No evidence for activation of T\textsubscript{H}1 or T\textsubscript{H}17 pathways in unstimulated peripheral blood mononuclear cells from children with β-cell autoimmunity or T1D

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**Introduction:** The balance between T\textsubscript{H}1, T\textsubscript{H}2, T\textsubscript{H}17, and regulatory T cells has been suggested to be disturbed in type 1 diabetes (T1D). We investigated this balance in peripheral blood mononuclear cells (PBMC) from children at risk of developing T1D and children with T1D.

**Methods:** We studied PBMC expression levels of markers related to T\textsubscript{H}1 (T-bet, IL-12Rβ\textsubscript{1}, IL-12Rβ\textsubscript{2}), T\textsubscript{H}2 (GATA-3, IL-4Rα), T\textsubscript{H}17 (IL-17A), and regulatory T cells (Foxp3, ICOS, and CTLA-4) with real-time polymerase chain reaction from 17 children with T1D, 13 children with β-cell autoimmunity, 15 children with T1D risk-associated human leukocyte antigen (HLA) haplotypes, and 24 healthy, control children.

**Results:** We observed decreased expression levels of GATA-3 by PBMC of healthy children with autoantibodies compared to healthy, control children (p = 0.014) or children with HLA risk alleles (p = 0.032). Children with T1D demonstrated lower expression levels of T-bet, IL-12Rβ\textsubscript{1}, and IL-4Rα both at diagnosis and 12 months later.

**Conclusion:** We found no indication of aberrant activation of T\textsubscript{H}1, T\textsubscript{H}17, or Treg in peripheral blood from children with or without risk of T1D. The observed immunological differences between children at risk of and with T1D should be considered when immunopathogenesis of β-cell destruction is studied.

**Keywords:** transcription factor, type 1 diabetes, mononuclear cells

Introduction

The expression profile of T-cell transcription factors and cytokine receptors reflects the activation status of the immune system. The functional arms of the adaptive immune system includes T helper (T\textsubscript{H})1, T\textsubscript{H}2, T\textsubscript{H}17, and regulatory T cells (Treg), and the balance between these may be aberrant in immune-mediated disease.

Interleukin-12 (IL-12) is a strong promoter of T\textsubscript{H}1 immune responses and the T cell response to IL-12 is dependent on the expression of the high affinity IL-12 receptor (IL-12R). This receptor consists of β\textsubscript{1} and β\textsubscript{2} subunits, which are both necessary for signal transduction. The IL-12Rβ\textsubscript{1} subunit can be detected on resting T-cells, but the β\textsubscript{2} subunit is induced only upon antigen activation (Szabo et al 1997). Signals via the IL-12R upregulate T-bet, a protein primarily expressed in T\textsubscript{H}1 cells (Szabo et al 2000), but also in type 1 cytotoxic T (T\textsubscript{C}1) cells (Sullivan et al 2003) and dendritic cells (Lugo-Villarino et al 2003). In T\textsubscript{H}1 cells, T-bet, together with interferon-gamma (IFN-γ), forms an autoregulatory positive feedback loop to maintain a T\textsubscript{H}1 response and inhibit IL-5 production (Lametschwandtner et al 2004).

GATA-3 and the IL-4 receptor (IL-4R) are believed to be crucial for the development and maintenance of a T\textsubscript{H}2 phenotype. IL-4R regulates the production...
of cytokines such as IL-4, IL-5, and IL-13 through GATA-3 in Th2 cells (Szabo et al 2003), and a recent study shows that Tc2 cells also express GATA-3 (Chen et al 2005).

The ratio between T-bet and GATA-3 expression levels, as a measure of Th1 and Th2 immune responses, has been implicated in different autoimmune diseases, such as rheumatoid arthritis (RA), idiopathic thrombocytopenic purpura (ITP), and systemic lupus erythematosus (SLE) (Kawashima and Miossec 2005; Wang et al 2005; Chan et al 2006).

