ORIGINAL ARTICLE

Dendritic cells frequency and phenotype in Egyptian type 1 diabetic patients

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Int J Diab Dev Ctries. 2014 ; 34: 10-17

Abstract This study was performed to investigate changes in the dendritic cells (DCs) frequency and phenotype in the peripheral blood in Egyptian Type 1 diabetic children. Also to study the level of B, T lymphocytes, activated T lymphocytes, and costimulatory molecules expression on B lymphocytes. Twenty five children with T1DM and 25 healthy controls were enrolled. Flow cytometric detection of DCs, B-lymphocytes and T-lymphocytes, $CD19^{+}CD80^{+}$, $CD19^{+}CD86^{+}$, $CD19^{+}$HLA-DR^{+} and $CD3^{+}$ HLA-DR^{+} was preformed. The frequencies of monocytoid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs) were significantly decreased in diabetic patients than the controls and the mDCs/pDCs ratio was significantly higher in diabetic patients. The expression of costimulatory molecules CD80 and CD86 on the entire DCs was significantly higher in diabetic children. The frequency of pDCs was nega-tively correlated with the age in diabetic patients and positively correlated with the level of insulin C-peptide. The percentage of CD80 expressing B lymphocytes and of activated T lymphocytes was significantly higher in the patients. Dendritic cells are re-duced in number and display more mature phenotype in T1DM children. The higher expression of CD80 on B lymphocytes and activation of T lymphocytes may reflect the ongoing autoim-mune process in this disease. Modulation of the DCs could have beneficial effect in T1DM.

Keywords Type 1 diabetes mellitus · Dendritic cells · Costimulatory molecules

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Introduction

Type 1 diabetes mellitus (T1DM) disease characterized by selective and progressive destruction of insulin-producing pancreatic beta (β) cells, results from both genetic and environmental factors [1]. Beta cell autoantigens, macrophages, dendritic cells (DCs), B lymphocytes and T lymphocytes are involved in the pathogenesis of autoimmune diabetes. β cell auto antigens are processed by macrophages, DCs, or B cells in the pancreatic islets and presented to auto reactive CD4+ T cells. These auto reactive CD4+ T cells are activated and secrete cytokines, which can activate β cell–specific cytotoxic CD8+ T cells contributing to the destruction of β cells [2]. B lymphocytes play a critical role in the development of insulitis and diabetes [3]. Self-reactive B lymphocytes play two main pathological roles one, as secretors of auto antibodies and the other as specialized antigen presenting cells (APCs) that present self-components to autoreactive T lymphocytes [4].

Dendritic cells are a heterogeneous group of APCs with an important role in the induction of immunity and maintenance of tolerance against self antigens [5]. DCs play a pivotal role in the pathogenesis/protection of autoimmune diseases because they are capable of either priming effector T cells or activating regulatory T cells depending on the maturation stimuli and/or DC subsets [6–9]. Two distinct subsets of DCs, monocytoid DCs (mDCs) and plasmacytoid DCs (pDCs) have been recognized in human [10]. Both popula-tions are distinguished from other lymphoid and myeloid cells by their expression of high levels of human leukocyte antigen (HLA)-DR and lack of expression of lineage specific markers (CD3, CD19, CD14, and CD16) [11]. Monocytoid DCs ex-press CD11c and promote differentiation of T helper (Th)-1 cells [12, 13]. Plasmacytoid DCs express CD123 and produce interferon (IFN) ∝/β upon activation with microbial products [14]. Plasmacytoid DCs were presumed to mature into DCs that drive Th2-cell differentiation [1], but recent evidence

indicates that both these DCs subsets can promote

differentiation of Th1 or Th2 cells depending on antigen dose and quality and the cytokine milieu in which DCs subsets mature [14–16]. In the steady state DCs are in an immature state and express low levels of major histocompatibility complex (MHC) class II and co-stimulatory molecules (B7 family members). The maturative state is associated with changes on DCs such as increased formation of MHC-peptide complexes, up-regulation of B7·1 and B7·2 (also known as CD80 and CD86) and other molecules that promote DCs survival, DC-T cell clustering and synthesis of cytokines and chemokines for T cell proliferation and differentiation [17]. Although the roles of various DCs subsets in T1DM received some attention in recent years [18, 19], their role in T1DM not fully studied in Egyptian patients..

