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 cells (CD45RO CD62L-) and T regulatory cells (CD4 CD25 Foxp3) 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.

Keywords: Type 1 diabetic children; Siblings; Dendritic cells; Monocytes; IL-17 responses

Abbreviations: T1D: Type 1 Diabetes; MDC: Myeloid Dendritic Cells; PDC: Plasmacytoid Dendritic Cells; T\textsuperscript{N}: T Naïve Cells; T\textsuperscript{CM}: T Central Memory Cells; T\textsuperscript{EM}: T Effector Memory Cells; T\textsuperscript{EM, T}: Terminally-differentiated effector cells; T\textsuperscript{TE}: 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 (TReg) cell response [6]. Interest has, therefore, been focused on the characterisation of blood MDC, plasmacytoid (P)DC and TReg 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 TReg cells has also been reported in children with recent-onset T1D, 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 TReg cell populations in adults with established T1D and HLA age-matched controls [11-13]. These differences between the reported blood DC and TReg numbers may result from different experimental conditions (e.g. study of cytokines produced by DC and monocytes [14-16] results in constant recruitment of CD4⁺T and CD8⁺T cells from the T naïve (Tₙ) and T central memory (Tₘ) pools into the T effector memory (TₑM) and T terminally-differentiated effector (TₑMRA) pools. As such, the effect of immune activation on the dynamics of blood Tₙ, TₑM, TₑMRA and Tₘ 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 awaiting elective operations, and their blood samples were collected:

| T1D Siblings Controls Significance Value |
|-----------------------------------------|----------------------------------------|
| Number                                  |                                       |
| Male                                     | 15 (58%)                               |
| Male                                     | 16 (50%)                               |
| Male                                     | 14 (48%)                               |
| Age (yrs)                                | 12.28 (6.18)                           |
| Age (yrs)                                | 11.75 (5.40)                           |
| Age (yrs)                                | 8.18 (7.60)                            |
| Disease duration (yrs)                   | 2.93 (4.14)                            |

| Autoantibody                             |
|------------------------------------------|
| GADA                                     | 15 (83%)                               |
| IA-2A                                    | 14 (82%)                               |
| GADA&IA-2A                               | 14 (82%)                               |

| Blood Count (x10⁶/ml)                     |
|------------------------------------------|
| RBC                                      | 4790 (200)                             |
| WBC                                      | 6.30 (1.37)                            |
| Lymphocytes                              | 0.20 (0.14)                            |
| Monocytes                                | 0.04 (0.04)                            |
| Neutrophils                              | 2.62 (0.89)                            |
| Eosinophils                              | 2.87 (0.98)                            |
| Basophils                                | 0.49 (0.12)                            |
| **Autoantibody**                         |
| GADA                                     | 4 (14%)                                |
| **IA-2A**                                |
| A1c of 8.1% ± 2.9%, insulin dosage of 0.95 ± 0.24 unit/Kg/day |

*Continuous variables: RBC, 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 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 was 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+ and CD8+ T cell subsets: Tn cells (CD45RO CD62L−), TCAM cells (CD45ROCD62L−), Treg cells (CD45ROCD62L−) and T naïve cells (CD45ROCD62L+) 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, CD127-Pe and CD25-APC.

A five-color immunostaining protocol was used to analyze intracellular Foxp3 expression in CD4+ T cells (CD4+Foxp3+ Treg 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: Treg, TCAM, TEM, T naïve, Treg 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 (Treg cells and cytokine expressing TEM cells) or association between continuous variables (Treg cells and cytokine expressing TEM 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+CD141−, CD16+CD141+, and 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 CD16+CD16- monocytes in diabetic children and siblings. These data suggest that diabetic children and their siblings share a decrease in CD16+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. To address this possibility, the proportions of $T_N$, $T_{CM}$, $T_{EM}$ and $T_{EMRA}$ cells within the CD4+ 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+CD127dim 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+CD127dim and CD4+Foxp3+ cells (data not shown), in disagreement to a previous report suggesting an association between CD4+CD25+CD127dim 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+CD127dim 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+CD127dim $T_{REG}$ 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+CD127dim 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,
an age-related increase in the proportion of CD4+CD25+CD127lo/- TReg 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/hi 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+ 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\textsubscript{N}, T\textsubscript{CM}, T\textsubscript{EM}, T\textsubscript{EMRA} and T\textsubscript{REG} cells in peripheral blood samples. (A) Gating strategy used to define T\textsubscript{N}, T\textsubscript{CM}, T\textsubscript{EM}, T\textsubscript{EMRA} subsets in CD4\textsuperscript{+} T and CD8\textsuperscript{+} T cell compartments based on CD45RO and CD62L expression. Left dot plot: events shown are gated upon CD45\textsuperscript{+} WBC. Middle and right dot plots: TN, TCM, TEM and TEMRA cells are gated upon CD4\textsuperscript{+} T cells (Gate 1) and CD8\textsuperscript{+} T cells (Gate 2). Percentages of CD4\textsuperscript{+} T cells expressing IL-17 and percentages of CD4\textsuperscript{+} T and CD8\textsuperscript{+} T cells expressing IL-2 correlated with age, age of diagnosis and disease duration in T1D children (Spearman P and r value).

Figure 4: Effector CD4\textsuperscript{+} T cells and CD8\textsuperscript{+} T cells in peripheral blood samples. (A) Scatter plot with median of the percentage of effector CD4\textsuperscript{+} T cells (left Y axis) and CD8\textsuperscript{+} T cells (right Y axis) that expressed IL-17, IL-2, IFN-\gamma, IL-6, TGF-\beta, TNF-\alpha, IL-10 or IL-1\beta in T1D children, siblings and controls. *, P < 0.05; **, P < 0.01. (B) Percentages of CD4\textsuperscript{+} T cells expressing IL-17 and percentages of CD4\textsuperscript{+} T and CD8\textsuperscript{+} 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 [31,32]. It is also possible that the over expression of Hox11 in islets of Langerhans can contribute to the destruction of β cells [33]. Recent studies also suggest that in diabetic children, distinction of Th17 immunity is not absolute, and IL-17 expression 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_em, T_em and T_EMA 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⁺CD127⁻ 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⁺CD127⁻ 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⁺CD127⁻ 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⁺CD127⁻ 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.

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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<sub>REG</sub> 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<sub>REG</sub> 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.

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