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Type 1 Diabetic Children and Siblings Share a Decrease in Dendritic Cell and Monocyte Numbers but are Differentiated by Expansion of CD4+T Cells Expressing IL-17

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Abstract

Type 1 diabetes (T1D) is an autoimmune disease characterised by multiple defects of immune cells which allow the expansion of pathogenic β cell-specific T effector cells and subsequent T1D development. We performed immunomonitoring assays on blood immune cells in T1D children, their siblings and controls with the supposition that the results would elucidate the sequence of abnormalities in peripheral immune cells leading to the progression of autoimmunity from non-diabetic siblings to diabetic children. We assessed myeloid dendritic cell (MDC), plasmacytoid (P)DC, monocyte, CD4+T and CD8+T cell compartments, and cytokine expression in CD4+T cells and CD8+T cells, by polychromatic flow cytometry in erythrocyte-lysed fresh blood. The numbers of CD16+MDC, PDC and CD16+ monocytes were similarly decreased in diabetic children and their siblings. Whereas the numbers of CD16-CD141-MDC, CD16-CD141+MDC, CD16- monocytes, T naïve (CD45RO-CD62L+), T central memory (CD45RO+CD62L+), T effector memory (CD45RO+CD62L-), T terminally-differentiated effector (CD45RO-CD62L-) and T regulatory cells (CD4+CD25+127lo/- or CD4+Foxp3+ cells) were not affected in diabetic children or their siblings. Furthermore, analysis of cytokine expression in CD4+T cells and CD8+T cell revealed an increased proportion of CD4+T cells expressing IL-17, differentiating diabetic children from their non-diabetic siblings. Our data suggest that diabetic children and their siblings suffer a considerable reduction of blood immune cells involving CD16+MDC, PDC and CD16+ monocytes that may result from shared genetic or environmental factors. However, this reduction of blood immune cells requires combination with the proinflammatory cytokine IL-17 to allow disease expression in diabetic children.

Abbreviations: T1D: Type 1 Diabetes; MDC: Myeloid Dendritic Cells; PDC: Plasmacytoid Dendritic Cells; T_N: T Naïve Cells; T_{CM}: T Central Memory Cells; T_{EM}: T Effector Memory Cells; T_{EMRA}:T terminally-differentiated effector cells; T_{REG}: T regulatory cells; NOD: non-obese diabetic; HLA: human leukocyte antigen; mAb: monoclonal antibodies; SD: standard deviation; GADA: glutamic acid decarboxylase autoantibodies; IA-2A: insulinoma-associated-2 autoantibodies ; RBC: red blood cells; WBC: white blood cells

Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterised by the expansion of pathogenic T effector cells which cause the irreversible destruction of insulin producing β cells [1]. However, these pathogenic T effector cells are not restricted to diabetic subjects, as they also persist in non-diabetic healthy controls [2]. It is believed that in healthy controls, peripheral tolerance mechanisms limit the pathogenic T effector cells whereas in diabetic subjects multiple immune defects breakdown this peripheral tolerance, permitting pathogenic T effector cell mediated T1D development [1].

Much of our understanding of peripheral immune cell defects in T1D comes from studies employing the non-obese diabetic (NOD) mouse model of T1D [1]. However, determining peripheral immune cell defects contributing to the development of T1D in humans remains a challenging task. The most promising approach in dentifying these defects is by directly comparing the blood immune cells from diabetic and non-diabetic subjects [2,3]. In particular, comparing diabetic patients with their non-diabetic siblings can provide major insight into disease specific defects as environmental factors and specific susceptibility genes are shared between these two groups [4]. Such studies can identify peripheral immune cell defects which are common to diabetic patients and their siblings, and as such, may result from shared genetic or environmental factors, as well as those that are disease specific.

