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Expansion of CD4+CD25+FOXP3+ regulatory T cells in infants of mothers with type 1 diabetes

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Abstract

Background—Reduced risk for type 1 diabetes (T1D) has been reported in the offspring of mothers with T1D when compared with children of affected fathers.

Objective—To evaluate the hypothesis that exposure of the offspring to maternal insulin therapy induces regulatory mechanisms *in utero*, we compared the FOXP3 expressing regulatory T cells in cord blood (CB) of infants born to mothers with or without T1D.

Subjects and Methods—Cord blood mononuclear cells (CBMCs) from 20 infants with maternal T1D and from 20 infants with an unaffected mother were analyzed for the numbers of CD4+CD25+FOXP3+ cells *ex vivo* and after *in vitro* stimulation with human insulin by flow cytometry. The mRNA expression of FOXP3, NFATc2, STIM1, interleukin (IL)-10, and transforming growth factor (TGF)- β was measured by real-time reverse transcription polymerase chain reaction.

Results—The percentage of FOXP3+ cells in CD4+CD25^{high} cells was higher in the CB of the infants with maternal T1D when compared with the infants of unaffected mothers ($p = 0.023$). After *in vitro* insulin stimulation an increase in the percentage of FOXP3+ cells in CD4+CD25^{high} cells ($p = 0.0002$) as well as upregulation of FOXP3, NFATc2, STIM1, IL-10, and TGF- β transcripts in CBMCs ($p < 0.013$ for all; Wilcoxon test) was observed only in the offspring of mothers with T1D, in whom the disease-related *PTPN22* allele was associated with reduced STIM1 and NFATc2 response in insulin-stimulated CBMCs ($p = 0.007$ and $p = 0.014$).

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Conflict of interest

The authors declare that there is no conflict of interest associated with this article.

Conclusions—We suggest that maternal insulin treatment induces expansion of regulatory T cells in the fetus, which might contribute to the lower risk of diabetes in children with maternal vs. paternal diabetes.

Keywords

CB; insulin treatment; regulatory T cells; T1D

Type 1 diabetes (T1D) is a multifactorial disease involving genetic and environmental factors leading to the loss of insulin-producing β -cells in the pancreas. A number of studies have reported a reduced T1D risk in the offspring of affected mothers when compared with the offspring of fathers with T1D (1–4). The exposure to maternal diabetes *in utero* has been implicated to be important in modifying the risk of development of T1D and to represent a protective factor in the offspring.

Insulin antibodies (IA) have been detected in the circulation and in cord blood (CB) of the offspring of mothers with T1D as a consequence of transplacental transfer from the maternal circulation (5–7). Studies in mothers with T1D have shown that insulin complexed to immunoglobulin G (IgG)-class IA cross the placenta (8, 9) and may facilitate transplacental passage of insulin from the maternal circulation to the fetus. Thus, exposure to the exogenously administered autoantigen, insulin, may have an influence on insulin-specific immune responses in the developing fetus. Previously, we reported lower insulin-induced T cell proliferation in offspring of diabetic mothers than in children with an affected father or sibling (10). On the basis of these observations it is possible that the exposure of offspring to maternal insulin therapy results in tolerization to insulin and may decrease the risk of T1D by this mechanism.

Regulatory T cells (Tregs) are subsets of CD4⁺ T cells involved in the maintenance of peripheral tolerance by actively suppressing the activation and expansion of Th1 and Th2 type CD4⁺ T cells as well as CD8⁺ T cells and B cells. Natural CD4⁺CD25⁺ Tregs develop in the thymus and express the α chain of the interleukin (IL)-2 receptor, CD25 (11, 12). The insulin expression level in thymus has been associated with the insulin gene polymorphism that also affects the risk of T1D (13, 14). The genetic link could be explained by the effects of insulin expression levels on the induction of insulin-specific Tregs in thymus. In addition, adaptive Tregs are induced in the periphery (15). The transcription factor FOXP3 has been identified as a critical regulator of the development and function of the Tregs which secrete IL-10 and/or transforming growth factor (TGF)- β that mediate the suppression (16, 17). Other molecules involved in the activation of Tregs are NFATc2 (18) and STIM1 (19).

In this article, our aim was to analyze the possible effect of maternal T1D on the circulating CD4⁺CD25⁺FOXP3⁺ Tregs in the newborn infant. We also stimulated cord blood mononuclear cells (CBMCs) of infants with and without maternal T1D with human insulin *in vitro* and analyzed the numbers of CD4⁺CD25^{high}FOXP3⁺ Tregs by flow cytometry and the gene expression of molecules involved in the activation of Tregs with quantitative real-time polymerase chain reaction (RT-qPCR).

