4+Foxp3+ cells after stimulation with anti-CD3 antibody. Results from FACS analysis of 35 subjects with DM1 and 15 non-diabetic controls. The range (the 25th and 75th percentiles are indicated by the lower and upper border of the box) and the median value are indicated. Differences are significant with p < 0.001 (Mann-Whitney test)

Figure 4. The percentage of CD4+Foxp3+CD62L+ cells after stimulation with anti-CD3 antibody. Results from FACS analysis of 35 subjects with DM1 and 15 non-diabetic controls. The range (the 25th and 75th percentiles are indicated by the lower and upper border of the box) and the median value are indicated. Differences are significant with p < 0.001 (Mann-Whitney test)

have to be present in pancreatic lymph nodes in order to effectively suppress autoreactive cells. Adding anti-CD3 antibody to the *in vitro* culture we have induced the conditions that were to mimic those present *in vivo*. Thus we could observe how cells will have reacted. As it has been shown, CD4+Foxp3+ as well as CD4+Foxp3+CD62L+ T lymphocytes from diabetic patients were lower in number and percentage which may mirror their behavior *in vivo*. In case of decreased frequencies of regulatory T cells expressing CD62L our results are consistent with the

findings of Pop et al. [13] but they are in contrast with those obtained by Brusko et al. [14]. It may be because we investigated the cells after stimulation with anti-CD3 antibody and we used Foxp3 as a reliable marker instead of CD25. On the other hand our results showing the frequency of peripheral blood CD4+CD25highCD62Lhigh cells are still in contrast with studies of Brusko et al. [14]. It may be because of the number of subjects enrolled in the study. The group involved in their study comprised 12 diabetic patients and 8 healthy control subjects.

Studies concerning the functional abilities of CD62L+ regulatory T cells are contradictory. According to Fu et al. the CD62L+ subset is a more potent suppressor than the CD62L– subpopulation of Tregs [15] but studies done by Thornton and Shevach [17] and later by Szanya et al. [7] reveal that the CD62+ and CD62L– subsets of regulatory T cells are not different and are equally suppressive *in vitro*. Further studies are needed to determine the suppressive activity of this subset.

Lack of significant differences in percentage of CD4+ CD25high subset is consistent with studies of Brusko et al. [14] but in contrast to Kukreja et al. [17], which indicated differences in Treg frequency between type 1 diabetic patients and healthy control subjects. We would propose the same explanation as Brusko did, that these differences may be due to some differences in methodology. Regulatory T cells from human peripheral blood express very high levels of CD25 molecule [18].

Our results showing decreased numbers of CD4+Foxp3+ after *in vitro* stimulation with anti-CD3 seem to negate the suggestion that a defect in Treg function rather than number contributes to autoimmune diabetes [14]. According to

Brusko et al. [12] in case of diabetes, frequencies of Tregs in the periphery are not specifically associated with the disease and these cells act in pancreas and/or pancreatic draining lymph nodes.

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