The aim of the present study is to investigate changes in the DCs frequency and phenotype in the peripheral blood (PB) in the Egyptian Type 1 diabetic children . Also to study the level of B, T lymphocytes, activated T lymphocytes, and costimulatory molecules expression on B lymphocytes in diabetic children.

Subjects and method

Subjects The study group consisted of 25 children with T1DM attending the Pediatric Endocrinology and Diabetes Unit of Assiut University Children Hospital, Egypt. The control group consisted of 25 healthy subjects, matched for age and sex. Informed written consent was obtained from parents and the study was approved by the Faculty of Medicine Ethic Committee for the Scientific Research Conduct. The data presented were obtained at one time point.

Inclusion criteria

Definite diagnosis of T1DM according to the definition of the World Health Organization criteria [20].

On insulin replacement therapy, age range 2–16 years.

Exclusion criteria

Children with secondary forms of diabetes mellitus (DM), children with type 2 DM, active infection requiring antibiotic therapy or other concurrent diseases, other autoimmune dis-ease, age <2 years or >16 years.

Methodology

After collecting biographical informal (age, sex, residence, family history of diabetes), full clinical examination was performed along with collecting blood for complete blood count (Celltac E automated hematology analyzer, Tokyo, Japan), Serum insulin C-peptide levels [radioimmunoassay using commercial kits (Diagnostic Systems Laboratories Inc, Webster, Texas]. Fasting normal insulin C peptide=0.78– 5.19 ng/ml [21]. Glycosylated hemoglobin (HbA1c%) was measured using Hitachi autoanalyser (Roch, Germany) by turbidimetric inhibition immunoassay. The American Diabetes Association (ADA) published the target age-specific HbA1c% as follow; < 6 years =7.5 %–8.5 %; from 6 to 12 years=≤ 8 %; from 13 to 18 years=≤7.5 % [22]. Flow cytometric detection of DCs, B-lymphocytes and T-lymphocytes, CD19+CD80+,CD19+CD86+, CD19+HLA-DR+ and CD3+ HLA-DR+ was done.

Flow cytometric detection of DCs numbers and phenotype

Dendritic cells in whole peripheral blood PB samples were enumerated using fluoroisothiocyanate (FITC)-conjugated monoclonal antibodies (MoAbs) against lineage markers (includes anti-CD3, CD14, CD19), phycoerythrin (PE)-conju-gated anti-CD123, PE-conjugated anti-CD11c and Peridinium- chlorophyll-protein (Per-CP)-conjugated an-HLA-DR. All MoAbs were purchased from Becton Dickinson Biosciences, USA.

To determine blood DCs numbers, 100 μl of blood sample was stained with 10 μl of FITC-conjugated lineage specific markers (CD3, CD14 and CD19), 10 μl of Per-CP-conjugated anti-HLA-DR, and 10 μl of PE-conjugated DCs markers (anti-CD11c or anti-CD123). The expression of CD86 and CD80 on DCs was analyzed in whole blood samples in parallel with DCs numbers; 100 μl of blood sample was stained with 10 μl of FITC-conjugated lineage specific markers (CD3, CD14 and CD19), 10 μl of Per-CP-conjugated anti-HLA-DR, and 10 μl PE-conjugated CD86 or CD80. The tubes were incubated for 50 min at room temperature in the dark. RBC lysis was done. After one wash, the cells were resuspended in phosphate buffer saline (PBS), and analyzed by FACSCalibur flow cytom-etry with CellQuest software (Becton Dickinson Biosciences, USA). Fifty thousand events were analyzed, and an isotype-matched negative control was used with each sample. The numbers of $CD11c^+$ (mDCs) and $CD123^+$ (pDCs) and the expression of CD86 and CD80 on the entire DCs population ($HLA\text{-}DR^+$ lineage $specific^{-ve}$ events) were detected by flow cytometry as shown in Fig. 1. CD86 and CD80 levels were recorded as a percentage of expression and as a geometric mean of fluorescence intensity (MFI). Figure 1, shows a representative example of flow cytometric detection DCs.