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It has been proposed that dysregulation of the number and/or function of CD11c+ myeloid dendritic cells (MDC) and monocytes, which also express β cell-autoantigen [5], favors the differentiation and expansion of pathogenic T effector cells and/or results in failure to induce an effective T regulatory (T_{REG}) cell response [6]. Interest has, therefore, been focused on the characterisation of blood MDC, plasmacytoid (P)DC and T_{REG} cells in separate cohorts of diabetic subjects. However, such studies have produced discrepant results. In recent-onset T1D patients, increased blood MDC and PDC numbers have been reported relative to established T1D patients and controls [7], whereas other studies have shown no difference in the numbers of blood MDC and PDC between adult subjects with established T1D and age-matched controls [8]. We have previously reported that diabetic children, aged 2-10 years, exhibit a marked decrease in blood MDC and PDC numbers whereas this difference was less obvious when comparing older cohorts [3]. This finding of decreased MDC and PDC numbers in both recent-onset and established T1D subjects was interpreted as a primary manifestation of T1D [3]. A decreased number of T_{REG} cells has also been reported in children with recent-onset TID, adults with established T1D and in the offspring of parents who carry the high risk human leukocyte antigen (HLA) alleles [9,10], Other studies have found comparable T_{REG} cell populations in adults with established T1D and HLA /age-matched controls [11-13]. These differences between the reported blood DC and T_{REG} numbers may result from different experimental conditions (e.g. study of erythrocyte-lysed fresh blood samples [3,13] versus Ficoll-density gradient isolated blood mononuclear cells [7-12] or superficial analyses (e.g. restricted surface markers for identifying DC and T_{REG} cells, constraints of three- to four-color flow cytometry). In light of these limitations and the associated potential for data misinterpretation,

continuing efforts are required to re-evaluate blood DC and T_{REG} cell populations in diabetic and non-diabetic subjects. It is anticipated that ongoing immune activation induced by an array of proinflammatory cytokines produced by DC and monocytes [14-16] results in constant recruitment of CD4+T and CD8+T cells from the T naïve (T_N) and T central memory (T_{CM}) pools into the T effector memory (T_{EM}) and T terminally-differentiated effector (T_{EMRA}) pools. As such, the effect of immune activation on the dynamics of blood T_N, T_{CM}, T_{EM} and T_{EMRA} subsets is far from understood and may differ greatly between diabetic and non-diabetic subjects.

In this study, we examined quantitative changes in MDC, PDC, monocyte, CD4+T and CD8+T cell compartments and assessed cytokine expression in CD4+T cells and CD8+T cells from diabetic children relative to sibling and age-matched controls. To do this, we used minimally manipulated leukocytes from erythrocyte-lysed fresh blood stained with antibodies to cell surface markers, cytokines and the transcription factor Foxp3. Stained leukocytes were then assessed by polychromatic five- to seven-color flow cytometry which allowed the dissection of multiple subsets within the circulating DC, monocyte and T cell compartments.

Materials and Methods

Study subjects

Children with T1D (mean ± standard deviation [SD], hemoglobin A1c of 8.1% ± 2.9%, insulin dosage of 0.95 ± 0.24 unit/Kg/day) and their siblings with normal blood glucose levels were recruited from the Queensland Diabetes Centre at the Mater Children's Hospital. Age-matched, non-diabetic controls were recruited from children awaiting elective operations, and their blood samples were collected

	T1D	Siblings	Controls	Significance Value
Number	26	32	29	
&Male	15 (58%)	16 (50%)	14 (48%)	0.43
#Age (yrs)	12.28 (6.18)	11.75 (5.40)	8.18 (7.60)	0.25
#Disease duration (yrs)	2.93 (4.14)			
Autoantibody				
&GADA	15 (83%)	4 (14%)		
&IA-2A	14 (82%)	1 (4%)		
&GADA&IA-2A	14 (82%)	1 (4%)		
Blood Count (x10⁶/ml)				
#RBC	4790 (200)	4670 (330)	4560 (440)	a*
#WBC	6.30 (1.37)	6.23 (1.34)	9.05 (3.14)	a***, b***
#Lymphocytes	2.62 (0.89)	2.58 (0.65)	3.64 (1.04)	a***, b***
#Monocytes	0.49 (0.12)	0.48 (0.10)	0.64 (0.25)	a**, b***
#Neutrophils	2.87 (0.98)	2.85 (1.06)	4.34 (2.33)	a**, b**
#Eosinophils	0.20 (0.14)	0.14 (0.22)	0.25 (0.36)	0.26
#Basophils	0.04 (0.04)	0.05 (0.04)	0.06 (0.04)	0.13

&Categorical variables are presented using counts and percentages. #Continuous variables: WBC, Neutrophils, Lymphocytes and Monocytes are presented as mean (SD); Age, Disease duration, RBC, Eosinophils and Basophils are expressed as median (interquartile range). Denote: a, difference between T1D children and controls; b, difference between siblings and controls. *, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$.

