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Th1/Th17 Plasticity Is a Marker of Advanced β Cell Autoimmunity and Impaired Glucose Tolerance in Humans

Linnea Reinert-Hartwall,*1 Jarno Honkanen,*1 Harri M. Salo,* Janne K. Nieminen,* Kristiina Luopajärvi,*1† Taina Härkönen,† Riitta Veijola,† Olli Simell,§ Jorma Ilonen,†,§ Aleksandr Peet,§ Vallo Tillmann,*,1 Mikael Knip,†,*††‡‡,2 Outi Vaarala,†,§,2 and The DIABIMMUNE Study Group3

Upregulation of IL-17 immunity and detrimental effects of IL-17 on human islets have been implicated in human type 1 diabetes. In animal models, the plasticity of Th1/Th17 cells contributes to the development of autoimmune diabetes. In this study, we demonstrate that the upregulation of the IL-17 pathway and Th1/Th17 plasticity in peripheral blood are markers of advanced β cell autoimmunity and impaired β cell function in human type 1 diabetes. Activated Th17 immunity was observed in the late stage of preclinical diabetes in children with β cell autoimmunity and impaired glucose tolerance, but not in children with early β cell autoimmunity. We found an increased ratio of IFN-γ/IL-17 expression in Th17 cells in children with advanced β cell autoimmunity, which correlated with HbA1c and plasma glucose concentrations in an oral glucose tolerance test, and thus impaired β cell function. Low expression of Helios was seen in Th17 cells, suggesting that Th1/Th17 cells are not converted thymus-derived regulatory T cells. Our results suggest that the development of Th1/Th17 plasticity may serve as a biomarker of disease progression from β cell autoantibody positivity to type 1 diabetes. These data in human type 1 diabetes emphasize the role of Th1/Th17 plasticity as a potential contributor to tissue destruction in autoimmune conditions. The Journal of Immunology, 2015, 194: 68–75.

Type 1 diabetes is an autoimmune disease caused by T cell–mediated destruction of the pancreatic β cells. As the first marker of disease development, autoimmune antibodies against β cell Ags appear into the peripheral blood. During this prediabetic phase, multiple diabetes-associated autoantibodies emerge, such as islet cell Abs; insulin autoantibodies (IAA), glutamic acid decarboxylase Abs (GADA), insulinoma-associated-2 Abs (IA-2A), and zinc transporter 8 Abs (ZnT8A) (1, 2). Although individuals at risk for type 1 diabetes are recognized by screening for HLA-associated risk genotypes and β cell autoantibodies, there is a lack of biomarkers for progression to clinical type 1 diabetes in autoantibody-positive individuals.

Type 1 diabetes is mediated by IFN-γ–producing Th1 cells (3, 4), but recently also the role of IL-17–secreting Th17 cells has been implicated. Th17 immunity is upregulated in the course of insulitis in spontaneous autoimmune diabetes in the NOD mouse, and the neutralization of IL-17 has been observed to prevent diabetes (5). We have previously reported upregulation of Th17 immunity in stimulated PBMCs and in circulating memory T helper cells in children with type 1 diabetes (6). Marwaha et al. (7) showed a significant increase in the proportion of IL-17–secreting CD4+ but also CD8+ cells in patients with type 1 diabetes. Arif et al. (8) found upregulation of the IL-17 response in PBMCs stimulated by islet Ags, and a more recent study demonstrated increased IL-17 immunity in the pancreatic lymph nodes in patients with type 1 diabetes (9). Elevated plasma levels of IL-17 have also been observed in autoantibody-positive children when compared with autoantibody-negative children (10).

IL-17 in combination with IL-1β and IFN-γ reportedly mediates detrimental effects on human pancreatic islets and β cells in vitro.
IL-17 increased β cell apoptosis and upregulated the expression of stress response genes and proinflammatory chemokines in β cells (6, 8, 11). Accordingly, the upregulation of Th17 immunity could contribute to the destruction of β cells and the development of type 1 diabetes.