Methods

Subjects

CB EDTA samples were randomly received from newborn infants randomized for participation in the Trial to Reduce IDDM in Genetically at Risk (TRIGR study), which has been described in detail earlier (20), with a first-degree relative (full-sibling, mother, or father) with T1D, or from infants born in the Department of Obstetrics, Helsinki University Central Hospital. We studied CB samples from 20 infants with maternal T1D and 20 infants of unaffected mothers. The inclusion criterion was gestational age of ≥ 35 wk. The infants with signs of infection and infants of mothers with gestational diabetes were excluded from this study. CB from newborn infants was obtained from umbilical vein after delivery and was further analyzed within 24 h. Maternal and fetal characteristics for this cohort are presented in Table 1. The duration of diabetes in mothers with T1D ranged from 2 to 30 yr, the median being 18 yr. The pregnant mothers with T1D were categorized according to White classification: six were in class B, four in class C, eight in class D, one in class F, and the remaining one in class R. The median individual level of glycosylated hemoglobin (HbA1c) at the end of the pregnancy was 6.9% (range 5.8–8.4%). No data of HbA1c levels were available from the unaffected mothers. The median daily insulin dose was 0.88 IU/kg (range 0.58–1.48 IU/kg) at the end of pregnancy in the T1D mothers. The mothers were treated according to Finnish care guidelines in T1D treatment during pregnancy with NPH insulin or long-acting analog insulin glargine together with rapid-acting human insulin analogs (lispro or aspart).

The Ethics Committee for Pediatrics, Adolescent Medicine and Psychiatry, and the Coordinating Ethics Committee in the Hospital District of Helsinki and Uusimaa (Helsinki, Finland) have approved the study protocol being in accordance with the Declaration of Helsinki. Written parental consent was obtained from the parents of all children.

In vitro stimulation test of CBMCs

CBMCs were isolated from fresh CB by Ficoll-Paque density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden) and were suspended in RPMI-1640 containing 5% inactivated human AB⁺ serum (Sigma, St. Louis, MO, USA), L-glutamine (2 mmol/L; Gibco, Paisley, Scotland) and gentamicin (25 µg/mL; Sigma). CBMCs were cultured with 300 µg/mL human insulin (sterile yeast recombinant protein from Boehringer Mannheim, Mannheim, Germany) at 2×10^6 cells (2 mL) per well on 24-well cell culture plates (Costar, Corning Incorporated, NY, USA) for flow cytometry analyses and on U-bottomed 96-well culture plates (Costar), 2×10^5 cells (200 µL) per well in quadruplicates, for RT-qPCR analyses. After 72 h of incubation the supernatants and the cells were collected for analyses. For RT-qPCR, the cell pellets were frozen at -70°C in the lysis buffer.

Analysis of FOXP3 by flow cytometry

The fresh CBMCs and the cultured cells were resuspended in phosphate-buffered saline with 0.5% bovine serum albumin (staining buffer). CBMCs were stained for surface antigens with the following antibodies: Alexa488-conjugated anti-CD4 (clone OKT4; eBioscience, San Diego, CA, USA), PE-conjugated anti-CD25 (4E3; Miltenyi Biotec, Bergisch Gladbach,

Germany), PerCP-conjugated anti-CD4 [SK3; Becton Dickinson (BD) Biosciences, San Jose, CA, USA], and APC-conjugated anti-CD8 (SK1; BD). The following isotype control antibodies were used: Alexa488-conjugated mouse IgG1 (clone OKT4; eBioscience), PE mouse IgG2b (IS6-11E5.11; Miltenyi Biotec), PerCP mouse IgG1 (X40; BD), and APC mouse IgG1 (X40; BD). After the cell surface staining was completed, the cells were fixed, permeabilized, and stained with anti-human FOXP3Alexa488-conjugated mAb (clone 236A/E7; eBioscience) using the FOXP3 staining kit (eBioscience) according to the manufacturer's instructions. At least 1×10^6 events were acquired from each sample on a BD FACSCalibur and analyzed with FACSDiva (BD) software. To investigate the number of circulating CD4⁺CD25^{high}FOXP3⁺ T cells in CB, we gated first CD4⁺ cells, and then CD4⁺ cells with the highest CD25 intensity (2%). The expression of FOXP3 was analyzed in this cell population. When the clinical study was initiated, CD127 was not generally used as a marker for Tregs. Our later studies show that the CD4⁺ cells with the highest CD25 intensity (2%) are CD127^{low} cells. CD4⁺CD25^{high}FOXP3⁺ mean fluorescence intensity (MFI) data were normalized between experiments by determining the MFI increase. First, the geometric MFI of isotype was subtracted from the geometric MFI of CD4⁺CD25^{high}FOXP3⁺ cells. Then, the geometric MFI values of FOXP3 in CD4⁺CD25^{high} cells in the non-stimulated population were subtracted from geometric MFI values of FOXP3 in CD4⁺CD25^{high} cells the after stimulation with insulin.