Table 1: Characterisation of study subjects.

prior to surgery. Characteristics of all participants are shown in Table 1. Venous blood samples were collected in EDTA (K2E) Vacutainer tubes, processed for full blood counts on an automated blood analyzer (CELL-DYN Sapphire or CELL-DYN 3200, Abbott Diagnostics, USA) and analyzed by polychromatic flow cytometry within 12 hours of collection. This study was approved by the Mater Health Services Human Research Ethics Committee and all subjects participated after informed consent was given by the parents/guardians and assent by the children.

Cell staining and polychromatic flow cytometry

All staining protocols were performed using erythrocyte-lysed fresh blood. Monoclonal antibodies (mAb) were purchased from BD Bioscience (Sydney, NSW) unless otherwise specified. The protocol for surface antigen staining used 50 μl of whole blood. The Foxp3 and cytokine expression protocols used 200 μl of whole blood in which surface antigen staining was followed by fixation, permeabilization (Fixation&Permeabilization buffer, eBioscience, Sydney, NSW) and intracellular staining for Foxp3 or cytokines.

A seven-color flow cytometry assay was used to analyze total MDC (CD11c$^+$MDC) and their subsets (CD16$^+$CD141$^-$MDC, CD16$^-$CD141$^-$MDC and CD16$^-$CD141$^+$MDC) and PDC (CD123$^+$PDC) in the same Trucount tube (BD Bioscience). This assay used a mAb mix consisting of CD45-APCH7, CD3, 19, 20, 14, 56, 34-FITC, HLA-DR-PECy7, CD11c-APC, CD123-PercPCy5.5, CD141-PE (Miltenyi Biotech, Sydney, NSW) and CD16-Pacific Blue. Total CD14$^+$ monocytes, B cells and their respective subsets (CD16$^+$ and CD16$^-$ monocytes; CD27$^+$ and CD27$^-$ B cells) were analyzed in the same Trucount tube using a five-color flow cytometry assay with a mAb mix consisting of CD45-APCH7, CD14-FITC, CD20-APC, CD27-PE and CD16-Pacific Blue. Data relating to B cells have not been included in this study. CD4$^+$T and CD8$^+$T cell subsets: T_N cells (CD45RO$^-$CD62L$^+$), T_{CM} cells (CD45RO$^+$CD62L$^+$), T_{EM} cells (CD45RO$^+$CD62L$^-$) and T_{EMRA} cells (CD45RO$^-$CD62L$^-$) were defined in the same FACS tube using a five-color flow cytometry assay with a mAb mix consisting of CD45-APCH7, CD4-FITC, CD8-PECy7, CD45RO-PE and CD62L-APC. T_{REG} cells (CD4$^+$CD25$^+$CD127$^{lo/-}$) were defined using a five-color flow cytometry assay with a mAb mix consisting of CD45-APCH7, CD4-FITC, CD8-PECy7, CD127-PE and CD25-APC.

A five-color immunostaining protocol was used to analyze intracellular Foxp3 expression in CD4$^+$T cells (CD4$^+$Foxp3$^+$ T_{REG} cells) and cytokine expression in CD4$^+$T and CD8$^+$T cells. This protocol used a mAb mix consisting of CD45-APCH7, CD4-FITC, CD8-PECy7, CD25-APC in conjunction with either Foxp3-PE (Foxp3 Assay staining kit, eBioscience), IL-17A-PE, IL-2-PE (eBioscience), IFN-γ-PE, TGF-β-PE (IQ products, Groningen, the Netherlands), IL-6-PE, TNF-α-PE, IL-10-PE or IL-1β-PE.