Animal studies suggest that plasticity of Th17 cells, and the development of IFN-γ and IL-17 coproducers in particular, is associated with autoimmunity. Th17 cells from BDC2.5 mice induced autoimmune diabetes in healthy recipients after their conversion into Th1 cells in vivo. The expression of IL-17 was downregulated and IFN-γ was upregulated in vivo in purified BDC2.5 Th17 cells, which infiltrated the islets and transferred diabetes (12, 13). Novelization of IFN-γ with Abs inhibited diabetes (12, 13), suggesting that the development of a Th1-type response in Th17 cells was essential for the initiation of β cell destruction. In humans, the conversion of Th17 cells into Th1/Th17-type cells has been reported in the synovial fluid of children with juvenile arthritis (14), and in patients with Crohn’s disease IFN-γ-expressing Th17 cells have been demonstrated in the gut (15). These results suggest that the plasticity of Th17 cells is promoted by the inflammatory cytokine milieu in the target tissue in autoimmune conditions.

There is some evidence of T cell plasticity in human type 1 diabetes. Marwaha et al. (7) reported that Th17 cells in type 1 diabetes also expressed FOXP3, which might imply regulatory activity. Beriou et al. (16) found that subjects with type 1 diabetes had a higher frequency of memory CD4+ cells with the capacity to transition into Th17 cells positive for IL-9. Additionally, plasticity of regulatory T cells (Tregs) has been observed in diabetic patients. Purified FOXP3+ Tregs producing IFN-γ showed, however, low expression of Helios, suggesting that the origin of the Tregs with characteristics of a Th1 effector phenotype was probably not from thymus but from the periphery (17).

In this study, we aimed to examine the timing of the upregulation of Th17 immunity and assess the degree of plasticity of Th17 cells in the development of type 1 diabetes, and accordingly we investigated samples from children in various phases of diabetes-associated autoimmunity up to clinical disease. Upregulation of IFN-γ, IL-9, and IL-17 and plasticity of Th17 cells were only seen in children with advanced β cell autoimmunity and impaired glucose tolerance or clinical type 1 diabetes. Interestingly, the IFN-γ/IL-17 mRNA ratio in Th17 cells correlated with glycosylated hemoglobin (HbA1c) and plasma glucose concentrations in an oral glucose tolerance test performed in the children with advanced β cell autoimmunity, suggesting that the increased degree of Th1/Th17 type of plasticity could reflect impaired β cell function and the degree of β cell destruction. Our results show that the upregulation of Th1/Th17 immunity is a late phenomenon in the preclinical phase and could serve as a biomarker for the development of clinical type 1 diabetes in children with diabetes-associated autoantibodies.

Materials and Methods

Study subjects

Altogether we studied 159 children: 28 Finnish children with type 1 diabetes, 90 children (57 Finnish and 33 Estonian children) from the DIABMINI study (5.1 y, 9 males), and 41 children from the Finnish Type 1 Diabetes Prediction and Prevention study (19).

Upregulation of Th17 immunity in PBMCs was studied in 29 children with early β cell autoimmunity (autoantibody [AAb]+), average age 5.7 ± 3.1 y, 13 males), 9 children with advanced long-term β cell autoimmunity and impaired glucose tolerance demonstrated in at least once in an oral glucose tolerance test (OGTT) (impaired glucose tolerance [IGT], average age 7.7 ± 4.3 y, 7 males), 80 children negative for β cell autoantibodies (AAb−, average age 4.8 ± 2.3 y, 30 males), and 15 children with type 1 diabetes (average disease duration 289 ± 586 d, median disease duration 31, average age 8.3 ± 5.5 y, 9 males).

Nine of the 29 children (31%) with early β cell autoimmunity had one autoantibody at the study visit, 7 children (24%) tested positive for two autoantibodies, 6 children (21%) were positive for three autoantibodies, and 7 children were positive for four autoantibodies. One of nine children with advanced β cell autoimmunity and IGT tested positive for one biochemical autoantibody (11%), two children were positive for two autoantibodies (22%), four children were positive for three autoantibodies (44%), and two children were positive for four biochemical autoantibodies (22%) at the time of assessing the activation of Th17 immunity.

Plasticity of Th17 cells was studied in 8 autoimmune-negative children (AAb−, average age 5.5 ± 2.3 y, three males), 5 healthy children with early β cell autoimmunity (AAb+, average age 5.8 ± 3.5 y, four males), 6 children with advanced long-term β cell autoimmunity (average age 9.2 ± 4.5 y, six males), and 13 children with type 1 diabetes (average disease duration 508 ± 166 d, median disease duration 756 d, average age 10.2 ± 5.1 y, 7 males).