Quantitative real-time PCR

Analyses of target gene expression were performed with RT-qPCR as earlier described (21). Briefly, total RNA was isolated with RNeasy Mini Kit (Qiagen, Hilden, Germany) with DNase treatment to eliminate genomic DNA. Concentration and purity of RNA was measured by a spectrophotometer. Reverse transcription was performed using TaqMan Reverse Transcription reagents (Applied Biosystems, Foster City, CA, USA). qPCR was performed using TaqMan fast universal master mix together with predesigned FAM-labeled TaqMan Gene Expression Assay reagents (Applied Biosystems) in triplicate wells. Determined targets were FOXP3 (cat. no Hs00203958_m1), NFATc2 (Hs00905451_m1), STIM1 (Hs00162394_m1), IL-10 (Hs00174086_m1), and TGF- β (Hs00171257_m1). Ribosomal 18s RNA (Hs99999901_s1) was used as an endogenous control.

The quantities of the target gene expression were analyzed by a comparative threshold cycle (Ct) method (as recommended by Applied Biosystems). An exogenous cDNA pool calibrator was collected from phytohemagglutinin (Sigma) stimulated peripheral blood mononuclear cells and considered as an interassay standard, to which normalized samples were compared. ΔCt stands for the difference between Ct of the marker gene and Ct of the 18S gene, whereas $\Delta\Delta Ct$ is the difference between the Ct of the analyzed sample and Ct of the calibrator. Calculation of $2^{-\Delta\Delta Ct}$ then gives a relative amount of the target gene in the analyzed sample compared with the calibrator, both normalized to an endogenous control (18S). The relative amount ($2^{-\Delta\Delta Ct}$) of FOXP3, IL-10, and TGF- β was multiplied by 1000 and the relative amount of NFATc2 and STIM1 was multiplied by 100. The fold change was calculated by dividing the relative mRNA level of human insulin-stimulated CBMCs by the relative mRNA level of non-stimulated CBMCs.

Disease-associated antibodies

From the CB samples IA, autoantibodies to the 65-kDa isoform of glutamic acid decarboxylase (GADA), and autoantibodies to the protein tyrosine phosphatase-related IA-2 molecule (IA-2A) were measured by specific radiobinding assays. Radioimmunoassay for antibodies to insulin does not differentiate autoantibodies to insulin (IAA) from antibodies induced by exogenous insulin treatment, i.e., IA (6). In the CB samples from infants with diabetic mother the antibodies detected by radioimmunoassay for IAA are not necessarily autoantibodies any longer but induced by exogenous insulin treatment and thus called IA. Islet cell antibodies (ICA) were measured by a standard immunofluorescence assay, as described earlier (20, 22).

Genetic analysis

Human leukocyte antigen (HLA) genotyping was performed according to the screening protocol in the TRIGR study (20). The initial HLA-DQB1 typing for risk associated (*DQB1**02, *DQB1**0302) and protective (*DQB1**0301, *DQB1**0602, *DQB1**0603) alleles was complemented with DQA1 typing for *DQA1**0201 and *DQA1**05 alleles in those with *DQB1**02 without protective alleles or the major risk allele *DQB1**0302. This two-step screening technique is based on the hybridization of PCR products with lanthanide-labeled probes detected by time-resolved fluorometry as described earlier (23, 24).

Similarly to the HLA assays, the principle of microtitration-plate-bound biotinylated amplification products and lanthanide-labeled probes was applied in the single nucleotide polymorphism assays for the *INS* -23A/T (rs689) polymorphism (25). For *PTPN22* 1858C/T (rs2476601) analysis, a one-step assay based on asymmetric amplification and subsequent time-resolved fluorescence measurement was used. Upon hybridizing to the PCR-product the probes dehybridize from their complementary quenchers and become capable for emitting fluorescence (26, 27).