Flow cytometric detection of B-lymphocytes, T-lymphocytes, $CD19^+CD80^+$, $CD19^+CD86^+$, $CD19^+HLA\text{-}DR^+$ and $CD3^+$ $HLA\text{-}DR^+$

One hundred μl of blood sample was stained with 10 μl of Per-CP-conjugated CD19 (Becton Dickinson Biosciences,

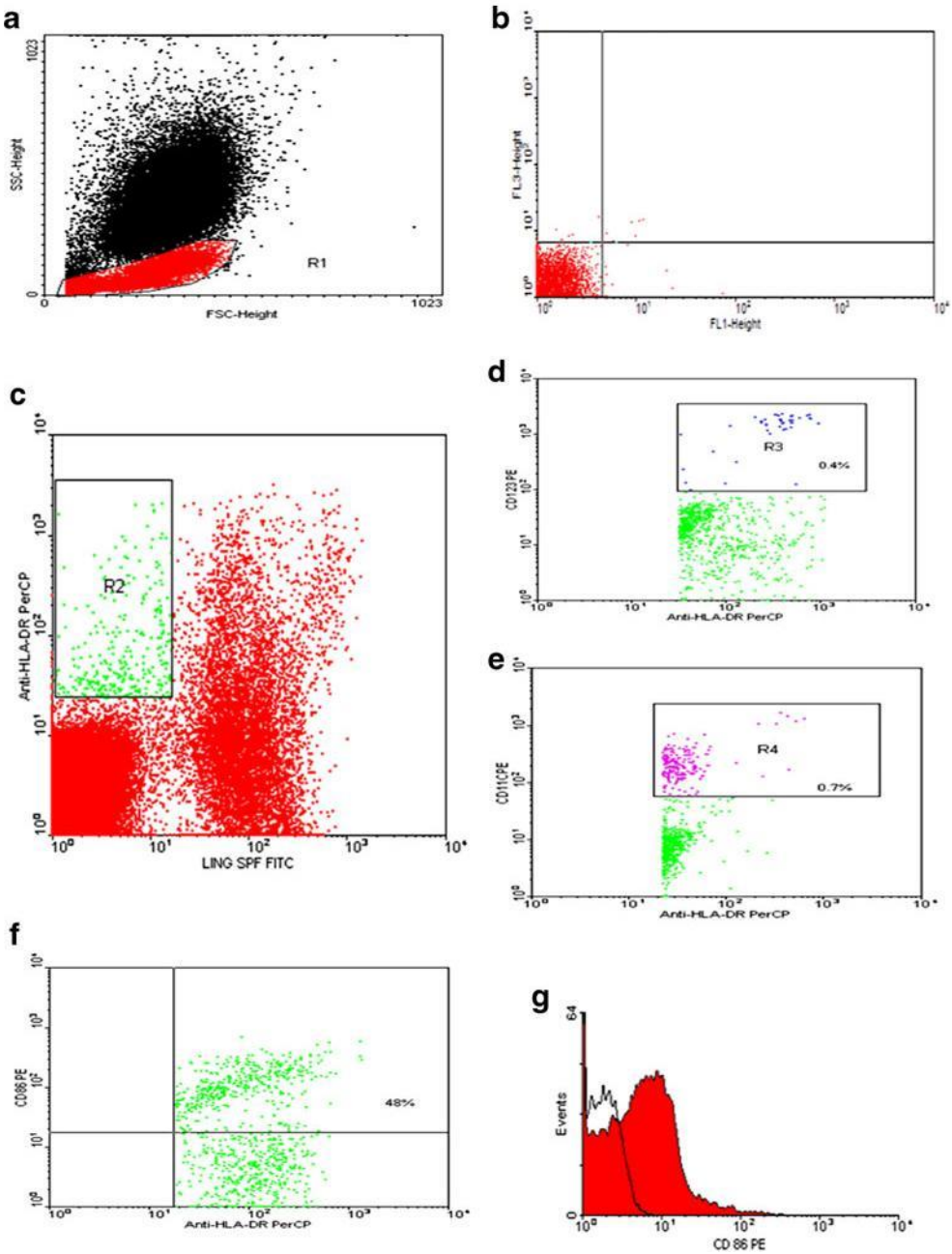

Fig. 1 Flow cytometric detection of DCs. Forward and side scatter histogram was used to define the lymphocyte and monocytes population (R1) (a). R2 gate containing entire dendritic cells (HLA-DR$^+$ lineage specific$^-$ populations) within the lymphocyte and monocytes population was selected (c), compared with the negative isotype control (b). Then, the percentage of the pDCs (HLA-DR$^+$ lineage specific$^-$CD123$^+$) (R3) and mDCs (HLA-DR$^+$ lineage specific$^-$ CD11c$^+$) (R4) were determined (d, e). The expression of CD86 and CD80 on the entire DC population was detected. CD86 and CD80 levels were recorded as a percentage of expression (f) and as a geometric mean of fluorescence intensity (MFI) (g). The positivity was defined as fluorescence (red histogram) higher than that of the isotype control (open histogram)

USA), 10 μl of PE-conjugated CD86 and 10 μl of FITC-conjugated CD80 (Invitrogen, USA) in one tube, and with 10 μl of Per-CP conjugated CD19, PE-conjugated CD3 (IQ Product, the Netherland) and FITC-conjugated anti-HLA-DR (Becton Dickinson Biosciences, USA), in another tube. The procedure was completed as the same procedure in DCs detection. The percentage of CD19$^+$ (B-lymphocytes) and CD3$^+$ (T-lymphocytes), CD19$^+$CD80$^+$, CD19$^+$CD86$^+$, CD19$^+$ HLA-DR$^+$ and CD3$^+$ HLA-DR$^+$ (activated T lymphocytes) were detected.

Statistical analysis

Data analyzed by statistical package for social sciences (SPSS), version 16. All data were expressed as the mean ± standard error of mean (SEM). Differences between the groups were examined for statistical significance using the Mann–Whitney analysis, because of the small sample size and a propensity for outliers in some of the variables. A P value of ≤0.05 denoted the presence of a statistically significant difference. Spearman correlation coefficient was used to examine the correlation among different studied parameters.

Results