A new subset of T cells, distinct from Th1 and Th2, has recently been classified and named Th17. This subset of cells develops from naïve T cells in response to IL-23 (Harrington et al 2005; Park et al 2005; Wynn 2005) and has the ability to produce IL-17A, IL-17F, and IL-22 (Aggarwal et al 2003; Zheng et al 2007), which are potent proinflammatory cytokines (Kolls and Linden 2004). Th17 cells seem to be central in the development of pathogenic tissue destruction in multiple sclerosis and rheumatoid arthritis (Kotake et al 1999; Hwang and Kim 2005; Kebir et al 2007).

Regulatory T cells (Treg) are characterized by expression of CD25, CTLA-4, and Foxp3 and play an active role in downregulating immune responses. Foxp3 seems to be a key factor in the development and function of naturally occurring Treg (Hori et al 2003), and decreased Foxp3 mRNA levels have been observed in patients with autoimmune diseases (Huan et al 2005). Recent studies have, on the other hand, shown that Foxp3 is upregulated upon T-cell activation and results in hyporesponsiveness of the activated T-cell (Morgan et al 2005; Wang et al 2007).

In type 1 diabetes (T1D), a functional imbalance between Th1, Th2, and Treg cells has been suggested by several research groups, including our own (Rabinovitch et al 1996; Shimada et al 1996; Halminen et al 2001; Kukreja et al 2002; Faresjo et al 2006), whereas the role of Th17 cells in human T1D is not known. Since hyperglycemia, ketoacidosis, or insulin treatment may affect immune cells, we studied the balance between markers of Th1, Th2, Th17, and Treg cells in peripheral blood mononuclear cells (PBMC) from children at risk of T1D and diabetic children followed during the first year after diagnosis.

**Materials and methods**

**Subjects**

The study includes a total of 69 children. Forty-four children participated in the population-based ABIS study (All Babies in south-east Sweden) (mean age 5.3 years; range 5.0–6.0 years). The ABIS study is a population-based follow-up study of all infants born between October 1st, 1997 and October 1st, 1999, in which newborns have been followed prospectively. Among the children participating in the ABIS study we selected 15 children (mean age 5.4 years; range 5.0–6.0 years) expressing human leukocyte antigen (HLA) class II haplotypes associated with an increased risk for T1D development (HLA-DR3-DQ2 or HLA-DR4-DQ8), without autoantibody production, and 13 children (mean age 5.3 years; range 5.1–5.7 years) who were autoantibody positive for GADA and/or IA-2A and/or IAA (11 of whom also expressed HLA haplotypes associated with increased risk for T1D). Antibody levels above the 98th percentile for GADA and IAA and the 99th percentile for IA-2 were regarded as positive values.

Sixteen ABIS children (mean age 5.3 years; range 5.0–5.6 years) who did not express the T1D risk-associated HLA alleles and were negative for autoantibodies were used as healthy controls for the children at risk for T1D development.

The T1D patients consisted of 17 children (mean age 10.4 years; range 6.0–15.6 years) treated at the Pediatric Clinic, Linköping University hospital. Children with T1D were followed with samples taken ten days, three months (range 1–3 months), and one year (range 9–18 months) after diagnosis. At the same time points glycosylated hemoglobin (HbA1c) values were recorded. Eight children with a mean age of 11.7 years (range 11.0–12.8 years) were enrolled from a nearby school and used as controls for the diabetic children. These children did not have any family members with autoimmune disease or allergy and were regarded as healthy.

**Measurement of autoantibodies**

Levels of autoantibodies against glutamic acid decarboxylase (GAD), tyrosine phosphatase-like insulinoma antigen 2 (IA-2) and insulin were analyzed in serum by radiobinding assay described previously (Holmberg et al 2006).

In the Diabetes Autoantibody Standardization Program (DASP) 2005 for GADA, we observed a specificity of 96% and sensitivity of 76%, for IA-2A, a specificity of 100% and sensitivity of 72%, and for IAA, a specificity of 100% and a sensitivity of 30%.