Statistical analysis

The nonparametric Mann–Whitney *U* test was applied for comparisons between the groups. Wilcoxon test was used for comparisons between samples collected after stimulation with and without insulin. Correlation analyses were performed with the Spearman rank correlation test (*r*). The statistical analyses were performed with SPSS 17.0 for Windows (SPSS, Chicago, IL, USA). A *p* value less than 0.05 was considered significant.

Results

Prevalence of diabetes-associated antibodies in CB of offspring of T1D mothers

Seventeen of 19 (89%) offspring of mothers with T1D tested positive for ICA in CB with levels ranging from 3 to 380 (median 7 JDF units). Fifteen infants of 20 (75%) had IA in their CB sample, with a median level of 14.6 (range 3.0–77.7) relative units (RU). GADA were detected in 15 CB samples (75%) with a median of 40.2 (range 6.2–15.734) RU. IA-2A were observed to be positive in seven CB samples (35%) with a median of 22.8 (range 6.2–55.0) RU. One of the infants was positive for only one antibody, four were positive for two antibodies, nine were positive for three antibodies, and four were positive

for all four antibodies analyzed. There was an inverse correlation between the IA levels and the duration of maternal T1D ($r = -0.56$; $p = 0.010$). In contrast, no infants of the unaffected mothers had IA, GADA, or IA-2A in their CB, while 6 of 20 (30%) tested positive for ICA, all of them with a level of 3 JDF units.

Increased numbers of FOXP3 expressing CD4+CD25^{high} cells in infants of T1D mothers

The number of CD4+ T cells ($p = 0.14$, median levels 39.2 and 42.3%, respectively) or the number of CD4+CD25+ T cells ($p = 0.28$, median levels 12.5 and 11.4%, respectively) did not differ between the groups. The percentage of FOXP3-positive cells among CD4+CD25^{high} T cells was increased in freshly isolated CBMCs from offspring of mothers with T1D compared with infants of unaffected mothers [$p = 0.023$; median values 31.5% (range 6.1–63.1%) and 16.4% (range 2.0–36.3%), respectively] (Fig. 1). The percentages of FOXP3-positive CD4+CD25^{high} cells tended to be higher in those infants of diabetic mothers who carried the -23 HphI AT genotype of the insulin gene when compared with the infants who carried the T1D-associated AA genotype variant ($p = 0.07$). None of the children had the TT genotype. The percentage of FOXP3+ cells in CD4+CD25^{high} cells were not significantly related to maternal age, gestational age, or the IA levels in the two groups of infants. In addition, neither maternal HbA1c (analyzed at the end of the pregnancy) nor the duration of maternal T1D correlated with the proportion of CD4+CD25^{high} FOXP3+ cells (data not shown).

In vitro insulin-induced FOXP3 in CD4+CD25^{high} cells in the infants of T1D mothers

In infants with maternal T1D, the increase in the percentage of FOXP3+ cells in CD4+CD25^{high} cells among insulin-stimulated CBMCs was higher when compared with unstimulated CBMCs cultured *in vitro* for 72 h ($p = 0.0002$; Wilcoxon test). In infants of non-diabetic mothers there was no difference between FOXP3 expression in CD4+CD25^{high} T cells after stimulation with insulin compared with unstimulated CBMCs ($p = 0.21$, Wilcoxon test). Median change in the proportion of FOXP3+ cells in CD4+CD25^{high} cells induced by *in vitro* insulin stimulation was 9.6% in the infants of diabetic mothers and 3.7% in the infants of non-diabetic mothers ($p = 0.008$; Mann–Whitney U test) (Fig. 2). FOXP3 median fluorescence intensity in CD4+CD25^{high}FOXP3+ cells in CBMCs increased also after stimulation with insulin in infants with maternal T1D compared with CBMCs in infants of non-diabetic mothers ($p = 0.042$).

In infants with maternal diabetes FOXP3, NFATc2, STIM1, IL-10, and TGF- β -specific mRNA increased significantly in CBMCs in response to insulin (Wilcoxon test $p < 0.001$, $p = 0.002$, $p = 0.003$, $p = 0.003$, and $p = 0.013$, respectively) (Fig. 3A), whereas no such increase was seen in the infants of non-diabetic mothers (Fig. 3B).