Samples that were stained in Trucount tubes were acquired within 2 hours and in FACS tubes within 12 hours of staining (LSR II Flow Cytometer, BD). Data were analyzed using CellQuest (BD Bioscience) and FlowJo (TreeStar, Ashland, OR) software. The absolute numbers of MDC, PDC and monocytes (cells/μl blood) were calculated from the data acquired from Trucount tubes [3]. Proportions of: T_N, T_{CM}, T_{EM}, T_{EMRA}, T_{REG} cells, and CD4$^+$T and CD8$^+$T effector cells expressing specific cytokines were calculated from FACS tubes.

Statistical analysis

Characteristics of study subjects are presented as categorical and continuous variables (Table 1). Categorical variables are presented using counts and percentages. Fisher's exact test or the chi-squared test was used to compare categorical variables between the three groups of subjects. Continuous variables are presented using mean and SD or median and interquartile range depending on the normality of the data (assessed using the Shapiro-Wilk normality test with results confirmed using visual inspection of histograms). Analysis of variance or Kruskal-Wallis test was used to compare continuous variables between the three groups of subjects applying Bonferroni's adjustment for multiple comparisons in post-hoc testing using Tukey's and Dunn's tests respectively. Spearman's correlation test was used to analyze the association between clinical variables (age, age of diagnosis and disease duration) and continuous variables (T_{REG} cells and cytokine expressing T_{EM} cells) or association between continuous variables (T_{REG} cells and cytokine expressing T_{EM} cells). Analysis was performed using GraphPad Prism 5.0 software (San Diego, California). Differences were considered statistically significant at the 0.05 level.

Results

Numbers of CD11c$^+$CD16$^+$ MDC and PDC are decreased in diabetic children and their siblings

We previously reported decreased blood MDC and PDC numbers in diabetic children [3], however, whether this finding differentiated diabetic children from their siblings was not assessed. In this study, we compared the absolute number of multiple DC subsets in blood samples of diabetic children, their siblings and age-matched controls (Figure 1). In contrast to our earlier report, which employed four-color flow cytometry [3], this study used seven-color flow cytometry, again, in conjunction with Trucount beads. This increased the data-capturing throughput, and more importantly, enabled concurrent analysis of the absolute numbers of three subsets of CD11c$^+$MDC (CD16$^+$MDC, CD16$^-$CD141$^-$, CD16$^-$CD141$^+$) and CD123$^+$PDC in the same reaction tube (Figure 1A, representative dot plots). As such, this approach eliminated factors that affect accurate quantification of DC subsets from multiple reaction tubes [3,17]. Using this improved analysis, we found that both diabetic children and siblings displayed a similar decrease in the total numbers of CD11c$^+$MDC and CD123$^+$PDC compared to controls (Figure 1B). The decrease in total CD11c$^+$MDC number was specifically a result of a decreased CD16$^+$MDC number (Figure 1B). The comparable age range between cohorts of diabetic children, siblings and controls (Figure 1B) ruled out the possibility that age differences contributed to the observed decrease in CD16$^+$MDC and PDC numbers in diabetic children and siblings. In contrast to CD16$^+$MDC and PDC, the numbers of CD16$^-$CD141$^-$MDC and CD16$^-$CD141$^+$MDC were comparable across the three study groups (Figure 1B). These findings suggest that a decrease in CD16$^+$MDC and PDC lineages is common to both diabetic children and their siblings.

Number of CD14$^+$CD16$^+$ monocytes is decreased in diabetic children and their siblings

We hypothesized that monocytes, which are developmentally connected to DC [18], would also be altered in diabetic children or their siblings. We initially used full blood counts to examine the absolute