Results were expressed as concentrations of autoantibodies calculated in relation to a standard curve.

**HLA genotyping**

HLA genotyping was done using biotinylated primers for DQB1, DQA1, or DRB1 genes. The amplified regions were, after denaturation, hybridized with a mix of sequence specific
probes labeled with various lanthanide (europium, samarium, and terbium) chelates.

Specific signals for each label were measured by time-resolved fluorometry after washes and addition of enhancement solution. Details of the method, including primer and probe sequences, have been described previously (Sjoroos et al 1998; Nejentsev et al 1999).

Preparation of samples
Human PBMC were isolated from peripheral blood by Ficoll-Paque™ (Amersham Biosciences AB, Uppsala, Sweden) density gradient centrifugation and stored in liquid nitrogen until further analysis.

Real-time polymerase chain reaction
PBMCs were analyzed for expression of GATA-3, T-bet, IL-4Rα, IL-12Rβ1, IL-12β2, IL-17A, Foxp3, ICOS, and CTLA-4 using real-time polymerase chain reaction (PCR).

Briefly, total ribonucleic acid (RNA) was extracted from unstimulated PBMC using the GenElute Mammalian total RNA kit® (Sigma, St. Louis, MO, USA). Genomic DNA were eliminated by an additional treatment with DNase I (0.01 U/µL) (Roche Diagnostics, Mannheim, Germany), after which RNA was converted to complementary DNA (cDNA) according to the manufacturer’s protocol, using random hexamers and Multiscribe Reverse transcriptase (Applied Biosystems, Foster City, CA, USA). Reactions were carried out in a total volume of 20 µL.

mRNA expression was determined by the TaqMan method of real-time PCR, with ribosomal 18s RNA (rRNA) as endogenous control. TaqMan Universal PCR master mix with AMPerase UNG and the primers and probes for rRNA (cat.no. Hs99999901_s1), GATA-3 (cat.no. Hs00231122_m1), T-bet (cat.no. Hs00203436_m1), IL-4Rα (cat.no. Hs00166237_m1), IL-12Rβ1 (cat.no. Hs00234651_m1), IL-12Rβ2 (cat.no. Hs00155486_m1), IL-17A (cat.no. Hs00174383_m1), ICOS (cat.no. Hs00359999_m1), CTLA-4 (cat.no. Hs00175480_m1), and Foxp3 (cat.no. Hs00203958_m1) were purchased from Applied Biosystems.

Each measurement was performed in triplicate with 1.8 µL of cDNA, except for IL-17A where 18 µL cDNA was used. The thermal cycle conditions were set with an initial step of 2 min at 50 °C and 10 min at 95 °C, followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. Water was included as no template control (NTC).

The comparative Ct method described by the manufacturer is used to calculate a relative transcription value. For presentations, the relative amount of target genes was multiplied by 100.

Characteristics of subjects
Children participating in the ABIS study had an overall mean age of 5.4 years (range 5.0–6.0 years) and included 18 girls and 26 boys.

Diabetic children included nine girls and eight boys with a mean age of 10.4 years (ranging from 6.0 to 15.6 years). At diagnosis, the median value of HbA1c was 10.3% (range 8.4–11.7) but improved during follow-up. At three months, the median was 5.1% (range 3.9–8.5) and at twelve months the median was 6.2% (range 4.7–8.2). Insulin requirements were between 0.4 and 1.4 U/kg/24 h at three months, and between 0.5–1.7 U/kg/24h at twelve months. The eight schoolchildren enrolled as controls included three girls and five boys, with the mean age of 11.7 years (range 11.0–12.8).