The relative mRNA level of FOXP3 correlated with the mRNA level of NFATc2 ($r = 0.88$, $p < 0.001$), and TGF- β and IL-10 transcripts correlated with each other ($r = 0.48$, $p = 0.038$) after stimulation with insulin. In the offspring of mothers with T1D the IA levels in the CB did not correlate with the number of insulin-induced CD4+CD25^{high}FOXP3+ cells ($r = 0.007$, $p = 0.98$).

In the infants with maternal diabetes and the disease-associated T allele at the *PTPN22* 1858 position the upregulation of *STIM1* and *NFATc2*-specific mRNA in CB cells on insulin stimulation was reduced compared with infants carrying the CC genotype ($p = 0.007$ and $p = 0.014$, Mann–Whitney *U* test; Fig. 4). No such associations with the *PTPN22* genotypes were seen in the infants of non-diabetic mothers. No associations were observed with the *HLA-DQ* genotypes nor were any gender-specific differences seen.

Discussion

We observed that the proportion of circulating CB CD4+CD25^{high}FOXP3+ T cells was increased in offspring of mothers with T1D when compared with infants of unaffected mothers. Interestingly, the percentage of FOXP3+ cells in CD4+CD25^{high} cells tended to be higher in those infants with maternal diabetes who carried the protective AT insulin genotype than in the infants who carried the AA genotype. The presence of the T allele has been reported to associate with higher expression levels of insulin in thymus and is known to be protective for T1D (13, 14). The expression level of insulin in thymus may thus regulate the induction of natural insulin-specific Tregs. Our results give support to this view because the highest numbers of Tregs were associated with the AT genotype in the infants with maternal diabetes.

The risk of T1D should be similar in children of diabetic mothers and fathers if only genetic factors are involved. The risk is, however, reduced in offspring of diabetic mothers as compared with the risk in offspring of diabetic fathers (1–4). This indicates that there are non-genetic factors which protect the children of diabetic mothers despite of the increased genetic risk. We hypothesized that exposure to insulin due to the maternal insulin treatment could induce regulatory T cells and explain the reduced risk. This was the reason why we focused on insulin as an antigen. In offspring of healthy mothers, exposure to exogenous insulin is not present and thus the expansion of insulin-specific Tregs should not be triggered.

Our results indicate firstly, that expansion of Tregs occurs *in utero* in infants of mothers with T1D, which may explain the decreased risk of T1D in the offspring of affected mothers compared with children of fathers with T1D. Secondly, as the insulin genotype showed association with the numbers of Tregs-induced *in utero* in the infants with maternal diabetes, maternal exogenous insulin treatment may have induced the expansion of insulin-specific Tregs. It has been implicated that insulin is transferred to the fetus via IA complexes and bioactive insulin is released into the circulation of the fetus (8, 28). IA in insulin-treated patients are of IgG class and are actively transported through the placenta (29). It is possible that either transplacentally transferred insulin bound to IA or IA as such are responsible for the induction of insulin-specific tolerance in the infants of diabetic mothers. The IA titers in the offspring of the T1D mothers did not, however, correlate with the number of insulin-induced CD4+CD25^{high}FOXP3+ cells. The IA may not directly correlate with the amount of transplacentally transferred insulin, because it has been reported that IA have no influence on insulin concentration in CB (7). It is also possible that similar kind of tolerance to other autoantigens is induced in the infants of diabetic mothers due to the transplacental transfer of autoantigens, e.g., GAD, in autoantibody–antigen complexes. We focused, however, on

insulin due to the fact that circulating insulin is seen at high levels after insulin treatment. Interestingly, the risk for T1D has been reported to be higher in children born before maternal onset of diabetes than in infants born after disease onset (30). This epidemiological observation also supports the view that the maternal insulin therapy is the inducer of the regulatory mechanisms during pregnancy. The present results of enhanced regulatory activity in the infants of diabetic mothers are also in agreement with our previous report of decreased insulin-specific T cell proliferation responses at the age of 9 months in offspring of diabetic mothers (10). Accordingly, early exposure to human insulin could induce FOXP3 expressing Tregs with potential to downregulate β -cell autoimmunity later in life.

To evaluate the presence of insulin-specific T cells in CB we stimulated CBMCs with human insulin *in vitro*. Increased expression of FOXP3 in the CD4+CD25^{high} cells and upregulation of FOXP3, NFATc2, STIM1, IL-10, and TGF- β -specific mRNA were seen after *in vitro* stimulation of CBMCs with human insulin in infants with maternal T1D, but not in the infants of non-diabetic mothers. This suggests that the *in vitro* stimulation with insulin resulted in further expansion of insulin-specific Tregs induced *in utero*.