Demographic and clinical data of diabetic children and controls are presented in Table 1, and laboratory data in Table 2. There were no significant differences in white blood cells (WBC) count, platelet count and hemoglobin concentration between cases and controls. Insulin C peptide was significantly lower in children with T1DM than the controls.

The frequencies of mDCs and pDCs were decreased in diabetic patients than the controls and the mDCs/pDCs ratio was significantly higher in diabetic patients. The expression of costimulatory molecules CD80 and CD86 on the entire DCs (HLA-DR^{+} lineage specific^{-ve} events) was significantly higher in diabetic children. Also the amount of CD80 and CD86 per cell, represented by the MFI was significantly higher in the patients (Table 3). The frequency of pDCs was negatively correlated with age in diabetic patients ($r=-0.601$; $P=0.002$), and positively correlated with the level of insulin C-peptide ($r=0.825$; $P=0.000$) (Figs. 2 and 3). There is no correlation between mDCs and any of the studied parameters.

Regarding the lymphocyte subsets, there was no significant difference in the percentages of T lymphocytes (CD3^{+}) or B lymphocytes (CD19^{+}) between diabetic patients and controls. The percentage of CD80 expressing B lymphocytes was significantly higher in the patients compared to the controls, while the percentages of CD86 and HLA-DR expressing B lymphocytes were not significantly different between the patients and the controls. The percentage of HLA-DR expressing T lymphocytes (activated T lymphocytes) was significantly higher in the patients com-pared to the controls (Table 4).

Discussion

Dendritic cells and macrophages play an important role in all three phases of T1DM pathogenesis. In phase 1, DCs and macrophages accumulate at the islet edges. In phase 2, DCs and macrophages activate the auto reactive T cells that accumulate in the pancreas. In the third phase the islets are invaded by macrophages, DCs and NK cells followed by the destruction of the β cells [23]. Many factors are involved in pathogenesis of T1DM, including reduced numbers of dendritic cells, increased myeloid: lymphoid DCs ratios and DCs maturation and activation states [24–27].