Eighty-seven of the studied children (55%) carried risk-associated HLA-DR/DQ genotypes (14 strongly, 52 moderately, and 21 slightly increased risk) and 66 (42%) carried genotypes conferring neutral or decreased risk for type 1 diabetes. Risk groups were defined as described earlier in Hekkala et al. (20). The risk-associated HLA genotype of five children (3.0%) with type 1 diabetes was not known at the time of analysis. Detailed characteristics of the study subjects are presented in Supplemental Table 1.

This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the local Ethical Committees. The parents and/or study subjects gave their written informed voluntary consent prior to blood sample collection.

HLA genotyping and analysis of diabetes-associated autoantibodies

HLA genotyping of major type 1 diabetes risk DR-DQ haplotypes of the study subjects was performed with a PCR-based lanthanide-labeled hybridization method using time-resolved fluorometry for detection as described before (21).

The diabetes-associated Abs IAA, GADA, IA-2A, and ZnT8A were quantified with specific radiobinding assays as previously described (22). The cutoff level was 2.80 relative units for IAA, 5.36 for GADA, 0.78 for IA-2A, and 0.61 relative units for ZnT8A, representing the 99th percentiles in >350 Finnish nondiabetic children. Assays were performed at least at two time points to ensure persistent autoantibody formation. Islet cell Abs were detected with the use of indirect immunofluorescence with a cutoff limit for positivity of 2.5 Juvenile Diabetes Foundation units.

T cell preparations and cell cultures

Plasma was separated from the heparinized blood samples by centrifugation, and PBMCs were isolated by Ficoll isogradient centrifugation (GE Healthcare, Uppsala, Sweden). PBMCs were washed three times with PBS (Lonza, Verviers, Belgium) and resuspended in serum-free X-VIVO 15 culture medium (Lonza). For studying the activation of Th17 immunity, PBMCs were cultured at the density of 2 × 10^6 cells/200 μl/well in triplicates for 40 h in 96-well round-bottom culture plates (Thermo Scientific, Waltham, MA). The cells were stimulated with plate-bound anti-CD3 (precoating with anti-CD3 at 5 μg/ml in PBS, BD Pharmingen, San Diego, CA) and soluble anti-CD28 (1 μg/ml, BD Pharmingen). Stimulated cells were lyzed in Qiagen RLT lysis buffer (Qiagen, Hilden, Germany) for reverse transcription–quantitative PCR (RT-qPCR).

Sorting of activated Th17 cells

For studying plasticity of activated Th17 cells, PBMCs were stimulated for 72 h as described in the previous section. Activated Th17 cells were labeled with the IL-17 secretion assay kit (Miltenyi Biotec, Gladbach, Germany) with optional staining with anti-CD4-allophycocyanin. Th17 cells were sorted with FACSAria II (BD Biosciences). CD4+ cells with high or intermediate expression of IL-17 (Th1-high and Th17-med) were collected (Fig. 2) and lysed in RLT buffer (Qiagen) for RT-qPCR analysis.

RT-qPCR

Total RNA from stimulated PBMCs was isolated with the Qiagen RNeasy Mini kit (Qiagen). An RNeasy Plus Micro kit (Qiagen) was used to isolate total RNA from FACS-purified Th17 cells. cDNA was synthesized using the random hexamer priming of a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). qPCR was performed with TaqMan Fast Master Mix and a StepOne Plus instrument (Applied Biosystems). TaqMan gene expression assays (Applied Biosystems) were used for real-time PCR. Ribosomal 18S was used as an endogenous reference. Relative expression was calculated by the 2^−ΔΔct method.
Analysis method of IFN-γ transcripts in FACS-sorted IFN-γ and IL-17–secreting CD4+ cells

To evaluate the correlation between the expression of IFN-γ mRNA and protein we sorted IFN-γ and/or IL-17A–secreting CD4+ cells from two autoantibody-negative children, three children with advanced β cell autoimmunity, and two children with type 1 diabetes. Briefly, frozen PBMCs were thawed and allowed to recover in X-VIVO 15 culture medium for 4 h in a CO2 incubator at 37°C. Then, the cells were stimulated for 72 h as freshly prepared PBMCs. Stimulated PBMCs were labeled with IL-17–allophycocyanin and IFN-γ–PE secretion assay kits (two-color cytokine secretion assays, Miltenyi Biotec) with CD4-FITC and 7-aminoactinomycin D staining. Viable CD4+ T cells were analyzed, and double-positive and IL-17 or IFN-γ single-positive cells were sorted and subjected to RT-qPCR analysis.