number of total blood monocytes in larger cohorts of diabetic children, siblings and controls (Figure 2A). This experimental approach revealed a decrease in total blood monocyte number in diabetic children and siblings compared to controls (Figure 2A). These cohorts of diabetic children, siblings and controls were comparable in age range (Figure 2A), therefore, ruling out the possibility that age differences contributed to the observed decrease in monocytes in diabetic children and siblings. In a smaller cohort of diabetic children, siblings and controls, total monocytes and their CD14+CD16+ and CD14+CD16- subsets were assessed by polychromatic flow cytometry (Figure 2B). This analysis revealed that the CD16+ monocyte subset exclusively accounted for the decrease in the number of total monocytes, while the number of CD16- monocytes was comparable between diabetic children, siblings and controls (Figure 2B). The subjects in these smaller cohorts were again comparable in age range (Figure 2B), therefore, ruling out the possibility of age as a contributing factor to the observed decrease in CD14+CD16+ monocytes in diabetic children and siblings. These data suggest that diabetic children and their siblings share a decrease in CD14+CD16+ monocytes, similar to what was noted for CD16+MDC and PDC.

Proportions of T_N, T_{CM}, T_{EM}, T_{EMRA} and T_{REG} cells are similar in diabetic children and their siblings

We reasoned that in diabetic children, ongoing immune activation could alter the dynamics of T_N, T_{CM}, T_{EM} and T_{EMRA} and T_{REG} cells

[9,19]. To address this possibility, the proportions of T_N, T_{CM}, T_{EM}, and T_{EMRA} cells within the CD4+T and CD8+T cell compartments and the proportion of T_{REG} cells within the CD4+T cell compartment of diabetic children, their siblings and controls were compared. The CD4+T and CD8+T cell compartments of diabetic children, siblings and controls contained similar proportions of T_N, T_{CM}, T_{EM}, and T_{EMRA} cells (Figure 3A). Proportion of T_{REG} cells, assessed by two commonly used marker combinations (CD4+CD25+CD127lo/- and CD4+Foxp3+) [20,21], were also comparable in diabetic children, siblings and controls (Figure 3B). In all three subject groups, there was no association between T_{REG} cells defined as CD4+CD25+CD127lo/- and CD4+Foxp3+ cells (data not shown), in disagreement to a previous report suggesting an association between CD4+CD25+CD127lo/- T cells and CD4+Foxp3+ T cells in adult controls [20].

We also performed association analyses in diabetic children correlating age (at time of sampling), age of diagnosis and disease duration with the proportions of CD4+CD25+CD127lo/- and CD4+Foxp3+ T_{REG} cells. In diabetic children, age and age of diagnosis was associated with an increase in the proportion of CD4+CD25+CD127lo/- T_{REG} cells but was not associated with the proportion of CD4+Foxp3+ T_{REG} cells (Figure 3C). In contrast, disease duration was not associated with the proportion of CD4+CD25+CD127lo/- or CD4+Foxp3+ T_{REG} cells (Figure 3C). Overall, these results suggest that disease development in diabetic children does not influence the dynamics of blood T_N, T_{CM}, T_{EM}, T_{EMRA} and T_{REG} cells. In addition, our study indicates that in diabetic children,

Figure 1: Enumeration of blood MDC and PDC. (A) Gating strategy for defining multiple blood MDC subsets and PDC. Left dot plot: events shown are gated upon CD45+ WBC. Middle dot plot: total CD11c+MDC (Gate 2) and CD123+PDC (Gate 3) are gated upon Lin-HLA-DR+ cells (Gate 1). Right dot plot: CD16-CD141+MDC (Gate 4), CD16+CD141-MDC (Gate 5) and CD16-CD141-MDC (Gate 6) are gated upon CD11c+MDC (Gate 2). **(B)** Scatter plot of the absolute number of total CD11c+MDC (median), CD16+MDC (median), CD16-CD141-MDC (median), CD16-CD141+MDC (mean) and CD123+PDC (mean). Absolute numbers of CD16+MDC and CD123+PDC correlated with age in T1D children, siblings and controls (Spearman P and r value). *, P < 0.05; **, P < 0.01.