Statistical analysis
Since the levels of mRNA were not normally distributed, non-parametric tests, corrected for ties, were used. Comparisons between unpaired groups were analyzed with Kruskal-Wallis as a pretest and Mann-Whitney U test was used for comparison of two unrelated groups. Friedman test was used for analysis between paired groups and correlations were analyzed with Spearman’s rank correlation test.

A p-value below 0.05 was considered significant without corrections for multiple comparisons (Perneger 1998). All calculations were performed with the statistical package SPSS 14.0 (SPSS Inc., Chicago, IL, USA).

Ethics
Informed consent was obtained from all children and parents, and the regional Research Ethics Committee of the Faculty of Health Sciences, Linköping approved the study.

Results
Activation stage of peripheral blood mononuclear cells in healthy children at risk of type 1 diabetes
PBMC of high-risk individuals, defined as children with β-cell autoantibodies and HLA risk genotype, demonstrated lower expression levels of GATA-3 in PBMC than healthy control children (p = 0.014) (see Figure 1) and children expressing an HLA risk genotype but without autoantibodies.
The children with genetic risk alone did not differ from the control children without genetic risk.

The amount of IL-17A expressed in PBMC was low but using ten times more cDNA, between 77%–88% of the individuals in each group had measurable levels but no differences in IL-17A expression or expression of T-bet, IL-4Rα, IL-12Rβ1, IL-12β2, Foxp3, ICOS, and CTLA-4 was found between healthy children at risk compared to those without risk (data not shown).

Expression of measured marker did not show significant changes in the T1D children during follow-up using paired analysis (data not shown).

The levels of IL-17A, GATA-3, or IL-12Rβ2 specific mRNA did not differ between samples taken from diabetic children and healthy controls (data not shown).

Foxp3 expression was lowest in samples taken at 12 months following diagnosis when it was significantly decreased when compared to the healthy control group (p = 0.027) (see Figure 3a). At 12 months, the expression was lower than at diagnosis, and also tended to be lower than in samples taken at three months after diagnosis (p = 0.038 and p = 0.056, respectively). The expression of ICOS or CTLA-4 did not differ between the groups (data not shown).

Expression of measured marker did not show significant changes in the T1D children during follow-up using paired analysis (data not shown).

### The relation of insulin dose, HbA1c, and autoantibodies to transcription factors in diabetic children

At three months of diabetes duration we observed a negative correlation between insulin dose per kg body weight,
Figure 2 Relative expression level of specific mRNA in PBMC from children with T1D at different duration and in healthy control children. The levels of IL-12Rβ1 mRNA (a) (type 1 marker), IL-4Rα mRNA (b) (type 2 marker) was lower at all time points, and T-bet mRNA (c) (type 1 marker) was decreased at diagnosis and after 12 month. Horizontal lines indicate median values and p-values of Mann-Whitney U-test are shown in the figure.

Abbreviations: HLA, human leukocyte antigen; PBMC, peripheral blood mononuclear cells; T1D, type 1 diabetes.

Figure 3 (a) Foxp3 mRNA expression (Treg marker) was lower after 12 month (M12) after diagnosis than earlier during the follow-up in children with T1D, and also when compared to healthy controls. (b) Relative expression level of Foxp3 mRNA shows a negative correlation with insulin dose (units/kg/24 h) at 3 months \( R = -0.541; p = 0.03 \).
that T1D is not a systemic autoimmune disease. To exhibit lower expression levels of T-bet, IL-12Rβ, immune aberrancies related to Type 1 diabetes is that unstimulated peripheral blood reflects poorly the major finding in individuals positive for autoantibodies (data not shown).

**Discussion**

Deviation of T cell responses, including Th1, Th2, and Th17 cells together with Treg activity, may contribute to the development of a chronic (auto)immune responses, as in T1D. We compared the expression of transcription factors, as markers for functional T cell subsets, in *ex vivo* derived peripheral PBMC from diabetic and healthy children. As our interest was to evaluate *ex vivo* expression levels of factors responsible for T cell differentiation in unstimulated PBMC, we were not able to measure secreted cytokines since in unstimulated circulating cells, cytokine secretion is low, or even undetectable, for most cytokines.