Percentages of mDCs and pDCs in diabetic patients were reduced and mDCs/pDCs ratio was higher in patients. This suggests that the decreased DCs numbers, especially pDCs, may play a critical role in the pathogenesis of T1DM. The accumulation of DCs in the pancreas in all phases of the pathogenesis of T1DM could be responsible for the decrease of DCs in the PB as a result of migration of DCs from PB to the pancreas. Consistent with our results, Vuckovic et al., [19] reported that the absolute blood mDCs and pDCs numbers were decreased in diabetic patients. Hinkmann et al., [28] observed a significant reduction of pDCs and mDCs in

Table 1 Some demographic and clinical data of diabetic children and control

	Patients (N=25)	Controls (N=25)
Age	11.51±0.76	10.63±0.66
Sex (male/female)	13/12	8/12
Family history of T1D	3/25	–
Duration of diabetes (months)	22.15±2.52	–
Diabetic ketoacidosis (N)	4.2±0.51	–
Insulin dose (unit/kg)	0.9 unit/kg	–

N number

Table 2 Some laboratory characteristics of diabetic patients and controls

	Patients (N=25)	Controls (N=25)	P-value
Blood glucose concentration (mg/dl)	278±1.54	77.67±1.02	0.003
HbA1c %	9.2±0.3	5.2±0.02	0.000
Insulin C peptide (ng/ml)	0.52±0.02	3.51±0.32	0.000
Platelets (10^9/L)	221.56±13.53	226.04±14.54	0.075
WBCs (10^9/L)	6.70±0.41	6.48±0.39	0. 475
Hemoglobin (gm/dl)	11.82±0.21	12.55±0.33	0.360

Mann–Whitney Test

Data represented as means ± SEM

$P \leq 0.05$ is significant

HbA1c glycosylated hemoglobin, WBCs white blood cells

Table 3 Frequency of Dendritic cell and their expression of costimulatory molecules in diabetic patients and controls

	Patients (25)	Controls (25)	P value
mDCs%	0.14±0.02	1.22±0.08	0.000
pDCs %	0.12±0.03	0.97±0.07	0.009
mDCs/pDCs ratio	1.72±0.11	1.28±0.04	0.000
% CD86^{+}expression on the entire DCs	20.38±1.6	14.37±0.93	0.004
MFI of CD86^{+} on the entire DCs	172.29±14.83	115.29±8.24	0.003
% CD80^{+}expression on the entire DCs	12.63±0. 61	11.45±0.54	0. 046
MFI of CD80^{+} on the entire DCs	87.17±4.08	69.14±3.45	0.003

Mann–Whitney Test

Data represented as means ± SEM

$P \leq 0.05$ is significant

DCs dendretic cells, mDCs monocytoid DCs, pDCs plasmacytoid DCs, MFI mean fluorescent intensity, % percentage

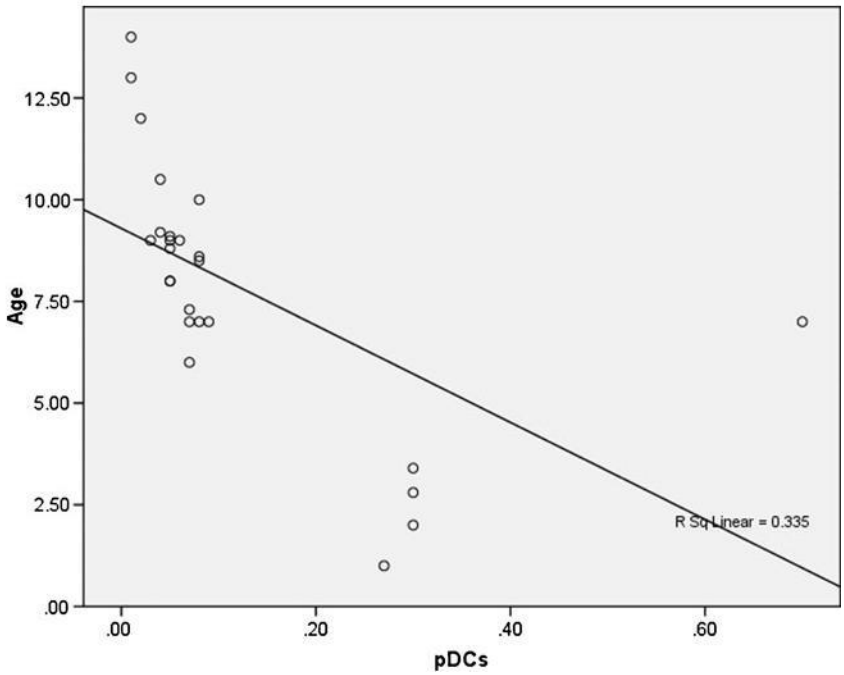

Fig. 2 A negative correlations between the frequency of pDCs and the age of diabetic patients (r=−0.601; P=0.002)