Figure 2: Enumeration of blood monocytes. (A) Scatter plot of the absolute number of monocytes obtained by full blood count (mean). Absolute number of monocytes obtained by full blood count correlated with age in T1D children, siblings and controls (Spearman P and r value). **, $P < 0.01$; ***, $P < 0.001$. **(B)** Dot plot: gating strategy used to define blood monocyte subsets. The CD14+CD16- (Gate 1) and CD14+CD16+ (Gate 2) monocyte subsets are gated upon CD45+ WBC. Scatter plot of the absolute numbers of total CD14+ monocytes (Gate 1+2, median), CD14+CD16+ monocytes (Gate 2, median) and CD14+CD16- monocyes (Gate 1, mean). Absolute numbers of total monocytes and CD14+CD16+ monocytes correlated with age in T1D children, siblings and controls (Spearman P and r value). *, $P < 0.05$; ***, $P < 0.001$.

an age-related increase in the proportion of CD4+CD25+CD127lo/- T$_{REG}$ cells exists.

Expansion of CD4+T cells expressing IL-17 differentiates diabetic children from their siblings

Despite our finding that diabetic children and their siblings did not display a difference in the proportion of specific subsets among CD4+T and CD8+T cells, it is clear that not all such T cells are equivalent in diabetic children and their non-diabetic siblings and, therefore, different effector functions should be elicited by such T cells. As a result, we proposed that function rather than quantity of T cells may represent the means by which T cells differentiate diabetic children from their non-diabetic siblings. In this study, functional attributes of CD4+T and CD8+ T cells from diabetic children, siblings and controls were assessed by expression of IL-17, IL-2, IFN-γ, IL-6, TGF-β, TNF-α, IL-10 and IL-1β cytokines. A discrete fraction of circulating CD4+T cells expressing IL-17 differentiated diabetic children from siblings and controls (Figure 4A, Supplementary Figure 1). In diabetic children, these CD4+T cells expressing IL-17 were accompanied by expansion

of CD4+T and CD8+T cells expressing IL-2 (Figure 4A, Supplementary Figure 1).

We next investigated the association between the proportion of CD4+T cells expressing IL-17 and the proportion of T$_{REG}$ cells, as these two categories of CD4+ T cells may show contrasting functions during immune responses [22]. In diabetic children, there was no association between the proportion of CD4+T cells expressing IL-17 and the proportion of T$_{REG}$ cells defined as CD4+CD25+CD127lo/- and CD4+Foxp3+ cells (data not shown). Also, there was no association between the proportion of CD4+T cells expressing IL-17 and the proportion of CD4+T or CD8+T cells expressing IL-2. In addition, there was no association between age, age of diagnosis or disease duration and the proportion of CD4+T cells expressing IL-17, or the proportion of CD4+T and CD8+T cells expressing IL-2 (Figure 4B).

Discussion

This study compared MDC, PDC, monocyte, CD4+T and CD8+T cell compartments in diabetic children, their siblings and controls, as well as immune activation as assessed by cytokine expression in

Figure 3: Proportions of T_N, T_{CM}, T_{EM}, T_{EMRA} and T_{REG} cells in peripheral blood samples. (A) Gating strategy used to define T_N, T_{CM}, T_{EM}, T_{EMRA} subsets □ in CD4+T and CD8+T cell compartments based on CD45RO and CD62L expression. Left dot plot: events shown are gated upon CD45+ WBC. Middle and right dot plots: T_N, T_{CM}, T_{EM} and T_{EMRA} cells are gated upon CD4+T cells (Gate 1) and CD8+T cells (Gate 2). Percentages of T_N, T_{CM}, T_{EM} and T_{EMRA} cells in CD4+T and CD8+T cell compartments in T1D children, siblings and controls (median with interquartile range). **(B)** Dot plots: gating strategy used to define CD25+127lo/- T_{REG} cells (Gate 3) and CD4+Foxp3+ T_{REG} cells (Gate 4) in CD4+T cell compartment. Percentages of CD25+127lo/- T_{REG} and CD4+Foxp3+ T_{REG} in T1D children, siblings and controls (mean ± SD). **(C)** Percentages of CD25+127lo/- T_{REG} and CD4+Foxp3+ T_{REG} cells correlated with age, age of diagnosis and disease duration in T1D children (Spearman P and r value).