Statistical analyses

Comparisons between groups were performed for log10-transformed data with one-way ANOVA and unpaired or paired Student t tests. Correlations were analyzed with the nonparametric Spearman test. A p value <0.05 was considered as significant. Statistical analyses were performed with the GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA), except for the correlation analyses, which were performed with the SPSS 20 software (IBM, Chicago, IL).

Results

Upregulation of the IL-17 pathway in anti-CD3– and anti-CD28–stimulated PBMCs in type 1 diabetes and in advanced β cell autoimmunity

Gene expression of IL-17A, IL-17F, IL-22, IFN-γ, and FOXP3 was higher in children with type 1 diabetes in comparison with autoantibody-negative children and to the children with early β cell autoimmunity (except for IL-22) (Fig. 1). The children with advanced β cell autoimmunity (IGT) showed an increased expression of IL-9 and IFN-γ when compared with autoantibody-negative children (Fig. 1D and 1E, respectively) and enhanced IL-17F levels when compared with the autoantibody-negative children and children with early β cell autoimmunity (Fig. 1B). No differences in the expression of IL-17A, IL-17F, IL-22, IL-9, IFN-γ, or FOXP3 mRNA in activated PBMCs were observed between the children with early β cell autoimmunity (AAb+) and children without any signs for β cell autoimmunity (AAb−) (Fig. 1). IL-22 expression correlated positively with age in children with advanced β cell autoimmunity (r = 0.80, p < 0.05, Spearman test). Age correlated with IL-17A, IL-17F, IL-22, IFN-γ, IL-9, and RORc in children with type 1 diabetes only. Correlations of expression levels of genes in stimulated PBMCs in different study groups are presented in Supplemental Table II. No differences were seen in the gene expression between children from Finland and Estonia (data not shown).

Detection of plasticity of Th17 cells in type 1 diabetes and in advanced β cell autoimmunity

We sorted IL-17–secreting T helper cells from the in vitro–stimulated PBMCs with FACS and evaluated the plasticity of Th17 cells by detecting the expression level of IL-17, IFN-γ, FOXP3, and Helios transcripts in these cells. Gating strategy for sorting of Th17 cells for plasticity analysis is presented in Fig. 2A. Dead cells were excluded from the analysis based on autofluorescence detected on

![FIGURE 1](http://www.jimmunol.org/ Downloaded from http://www.jimmunol.org/)

**70 Th1/Th17 PLASTICITY IN HUMAN TYPE 1 DIABETES** by guest on June 11, 2017 http://www.jimmunol.org/ Downloaded from http://www.jimmunol.org/
the FITC/FL-1 channel. Remarkable enrichment of IL-17A transcripts was evident in FACS-sorted Th17 cells with intermediate and high expression of the IL-17A protein (Fig. 2B). In contrast, enrichment of Helios was not seen in Th17int or Th17high cells (Fig. 2C).

For the method validation, we investigated the relationship between the secreted protein and its mRNA expression, that is, IFN-γ in the FACS-sorted CD4+ cells secreting IFN-γ only, cosecreting IFN-γ and IL-17, and secreting IL-17 alone (Fig. 3). Dead cells were excluded from the analysis by 7-aminoactinomycin D staining of the dual-labeled cells. We observed the highest expression level of IFN-γ transcripts in the FACS-sorted CD4+ cells secreting IFN-γ only whereas the levels were lower in IFN-γ and IL-17 cosecreting cells and lowest in the cells secreting IL-17 alone (Fig. 3), which confirms that the expression level of cytokine-specific transcripts correlates with the secretion of the corresponding cytokine in FACS-sorted cells.