In the group of at-risk individuals, ie, children with β-cell autoantibodies, a decreased expression of GATA-3 in PBMC was the only aberrancy found in comparison to healthy age-matched children. Inhibition of GATA-3 seems to be more important than increased levels of T-bet for the production of IFN-γ (Chakir et al 2003), and thus the low levels of GATA-3 could support the development of Th1 responses that may be capable of destruction of β-cells. However, we did not see any difference in expression levels of T-bet or IL-12Rγ, and thus this is rather speculative. The major finding in individuals positive for β-cell autoantibodies is that unstimulated peripheral blood reflects poorly the immune aberrancies related to β-cell autoimmunity leading to β-cell destruction. This is in agreement with the view that T1D is not a systemic autoimmune disease.

In contrast to children at-risk of T1D, diabetic children exhibited lower expression levels of T-bet, IL-12Rβγ, and IL-4Rα both at diagnosis and 12 months later compared to healthy children. These data suggest poor Th1 immune activation in peripheral blood from the children with recent-onset clinical diabetes and are in good agreement with earlier studies showing decreased expression of IFN-γ transcripts in peripheral blood of diabetic patients (Halminen et al 2001). Altogether, our results indicate that no general activation of type 1 immune responses are observed in peripheral blood of children with T1D or that of children at-risk of T1D, despite the earlier findings of high IFN-γ response to stimulation reported by some studies (Kallmann et al 1997).

The expression of IL-17A transcripts in PBMC was low in general and required ten times more templates in the PCR for IL-17A. This may indicate that the numbers of IL-17A positive cells or their functional upregulation are not increased in the peripheral blood in children with T1D or in risk individuals. If Th17 cells are involved in the pathogenesis of T1D, their role should, ideally, be studied locally in the population of T cells infiltrating the islets.

Foxp3 expression in unstimulated PBMC did not differ between healthy and newly diagnosed diabetic children, but the Foxp3 expression was decreased 12 months after diabetes diagnosis. Our earlier findings suggest that activation of Foxp3 occurs at the time of diagnosis as a response to insulin treatment (Tiittanen et al 2006). Interestingly, we observed a negative correlation between insulin dose and the Foxp3 levels at 3 months after diagnosis. This could indicate that the activation status of Treg cells near diagnosis may control the β-cell destruction and thus be related to the insulin dose needed. Since Foxp3 up-regulation is associated with T cell activation in humans (Wang et al 2007), the decrease of Foxp3 expression from diagnosis toward 12 months after diagnosis could be a result of fading autoimmune attack with final loss of β-cells.

We observed impaired type 1 responses in children with T1D, but this defect was not seen in children at risk of T1D, suggesting an immunological shift from prediabetes to diabetes. This raises the question of whether poor T cell activation in T1D is secondary to the metabolic disturbances in diabetes or whether it is related to the accelerated β-cell destruction seen at diagnosis. We did not observe any direct correlations between any of the measured markers and HbA1c values, suggesting that this aberrancy is not due to long-term hyperglycemia. It remains to be elucidated whether a peripheral decrease T cell activation is related to the initiation of autoimmune β-cell destruction in the pancreas, when the disease progresses from silent autoimmunity towards clinical diabetes.

Our findings do not support a general activation of Th1 or Th17 responsiveness in children at risk of developing T1D or in children diagnosed with T1D. Rather, in the latter subject group, the data support a global downregulation of Th1, Th2, and Th17 responses. It should nevertheless be emphasized that the circulating lymphocytes may not always reflect the infiltrating T cells in the target tissue, and thus our results do not demonstrate the local changes of insulitis. It is important to realize that an immunological shift takes place from prediabetes to manifest T1D, as we and others (Jain et al 2008) have demonstrated, and this should be considered when the immuno-pathogenesis of β-cell destruction is studied.

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