Figure 4: Effector CD4+T cells and CD8+T cells in peripheral blood samples. (A) Scatter plot with median of the percentage of effector CD4+T cells (left Y axis) and CD8+T cells (right Y axis) that expressed IL-17, IL-2, IFN-γ, IL-6, TGF-β, TNF-α, IL-10 or IL-1β in T1D children, siblings and controls. *, P < 0.05; **, P < 0.01. **(B)** Percentages of CD4+T cells expressing IL-17 and percentages of CD4+T and CD8+T cells expressing IL-2 correlated with age, age of diagnosis and disease duration in T1D children (Spearman P and r value).

CD4+T and CD8+T cells. This approach was based on the supposition that identified blood immune cell abnormalities may explain the progression of autoimmunity from non-diabetic siblings to diabetic children. Diabetic children and siblings living in the same family were recruited to minimize differences in age and environmental factors which could confound the immunological assessment of blood cells [3,23]. This study demonstrates two main points; firstly, diabetic children and siblings have decreased numbers of circulating blood immune cells involving CD16+MDC, PDC and CD16+ monocytes, and secondly, diabetic children have an expanded pool of CD4+T cells expressing IL-17 which differentiates diabetic children from their siblings and controls.

In humans, a quantitative deficiency in blood DC and monocyte lineages is a rare occurrence and has traditionally been interpreted as a consequence of disease, in particular to certain infections [24] and malignancy [17]. Our data suggest that a decrease in blood CD16+MDC, PDC and CD16+ monocyte numbers is not specific to disease status, rather it is common to both diabetic children and their healthy non-diabetic siblings and may signify a shared genetic or environmental origin. As such, a decrease in blood CD16+MDC, PDC and CD16+ monocytes is not sufficient by itself for disease development, as the vast majority of siblings (> 90%) do not develop T1D [25]. Therefore, the contribution of a quantitative defect in peripheral blood CD16+MDC, PDC and CD16+ monocytes in driving T1D pathology remains unclear. However it is worth noting that blood CD16+MDC, PDC and CD16+ monocytes belong to the family of blood CD11c+ myeloid cells which express the β cell-autoantigen proinsulin [5], a target autoantigen in T1D. Although expression of β cell-autoantigen(s) on discrete blood DC and monocyte subsets has yet to be defined, it is possible that CD16+MDC, PDC and CD16+ monocytes may also be an important source of β cell-autoantigen(s), and decreased numbers of these cells may impact on peripheral tolerance.

It is unclear what causes the decrease in CD16+MDC, PDC and CD16+ monocyte number in diabetic children and their siblings, nonetheless, it is possible that a dysregulated development from myeloid precursors or a reduced lifespan within the circulation can contribute. With regard to dysregulated myeloid cell development, the highly conserved transcription factor *Hox11* is of particular interest, as its over expression can immortalize or reprogram bone-marrow derived progenitors [26,27]. A recent study in NOD mice suggests that *Hox11*-derived organs are prone to developing primary structural and functional defects largely independent of autoimmune-mediated destruction of the pancreas [28]. It is equally possible that in diabetic children and their siblings, the lifespan of blood CD16+MDC, PDC and CD16+ monocytes is shortened due to their migration into the extra-vascular space. Consistent with this concept, DC and macrophages are attracted to the pancreas of NOD mice in the early stages of insulitis prior to disease onset [29]. Further studies are needed to address the mechanisms causing the decreased number of CD16+MDC, PDC and CD16+ monocyte in diabetic children and their siblings and how this may promote the breakdown of islet β cell tolerance. Of interest is our observation that diabetic children and siblings have a reduced numbers of neutrophils and lymphocytes in addition to the decreased CD16+MDC, PDC and CD16+ monocyte number (Table 1). In contrast, the red blood cell count was increased in diabetic children when compared to controls (Table 1). Our study complements previous reports suggesting decreased lymphocyte number (lymphopenia) in

patients with autoimmune diseases [30], and decreased lymphocytes, myeloid cells and DC but not erythromyeloid cells in NOD mice before the onset of insulitis [31,32].