We then compared the plasticity in Th17int and Th17high cells between the study groups. To assess the plasticity of Th17 cells, we calculated the ratio of IFN-γ, FOXP3, and Helios gene expression to IL-17 expression. When the highest IFN-γ/IL-17 mRNA ratio in Th17int and Th17high cells from the healthy children was used as a cutoff value, the children with advanced β cell autoimmunity showed a significantly increased IFN-γ/IL-17 mRNA ratio in Th17int and Th17high cells (Fig. 4). Both FOXP3/IL-17 and Helios/IL-17 ratios were comparable between the study groups in both Th17int and Th17high cells.

Correlations of mRNA levels in Th17 cells in different study groups are shown in Supplemental Table III. In contrast to stimulated PBMCs, only a few statistically significant correlations were observed in FACS-sorted Th17 cells. In healthy children, IL-17A and FOXP3 mRNA levels correlated significantly in Th17int cells, whereas in Th17high cells IL-17A levels correlated with IFN-γ and FOXP3. In children with advanced β cell autoimmunity (IGT), IL-17A and IFN-γ correlated in Th17int cells, whereas in Th17high cells a significant correlation was found between IL-17A and FOXP3. In patients with type 1 diabetes, IL-17A and IFN-γ showed a significant correlation in Th17high cells. Calendar age correlated with IFN-γ expression in Th17int cells in healthy children and with IFN-γ expression in Th17high cells in children with type 1 diabetes. IFN-γ expression did not correlate with age in Th17int or Th17high cells in the children with advanced β cell autoimmunity.

**Increased plasticity of Th17 cells correlated with impaired glucose control**

In children with advanced β cell autoimmunity and impaired glucose tolerance, we observed a strong correlation between plasma glucose concentrations and IFN-γ/IL-17 ratio in Th17high cells in the fasting state (Fig. 5A) and at 120 min in the OGTT (Fig. 5B). Additionally, HbA1c and the IFN-γ/IL-17 ratio in Th17high cells correlated strongly in children with advanced β cell autoimmunity (Fig. 5C). No correlation was seen between the IFN-γ/IL-17 ratio and the glucose concentrations or HbA1c levels in Th17int cells in children with advanced β cell autoimmunity. A statistically significant correlation between FOXP3 expression and the 120 min plasma glucose concentration was observed in the

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**FIGURE 2.** (A) Gating strategy for FACS sorting of Th17 cells. IL-17–secreting CD4+ cells were stained with a cytokine capture assay and identified based on the IL-17–PE intensity of the CD4+ gated lymphocyte population and sorted into Th17high and Th17int cells. Dead autofluorescent cells were excluded from the sorting based on autofluorescence signal detected on FITC/FL-1 channel. (B) IL-17A mRNA expression is remarkably higher in Th17int (gray bars) and Th17high (black bars) cell fractions in comparison with anti-CD3– and anti-CD28–stimulated PBMCs (white bars). (C) mRNA of Helios, the marker of Tregs, was not enriched in sorted Th17 cells. Bars represent mean and whiskers SD. Relative gene expression of the markers was calculated by comparing ΔCt values of IL-17A and Helios to the average ΔCt value of IL-17A in the stimulated PBMCs of healthy controls. Comparison of different cell populations within the study group was performed with the Student t test for paired samples. Differences between the study groups within a cell population were compared with the Student t test for unpaired samples. *p < 0.05, ***p < 0.001.
antibodies. The process is not linear but it accelerates close to the positive individuals.

function and progression to overt type 1 diabetes in autoantibody-appearance of the first marker of disease, namely secreting CD4$^+$ cells sorted from anti-CD3$^-$ and anti-CD28$^-$stimulated PBMCs. (A) Frozen PBMCs were thawed and stimulated with anti-CD3 and anti-CD28 for 72 h and sorted with FACS into CD4$^+$IFN-$\gamma^+$, CD4$^+$ IFN-$\gamma^+$IL-17$^+$, and CD4$^+$IL-17$^+$ cells. IFN-$\gamma$ mRNA expression in sorted cell fractions was analyzed with RT-qPCR. A representative FACS plot of IFN-$\gamma$ and IL-17 protein expression in CD4$^+$ cells of a child with advanced β cell autoimmunity and IGT is shown. (B) The IFN-$\gamma$ mRNA expression was highest in the FACS-sorted CD4$^+$ cells secreting IFN-$\gamma$ alone (n = 7), being somewhat lower in CD4$^+$ cells co secreting of IFN-$\gamma$ and IL-17 (n = 6) and lowest in the CD4$^+$ cells secreting IL-17 alone (n = 7). Dead cells were excluded from the analysis by 7-aminoactinomycin D staining of the dual-labeled cells. The p values were calculated with the Student t test for paired samples. *p < 0.05.