Insulin stimulation induced upregulation of several molecules involved in the T cell receptor (TCR) mediated activation of Tregs, such as FOXP3, NFATc2, STIM1, as well as Treg-related cytokines, IL-10 and TGF- β , exclusively in the offspring of mothers with T1D. In addition, the impaired insulin-induced upregulation of STIM1 and NFATc2 was associated with the predisposing *PTPN22* polymorphism in the infants with maternal T1D. The *PTPN22*-coded protein is a downstream signaling molecule in TCR-mediated T cell activation (31), and impaired T cell activation has been associated with the T1D predisposing *PTPN22* polymorphism (32), which is in agreement with our observations of impaired insulin-induced T cell signaling seen as weak STIM1 and NFATc2 responses. STIM1 is a sensor of endoplasmic reticulum calcium in T cell activation and tolerance (19). These findings strongly support the specificity of the Treg response to insulin. Interestingly, the strength of TCR signaling altered thymic T cell selection, regulatory T cell function and autoimmune phenotype in experimental studies (33). On the basis of these findings weak TCR signaling could lead to decreased regulatory T cell induction in thymus and/or defects in Treg function (34). We suggest that *PTPN22* risk variant of T1D could be associated with the impaired activation of insulin-specific regulatory T cells because of impaired TCR signaling and thus contribute to T1D.

Our observations indicate that expansion of Tregs occurs *in utero* in infants of diabetic mothers and is associated with the insulin genotype conferring protection against T1D. This may be due to the transplacental transfer of insulin-insulin antibody complexes. Accordingly early induction of autoantigen-specific immunological tolerance might protect against the induction of autoimmunity and progression to diabetes later in life. Our results are encouraging in terms of attempts to develop insulin-specific immune intervention for prevention of T1D.

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References

1. Warram JH, Krolewski AS, Gottlieb MS, Kahn CR. Differences in risk of insulin-dependent diabetes in offspring of diabetic mothers and diabetic fathers. *N Engl J Med*. 1984; 311:149–152. [PubMed: 6738600]
2. Tuomilehto J, Podar T, Tuomilehto-Wolf E, Virtala E. Evidence for importance of gender and birth cohort for risk of IDDM in offspring of IDDM parents. *Diabetologia*. 1995; 38:975–982. [PubMed: 7589885]
3. The EURODIAB ACE Study Group and the EURODIAB ACE Substudy 2 Study Group. Familial risk of type I diabetes in European children. *Diabetologia*. 1998; 41:1151–1156. [PubMed: 9794100]
4. Harjutsalo V, Reunanen A, Tuomilehto J. Differential transmission of type 1 diabetes from diabetic fathers and mothers to their offspring. *Diabetes*. 2006; 55:1517–1524. [PubMed: 16644714]
5. Palmer JP, Asplin CM, Clemons P, et al. Insulin antibodies in insulin-dependent diabetics before insulin treatment. *Science*. 1983; 222:1337–1339. [PubMed: 6362005]
6. Greenbaum CJ, Palmer JP. Insulin antibodies and insulin autoantibodies. *Diabet Med*. 1991; 8:97–105. [PubMed: 1827408]
7. Fineberg SE, Kawabata TT, Finco-Kent D, Fountaine RJ, Finch GL, Krasner AS. Immunological responses to exogenous insulin. *Endocr Rev*. 2007; 28:625–652. [PubMed: 17785428]
8. Knip M, Lautala P, Leppäluoto J, Åkerblom HK, Kouvalainen K. Relation of enteroinsular hormones at birth to macrosomia and neonatal hypoglycemia in infants of diabetic mothers. *J Pediatr*. 1983; 103:603–611. [PubMed: 6352886]
9. DiMario U, Fallucca F, Gargiulo P, et al. Insulin-anti-insulin complexes in diabetic women and their neonates. *Diabetologia*. 1984; 27 (Suppl):83–86. [PubMed: 6383924]
10. Paronen J, Knip M, Savilahti E, et al. Effect of cow's milk exposure and maternal type 1 diabetes on cellular and humoral immunization to dietary insulin in infants at genetic risk for type 1 diabetes. Finnish trial to reduce IDDM in the genetically at risk study group. *Diabetes*. 2000; 49:1657–1665. [PubMed: 11016449]
11. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol*. 1995; 155:1151–1164. [PubMed: 7636184]
12. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell*. 2008; 133:775–787. [PubMed: 18510923]
13. Pugliese A, Zeller M, Fernandez A, et al. The insulin gene is transcribed in the human thymus and transcription levels correlated with allelic variation at the INS VNTR-IDDM2 susceptibility locus for type 1 diabetes. *Nat Genet*. 1997; 15:293–297. [PubMed: 9054945]
14. Pugliese A, Brown D, Garza D, et al. Self-antigen-presenting cells expressing diabetes-associated autoantigens exist in both thymus and peripheral lymphoid organs. *J Clin Invest*. 2001; 107:555–564. [PubMed: 11238556]
15. Walker MR, Kasproicz DJ, Gersuk V, et al. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25– T cells. *J Clin Invest*. 2003; 112:1437–1443. [PubMed: 14597769]
16. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol*. 2003; 4:330–336. [PubMed: 12612578]
17. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science*. 2003; 299:1057–1061. [PubMed: 12522256]
18. Rudensky AY, Gavin M, Zheng Y. FOXP3 and NFAT: partners in tolerance. *Cell*. 2006; 126:253–256. [PubMed: 16873058]