T1DM compared with controls. Chen et al., [29] reported that the frequency of pDCs was significantly reduced in T1DM patients compared to controls whereas, no difference in mDCs was observed. Studies in the NOD mice indicated that the loss of pDCs is associated with accelerated insulitis in NOD mice [30]. It has also been shown that increased pDC by granulocyte colony stimulating factor (GCSF) treatment is correlated with reduced diabetes incidence in NOD mice [31]. Transfer of exogenous DCs, e.g. pancreatic lymph node DCs or in vitro-generated dendritic cells, into pre diabetic NOD mice was protective against T1DM [32, 33].

Regarding their pivotal role in regulating T-cell immunity, DCs would be expected to maintain the functional balance between immunity and tolerance in T1DM [34]. During

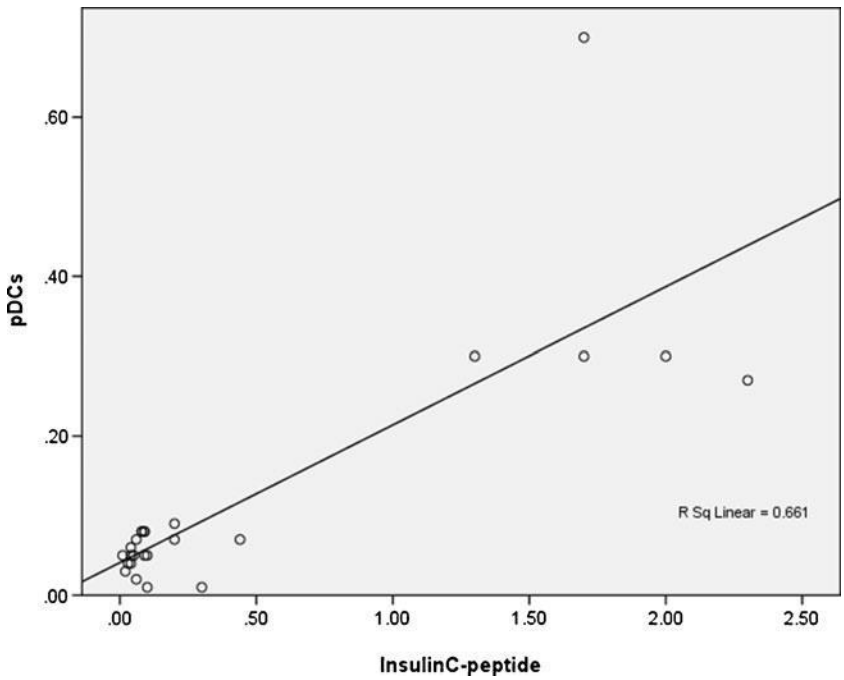

Fig. 3 A positive correlations between the level of insulin C-peptide and frequency of pDCs in diabetic patients (r=0.825; P=0.000)

Table 4 Frequency of B and T lymphocytes and the expression of costimulatory molecules and HLA-DR on B lymphocytes and HLA-DR on T lymphocytes in diabetic patients and controls

	Patients (25)	Control (25)	P value
$CD19^+$ %	11.88±0.61	12.62±0.55	0.298
$CD3^+$ %	58.38±2.30	63.45±1.67	0.063
$CD19^+$ $CD86^+$ %	5.11±0.22	5.40±0.32	0.481
$CD19^+$ $CD80^+$ %	8.17±0.69	5.39±0.25	0. 000
$CD19^+$ HLA-DR $\%^+$	7.76±0.49	8.66±0.59	0.291
$CD3^+$ HLA-DR^+ %	6.70±0.41	4.11±0.29	0.001