OGTT in Th17$^{\text{hig}}$ cells in children with advanced β cell autoimmunity (Fig. 5D). The FOXP3/IL-17A or Helios/IL-17A ratio did not show any association with HbA1c or plasma glucose concentrations in the fasting state or after 120 min of glucose challenge in the OGTT in children with advanced β cell autoimmunity.

Discussion

We evaluated the upregulation of IL-17 immunity in children at different stages of preclinical diabetes and in children with type 1 diabetes, and observed that the IL-17 pathway is upregulated in peripheral blood in children in the late phase of preclinical diabetes characterized by impaired glucose tolerance, as well as in children with clinical type 1 diabetes. We found no evidence of activated IL-17 immunity in the early stages of the disease process when β cell autoantibodies appear. Most interestingly, sorted Th17 cells showed plasticity characterized by increased expression of IFN-$\gamma$, and the degree of Th1/Th17 plasticity correlated with impaired glucose control in children with advanced β cell autoimmunity. These findings suggest that the development of Th17 cells secreting both IFN-$\gamma$ and IL-17 seems to associate with impaired β cell function and progression to overt type 1 diabetes in autoantibody-positive individuals.

The β cell destruction process leading to clinical type 1 diabetes is relatively slow and stretches often over several years from the appearance of the first marker of disease, namely β cell autoantibodies. The process is not linear but it accelerates close to the diagnosis (23). The detection of β cell autoantibodies, positivity for multiple autoantibodies in particular, is strongly predictive of type 1 diabetes, but there are no biomarkers for the prediction of the rate of progression from autoantibody positivity to clinical type 1 diabetes. Our results demonstrating a correlation between Th1/Th17 plasticity and IGT in children with advanced β cell autoimmunity suggest that the degree of Th1/Th17 plasticity could be a novel biomarker for disease progression and accelerated destruction of the insulin-producing cells in children with β cell autoimmunity. Our study is limited by the cross-sectional design, and these findings should be evaluated in a longitudinal observational study of children with β cell autoimmunity in whom β cell function and the degree of Th17 plasticity are monitored in parallel. The detection of accelerated impairment in the β cell function in autoantibody-positive individuals would facilitate the identification of likely progressors suitable for secondary intervention trials. It might also help in the timing of immune interventions at a stage when the endogenous insulin production is higher than that seen at the time of diagnosis when <30% of functioning β cells are left (24). There is also a need for a biomarker for monitoring the effects of interventions and helping in the assessment of residual β cell function and glycemic control both in children at increased risk for type 1 diabetes and in those with recently diagnosed type 1 diabetes.

Increased Th17 immunity in the peripheral blood has firmly been associated with type 1 diabetes in patients with clinical disease (6–8, 16). To our knowledge, this is the first study demonstrating that Th17 immunity appears in the late phase of preclinical disease in human type 1 diabetes, as earlier suggested in animal models of type 1 diabetes (12, 13, 25). We further demonstrate that Th17 cells express IFN-$\gamma$ in human type 1 diabetes, and this seems to be associated with the loss of β cell function as also indicated by studies in animal models of autoimmune diabetes (12, 13, 25). Th17 cells purified from diabetic mice and transferred to healthy recipients induced autoimmune diabetes in the recipients, but only when the conversion of Th17 cells into coproducers of IFN-$\gamma$ and IL-17 took place in vivo (12, 13). Th17 cells are known to show greater instability than other Th cell subsets. In experimental autoimmune encephalomyelitis, most pathogenic IFN-$\gamma$-producing cells were derived from Th17 cells in chronic inflammatory settings (26). In the present study, we observed a remarkable increase in the IFN-$\gamma$/IL-17 ratio in highly purified Th17 cells from children with advanced β cell autoimmunity and clinical type 1 diabetes, suggesting a similar shift from the Th17 phenotype toward the Th1/Th17 phenotype. The strong positive correlations of the IFN-$\gamma$/IL-17 ratio in Th17$^{\text{hig}}$ cells with the plasma glucose concentrations after the glucose challenge and with the HbA1c values in children with advanced β cell autoimmunity further support the association of Th1/Th17 cells and impaired β cell function in human type 1 diabetes. It is possible that Th17 cells cosecreting IFN-$\gamma$ and IL-17 are detrimental to β cells and mediate aggressive β cell destruction as indicated by in vitro studies showing an additive effect of IFN-$\gamma$ and IL-17 on β cell apoptosis (6, 8, 11).