19. Oh-hora M, Yamashita M, Hogan PG, et al. Dual functions for the endoplasmic reticulum calcium sensors STIM1 and STIM2 in T cell activation and tolerance. *Nat Immunol.* 2008; 9:432–443. [PubMed: 18327260]
20. TRIGR Study Group. Study design of the trial to reduce IDDM in the genetically at risk (TRIGR). *Pediatr Diabetes.* 2007; 8:117–137. [PubMed: 17550422]
21. Hölttä V, Klemetti P, Sipponen T, et al. IL-23/IL-17 immunity as a hallmark of crohn's disease. *Inflamm Bowel Dis.* 2008; 14:1175–1184. [PubMed: 18512248]
22. Kukko M, Virtanen SM, Toivonen A, et al. Geographical variation in risk HLA-DQB1 genotypes for type 1 diabetes and signs of beta-cell autoimmunity in a high-incidence country. *Diabetes Care.* 2004; 27:676–681. [PubMed: 14988284]
23. Sjöroos M, Iitiä A, Ilonen J, Reijonen H, Lövgren T. Triple-label hybridization assay for type-1 diabetes-related HLA alleles. *BioTechniques.* 1995; 18:870–877. [PubMed: 7619493]
24. Laaksonen M, Pastinen T, Sjöroos M, et al. HLA class II associated risk and protection against multiple sclerosis-a Finnish family study. *J Neuroimmunol.* 2002; 122:140–145. [PubMed: 11777553]
25. Laine AP, Holmberg H, Nilsson A, et al. Two insulin gene single nucleotide polymorphisms associated with type 1 diabetes risk in the Finnish and Swedish populations. *Dis Markers.* 2007; 23:139–145. [PubMed: 17473382]
26. Hermann R, Lipponen K, Kiviniemi M, et al. Lymphoid tyrosine phosphatase (LYP/PTPN22) Arg620Trp variant regulates insulin autoimmunity and progression to type 1 diabetes. *Diabetologia.* 2006; 49:1198–1208. [PubMed: 16614815]
27. Kiviniemi M, Nurmi J, Turpeinen H, Lövgren T, Ilonen J. A homogeneous high-throughput genotyping method based on competitive hybridization. *Clin Biochem.* 2003; 36:633–640. [PubMed: 14636879]
28. Menon RK, Cohen RM, Sperling MA, Cutfield WS, Mimouni F, Khoury JC. Transplacental passage of insulin in pregnant women with insulin-dependent diabetes mellitus. Its role in fetal macrosomia. *N Engl J Med.* 1990; 323:309–315. [PubMed: 2195347]
29. Potter KN, Wilkin TJ. The molecular specificity of insulin autoantibodies. *Diabetes Metab Res Rev.* 2000; 16:338–353. [PubMed: 11025558]
30. Warram JH, Martin BC, Krolewski AS. Risk of IDDM in children of diabetic mothers decreases with increasing maternal age at pregnancy. *Diabetes.* 1991; 40:1679–1684. [PubMed: 1756908]
31. Vang T, Congia M, Macis MD, et al. Autoimmune-associated lymphoid tyrosine phosphatase is a gain-of-function variant. *Nat Genet.* 2005; 37:1317–1319. [PubMed: 16273109]
32. Aarnisalo J, Treszl A, Svec P, et al. Reduced CD4+ T cell activation in children with type 1 diabetes carrying the PTPN22/Lyp 620Trp variant. *J Autoimmun.* 2008; 31:13–21. [PubMed: 18299186]
33. Tanaka S, Maeda S, Hashimoto M, et al. Graded attenuation of TCR signaling elicits distinct autoimmune diseases by altering thymic T cell selection and regulatory T cell function. *J Immunol.* 2010; 185:2295–2305. [PubMed: 20644168]
34. Stanford S, Mustelin T, Bottini N. Lymphoid tyrosine phosphatase and autoimmunity: human genetics rediscovers tyrosine phosphatases. *Semin Immunopathol.* 2010; 32:127–136. [PubMed: 20204370]