In diabetic subjects, chronic immune activation would be expected to influence the dynamics of blood CD4+ T and CD8+ T cell subsets, in particular, favoring an increased proportion of pathogenic T_{EM} cells [16,33,34]. In addition, with the expansion of pathogenic T_{EM} cells and the progression of β cell autoimmunity, a decreased number or diminished function of blood T_{REG} cells might occur [9,19]. However, we found that the CD4+ T and CD8+ T cell subsets demarked as T_N, T_{CM}, T_{EM} and T_{EMRA} cells were comparable in diabetic children, siblings and controls. There were also no detectable differences in the proportion of T_{REG} cells defined as CD4+CD25+CD127lo/- and CD4+Foxp3+ T cells between the three groups, a finding similar to other studies investigating diabetic subjects [11-13]. However, our study identified a strong association between the age at time of sampling and age of diagnosis and the proportion of CD4+CD25+CD127lo/- T_{REG} cells, suggesting in line with a previous study [19] that age matching between diabetic and non-diabetic subjects is required for the immunological assessment of blood T_{REG} cells. This age-related increase in CD4+CD25+CD127lo/- T_{REG} cells in diabetic children can mask any effect that disease duration may have on dynamics of blood T cells subsets including T_{REG} cells. Also, in diabetic children and non-diabetic children (siblings and controls), the lack of association between CD4+CD25+CD127lo/- T cells and CD4+Foxp3+ T cells, which are categorically accepted as T_{REG} cells in adult subjects [20,21], calls into question the use of these marker combinations in assessing T_{REG} cells in pediatric subjects.

Although T cell subsets are comparable in diabetic children, their siblings and controls, we observed an expansion of CD4+ T cells expressing IL-17 exclusively in diabetic children. Two recent articles suggest that CD4+T cells from diabetic children are capable of IL-17 production upon *in vitro* activation [33,34]. However, our study represents the first report that circulating CD4+T cells expressing IL-17 are detectable directly in unstimulated erythrocyte-lysed fresh blood samples of diabetic children. We detected circulating CD4+T cells expressing IL-17 in children with established T1D (1.3-10.9 yrs), suggesting that these cells may be associated with continuing progression of β cell autoimmunity. Certainly in inflammatory conditions, circulating CD4+T cells expressing IL-17 contribute to the proinflammatory responses, and in diabetes they may promote human islet cell destruction [33]. Recent studies also suggest that in diabetic children, distinction of Th17 immunity is not absolute, and IL-17 expressing cells may exhibit an intermediate pattern of cytokine and transcription factor production. For example, CD4+T cells expressing IL-17 coexpress IFN-γ and Foxp3, biomolecules originally described as being restricted to Th1 and T_{REG} cells respectively [33,34]. Studies using NOD mice also emphasize that coexpression of IL-17 and IFN-γ by pathogenic T cells contributes to the effector phase of disease [35,36]. In our study design we did not explore coexpression of IFN-γ and Foxp3 on CD4+T cells expressing IL-17, and it will be of interest to define whether distinctive Th17 patterns differentiate diabetic children from their siblings. Further research is required to establish whether these blood CD4+T cells expressing IL-17 are capable of directly reacting against self-tissue, including islet β cells. Such insights may provide the rationale for strategies aimed at selective elimination of tissue (islet) autoreactive T cells.

Increased proportions of CD4+T and CD8+T cells expressing IL-2 were also detected in diabetic children. The role of IL-2 in regulating autoimmunity appears to be complex [37,38]. On one side, IL-2 can prevent autoimmunity through the generation and maintenance of T_{REG} cells [37]. However, IL-2 has also been reported to promote some aspects of autoimmunity through stimulating T cell activation, perhaps in the same way that it promotes T cell adaptive immunity against foreign antigen [38]. Evidence from a human study supports a role for IL-2 in the development of IL-17 cells from T_{REG} cells [39]. Nevertheless, our data suggest that in diabetic children, combined IL-2 and IL-17 responses contribute to the inflammatory environment that sustains β cell damage and the clinical onset of T1D.

Our results, suggest that a decrease in circulating blood immune cells, in particular CD16+MDC, PDC and CD16+ monocytes, by itself is not sufficient for the development of T1D. However, the combination of decreased circulating immune cells and IL-17 proinflammatory responses appears to be important for the breakdown of peripheral tolerance and ultimately, disease expression in diabetic children.

Acknowledgements

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