In our approach, we did not detect autoreactivity but we detected emerging plasticity of IL-17-immunity, which reflects phenotype changes in IL-17-secreting T cells in the peripheral blood. Based on animal studies and research on several autoimmune diseases, this kind of change from a well-differentiated T cell phenotype to Th1/Th17 plasticity takes place in autoimmune conditions and could be related to the destruction of the target tissue (7–13, 25, 27, 28). Thus, we do not necessarily need to detect autoreactivity of T cells, as the Th1/Th17 plasticity can be used as a biomarker for the pathogenic β cell autoimmunity when the risk of type 1 diabetes is first identified through an HLA risk genotype and autoantibody positivity.

We cannot exclude the possibility that the upregulation of Th17 immunity and its plasticity are secondary to the subtle elevations in plasma glucose concentrations in children with advanced β cell autoimmunity and type 1 diabetes. Glucose may stimulate β cells to secrete IL-1β, a cytokine important in the activation of Th17 cells (29, 30). Metabolic pathways may affect T cell function and differentiation, and it is of special interest that optimal IFN-$\gamma$ induction in CD8$^+$ T cells has been shown to be glucose-dependent (31–33). The HbA1c values and fasting glucose concentrations were, however, in the normal range in most of the children with advanced β cell autoimmunity. Nevertheless, the role of glucose needs to be addressed in future studies.
In the present study, the children with type 1 diabetes and the children with advanced β cell autoimmunity had a higher proportion of IL-17–secreting CD4+ cells in stimulated PBMCs than did healthy children (median, 0.2%; range, 0.025–0.45 and 0.14%; 0.11–0.36 versus 0.10%, 0.043–0.3%). Comparable proportions of peripheral blood Th17 cells have been reported in the patients with type 1 diabetes by others. Marwaha et al. (7) demonstrated that the proportion of CD4+IL-17+ cells was 0.47% in children with type 1 diabetes and 0.18% in healthy children based on the intracellular cytokine staining of anti-CD3– and anti-CD28–stimulated and PMA plus ionomycin–restimulated PBMCs. Ferraro et al. (9) reported a slightly higher proportion of CD4+IL-17+ cells using intracellular cytokine staining of PBMCs stimulated with 2-O-tetradecanoylphorbol-13-acetate/ionomycin, that is, ~1.3 and 0.6% of CD4+IL-17+ cells in adult patients and healthy subjects, respectively. Annunziato et al. (15) observed consistently <1% of Th17 cells among the PMA plus ionomycin–stimulated PBMCs in healthy control subjects.

Tregs can reportedly shift their phenotype and acquire an effector phenotype, characterized by production of IFN-γ or IL-17 (17, 34, 35). We did not find any evidence that Th17 cells in type 1 diabetes would originate from thymic-derived Tregs because the expression of Helios, a marker of thymic-derived Tregs, was low in sorted Th17 cells and did not differ between the children with type 1 diabetes and healthy children. Also, the expression of FOXP3 was similar in Th17 cells from children with type 1 diabetes and in healthy children. However, we observed a strong correlation between FOXP3 expression in Th17int cells and plasma glucose concentration.