Fig. 1.

Flow cytometric analysis of the percentage of FOXP3+ cells among CD4+CD25^{high} regulatory T cells in cord blood. Cord blood mononuclear cells were stained with CD4, CD25, FOXP3, and isotype control. The expression of FOXP3 in CD4+CD25^{high} regulatory T cells is presented in infants with maternal type 1 diabetes ($n = 16$) and infants of non-diabetic mothers ($n = 12$). Values represent the proportion (%) of FOXP3-positive cells. Horizontal lines represent median values. p Value derived from the Mann–Whitney U test is shown.

Fig. 2.

The increase in the proportion of FOXP3-positive cells in CD4+CD25^{high} regulatory T cells in infants with maternal diabetes (n = 15) and infants of non-diabetic mothers (n = 17) after 72 h stimulation with human insulin. Cord blood derived mononuclear cells were stained with CD4, CD25, FOXP3, and isotype control and analyzed with flow cytometry. Horizontal lines represent median values. p Value comparing the *in vitro* insulin-induced change in the numbers of FOXP3 expressing CD4+CD25^{high} cells between the study groups (Mann–Whitney *U* test) is shown.

Fig. 3.

Expression of FOXP3, IL-10, NFATc2, STIM1, and transforming growth factor (TGF)-β specific mRNA in cord blood mononuclear cells in infants of mothers with type 1 diabetes (A) and of unaffected mothers (B) after 72 h stimulation with human insulin (hi) or medium alone (neg). Results of FOXP3, IL-10, and NFATc2 are presented as 1000× relative transcription and STIM1 and TGF-β are presented as 100× relative transcription. Median and interquartile range are shown. The asterisk indicates significant differences (* $p < 0.05$, ** $p < 0.01$; Wilcoxon test).

Fig. 4.

Upregulation of STIM1 and NFATc2-specific mRNA expressed as fold change in relation to the *PTPN22* genotype in infants of mothers with type 1 diabetes ($n = 19$). Fold change in STIM1 (A) and NFATc2 (B) was calculated by dividing the relative mRNA level of insulin-stimulated (72 h) cord blood mononuclear cells (CBMCs) by the relative mRNA level of non-stimulated CBMCs. Horizontal lines represent median values. p Values comparing the groups (Mann–Whitney *U* test) are shown.

Table 1

Characteristics of mothers with type 1 diabetes (T1D) and their offspring vs. control mothers and infants

	Maternal T1D	No maternal T1D	p [*]
n	20	20	—
Gestational age (wk)	37.4 (35.4–39.9)	39.6 (37.1–42.3)	<0.001
Maternal age (yr)	28.9 (23.8–44.7)	30.4 (24.6–42.1)	0.39
Children (female/male)	8/12	8/12	—
Caesarean section	15 (75%)	8 (40%)	0.027
Birth weight (g)	3870 (2550–5335)	3528 (2710–4270)	0.023
Birth length (cm)	50 (46–55)	50 (47–54)	0.62
T1D in father/sibling	0	14 (70%)	—
HLA genotype			
DR3-DQ2/DR4-DQ8	2	2	
DR4-DQ8/x	8	7	
DR3-DQ2/y	4	6	
z/z	6	5	
INS –23 HphI A/T			
AA	13	16	
AT/TT	7	4	
PTPN22 1858C/T			
CT	7	6	
CC	13	14	

DR3-DQ2, *DQA1**05-*DQB1**02; DR4-DQ8, *DQB1**0302; T1D, type 1 diabetes; x, non-DR3-DQ2; y, non-DR4-DQ8; z, neither risk haplotype.

Data are medians (with range) or n (%) unless otherwise indicated.

* Value of significance in the Mann–Whitney *U* test. HLA genotype indicates the presence of HLA-DQ haplotypes associated with T1D risk.