Data represented as means ± SEM

P≤0.05 is significant

HLA-DR human leukocyte antigen-DR, % percentage

development, deletion of high affinity auto reactive T cells occurred after presentation of self-peptides by DCs in the thymus [35]. Furthermore, DCs in the periphery can either immunize or tolerize T cells depending on their functional maturation, levels of antigen, costimulatory molecules, and other surface molecules [36–38]. Tolerance is associated with relatively immature DC that presents self-antigens to auto re-active Tcells. Immature DCs induce T-cell hypo responsiveness by anergy, apoptosis, or both, thereby silencing their auto reactive potential and could activate regulatory T cells [39, 40]. Conversely, activated mature DCs are potent T cell stimu-lators and capable of converting tolerance to immune activation [39].

We found higher expression of co stimulatory molecules CD80 (B7.1) and CD86 (B7.2) on DCs in diabetic children, along with MFI of CD80 and CD86 expression. This indicates that DCs in T1DM children display more mature phenotype than that of the controls and may become potent T cell stimu-lators. DCs maturation, specifically, elevated costimulator mol-ecule expression, has been associated with both heightened and prolonged T cell responses as well as induction of more strong-ly Th1-biased immunity [17]. The proinflammatory Th1 cells (producing IFN-γ) tend to be pathogenic whereas Th2 cells can protect from diabetes in the NOD mouse [41]. The ability of DCs to both immunize and tolerize T cells make them unique candidates for immunotherapy. Immunotherapy that promotes the differentiation of DCs to induce tolerance or a protective Th2 immune response may be an ideal treatment in T1DM [39]. Angelini et al., [42] studied the effect of stimulation of cultured mature DCs with lipopolysaccharide for at last 24 h in 18 children with T1DM and ten age-matched healthy children. They showed that the DCs derived from patients had reduced expression of B7.1and B7.2. The difference between our results and that of Angelini et al., could explained by the use of in vitro-generated DCs in the study conducted by Angelini and his collages.

We observed that the frequency of pDCs in PB decreased with age in diabetic patients, while no significant correlation with age was observed for mDCs, in conformity with previous studies [29, 43, 44]. Insulin C-peptide level decreased with the decrease in the frequency of pDCs in T1DM patients. Insulin C-peptide level is the most reliable factor evaluating the endogenous insulin secretion in patients with T1DM. The low level of C-peptide associated with severe T1DM. This may confirm that the decline in pDCs is associated with T1DM.

In our study, there was a difference in the expression of CD80 and CD86 on B lymphocytes, the percentage of CD80 expressing B lymphocytes was higher in patients, while the percentage of CD86 expressing B lymphocytes showed no difference between the patients and controls. The increased expression of CD80 might promote lymphocyte survival and interfere with the elimination of auto reactive lymphocytes. Co-stimulatory signals of CD80 and CD86 may lead to differential polarization of T helper cell responses. There is evidence that CD80 preferentially acts as a co stimulus for the generation of Th1 cells, whereas CD86 co stimulates and induces Th2 cells [45]. So the increased CD80 expression on B lymphocytes may lead to induction of Th1 which is more pathogenic.

The level of activated T-lymphocytes (HLA-DR expressing T lymphocytes) was higher than in controls. T lymphocyte activation could reflect an ongoing autoimmune process, because augmented expression of HLA-DR on $CD4^+$ cells suggests activation of a cellular immune response in T1DM. Our result is in agreement with Peakman et al., [46] who reported that levels of activated T-lymphocytes are increased in recently diagnosed patients with T1DM and remain elevated up to 6 months after diagnosis. Gessl and Walsha¨usl, [47] found increased HLA-DR expression on T-lymphocytes in long standing T1DM. Hoffman et al., [48] found increased activated T-lymphocytes in diabetic patients with ketoacidosis.

Limitations of the study This study is did not examine the natural history of the DCs during the progression to T1DM. It is unclear whether the observed reduction in DCs in the PB of T1DM patients is associated with the development of diabetes or it is a consequence of diabetes. Analyses of DCs in prospective studies will help to address this question. Also further study of functional activity of DCs is required.

Conflicts of interest None.

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