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** The plasticity of sorted Th17 cells was evaluated by calculating the ratio of IFN-γ/IL-17A, FOXP3/IL-17A, and Helios/IL-17A mRNA in Th17int (left column) and Th17high cells (right column) in healthy children (HC), children with advanced β cell autoimmunity and IGT, and in children with type 1 diabetes. Red symbols in the HC group denote the subjects with early β cell autoimmunity. (A and B) The highest IFN-γ/IL-17 ratio in Th17 cells of the healthy children was used as a cutoff for increased Th17 plasticity (dashed line). An increased ratio of IFN-γ/IL-17 in Th17high and Th17int cells was seen in the children with advanced β cell autoimmunity and in the children with type 1 diabetes in comparison with HC. (C–F) FOXP3/IL-17 and Helios/IL-17 ratios in sorted Th17 cells were comparable between the study groups. Statistical significance was calculated with the Fisher exact test. Horizontal lines represent the median ratio. **p < 0.01, ***p < 0.001.

![FIGURE 5](http://www.jimmunol.org/)

**FIGURE 5.** Children with advanced β cell autoimmunity and IGT have an increased IFN-γ/IL-17 ratio in Th17- cells, which correlates with the parameters reflecting β cell function (A–C). (A) Fasting plasma glucose concentration correlated with IFN-γ/IL-17 ratio in Th17high cells in children with advanced β cell autoimmunity. (B) IFN-γ/IL-17 ratio in Th17high cells correlated with plasma glucose concentrations at 120 min in the OGGT in children with advanced β cell autoimmunity. (C) HbA1c levels correlated with the IFN-γ/IL-17 ratio in Th17high cells in children with advanced β cell autoimmunity. (D) Statistically significant correlation between the expression of FOXP3 mRNA in Th17int cells and the 120 min glucose concentration in the OGGT was observed in children with advanced β cell autoimmunity. Correlations were calculated with a nonparametric Spearman’s test.
concentrations at 120 min in OGTT in children with advanced β cell autoimmunity. FOXP3 is a transcription factor of Tregs, but it is also upregulated upon TCR-mediated activation of T cells, and thus upregulation of FOXP3 in Th17high cells might reflect the degree of activation of these cells. Accordingly, FOXP3 expression in Th17 cells may reflect the activation of Th17 immunity and show an association with β cell function.

Li and colleagues have demonstrated upregulation of IFN-γ in IL-17-producing invariant NKT cells upon IL-1β in vitro stimulation of PBMCs from patients with type 1 diabetes (36). Because some of the invariant NKT cells express CD4+ cell surface Ag, these cells might be involved in the plasticity observed in our study. Interestingly, we observed upregulation of IL-17F, IFN-γ, and IL-9 in the activated peripheral blood T cells in children with advanced β cell autoimmunity. This might indicate that the upregulation of IFN-γ and IL-9 precedes the full-blown activation of the Th17 pathway. IL-9 is considered to be a driver for Ag-specific Th17 cells (37). It is also known that IL-17F is expressed earlier than IL-17A during Th17 cell development (38). A recent study showed that IL-9 signaling was associated with type 1 diabetes in two of monozygotic quadruplets (39). Increased numbers of TGF-β1-induced IL-9–secreting Th17 cells have been reported in type 1 diabetes, too (16). Accordingly, IL-9 upregulation could be considered as a marker of Th17 cells also in children with advanced β cell autoimmunity.

In conclusion, upregulation of IFN-γ, IL-9, and IL-17 and plasticity of Th17 cells characterized by coexpression of IFN-γ was only seen in the late phase of the disease process resulting in overt type 1 diabetes. More importantly, the IFN-γ/IL-17 ratio in Th17high cells correlated strongly with plasma glucose concentrations at 120 min in OGTT in children with advanced β cell autoimmunity. This might indicate that the upregulation of IFN-γ and IL-9 precedes the full-blown activation of the Th17 pathway. IL-9 is considered to be a driver for Ag-specific Th17 cells (37). It is also known that IL-17F is expressed earlier than IL-17A during Th17 cell development (38). A recent study showed that IL-9 signaling was associated with type 1 diabetes in two of monozygotic quadruplets (39). Increased numbers of TGF-β1-induced IL-9–secreting Th17 cells have been reported in type 1 diabetes, too (16). Accordingly, IL-9 upregulation could be considered as a marker of Th17 cells also in children with advanced β cell autoimmunity.

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Disclosures

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