Original Article

Decreased In Vitro Type 1 Immune Response Against Coxsackie Virus B4 in Children With Type 1 Diabetes

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Enteroviruses, particularly Coxsackie virus B4 (CVB4), are considered to be involved in the pathogenesis of type 1 diabetes. We wanted to compare the characteristics of T-cell immune response to CVB4 in children with type 1 diabetes and healthy children with and without HLA risk-associated haplotypes (HLA-DR3-DQ2 or HLA-DR4-DQ8) for type 1 diabetes. Peripheral blood mononuclear cells (PBMCs) were isolated and cultured with CVB4 and analyzed for cytokine and chemokine receptors by flow cytometry and for expression of transcription factors Tbet and GATA-3 by RT-PCR and Western blot. Culture supernatants were analyzed for secretion of γ-interferon (IFN-γ). In children with type 1 diabetes, a decreased percentage of T-cells expressed CCR2, CXCR6, interleukin (IL)-18R, and IL-12Rβ_2-chain after in vitro stimulation with CVB4 in comparison with healthy children with or without HLA risk genotype. Moreover, we found that children with type 1 diabetes had decreased IFN-γ secretion and expression of Tbet, both on mRNA and protein level, in CVB4-stimulated PBMCs. Accordingly, children with type 1 diabetes show an impaired type 1 immune response against CVB4 compared with healthy children. This may lead to a delayed clearance of the virus and, at least partly, explain why children with type 1 diabetes may be more prone to CVB4 infections and related complications, such as β-cell damage. *Diabetes* 55: 996–1003, 2006

Type 1 diabetes is an autoimmune disease caused by destruction of the insulin producing β-cells in pancreas, which is probably mediated by autoreactive T-cells. The risk of developing type 1 diabetes is to some extent genetically determined, but environmental factors are also involved in the autoimmune process. This is demonstrated, e.g., by the rapid increase of the disease incidence during the last decades (1).

Enteroviruses, in particular Coxsackie virus B4 (CVB4), have been proposed to be an environmental trigger of β-cell autoimmunity and type 1 diabetes. An increased number of enterovirus infections have been shown in pre-diabetic individuals in a number of prospective studies using serological tests and enterovirus RNA detection (2–5). Enterovirus infections during pregnancy have also been suggested to be associated with an increased risk of developing type 1 diabetes in the offspring (5–7); however, the results are contradictory (8,9).

Several case-control studies have reported serological evidence for recent enterovirus infection (10–13) and enterovirus RNA (14–17) in the peripheral circulation in patients with type 1 diabetes. Moreover, cellular and humoral immune responses to enteroviruses have been reported to be aberrant in children with type 1 diabetes carrying risk genotype for type 1 diabetes (18–21). Despite these findings, more studies and especially prospective follow-up studies are still needed to determine whether there is a causal association of CVB4 infection and type 1 diabetes (22).

Several mechanisms for the induction of β-cell destruction by viruses have been suggested. Viral antigens may show molecular mimicry with β-cell antigens and induce autoreactivity, viruses may also cause a direct cytolysis of infected β-cells or induce bystander activation of autoreactive T-cells due to the inflammatory mediators released in infected islets.

The balance of type 1 and type 2 T-cells plays a major role in the regulation of cellular and humoral immune responses to give rise to an appropriate immune response against foreign antigens, e.g., viruses. Naïve T-cells express the chemokine receptors CXCR4 and CCR7 (23). Type 1 T-cells are characterized by expression of the cytokine receptors interleukin (IL)-12Rβ_2-chain and IL-18R (24) and the chemokine receptors CXCR3, CCR5, CCR2 (23), and CXCR6 (25). The transcription factor Tbet is associated with type 1 response, such as IFN-γ activation (24). The chemokine receptors CCR3 and CCR4 (23) and the transcription factors GATA-3 and c-maf are associated with type 2 T-cells (24).

To study whether the immune response to CVB4 differs between children with type 1 diabetes and healthy children, we stimulated peripheral blood mononuclear cells (PBMCs) with inactivated CVB4 and studied expression of intracellular transcription factors, secretion of IFN-γ, and the expression of cytokine and chemokine receptors on T-cells.

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CVB4, Coxsackie virus B4; IFN-γ, γ-interferon; IL, interleukin; PBMC, peripheral blood mononuclear cell.

RESEARCH DESIGN AND METHODS

Cell culture. After informed consent from the participants, venous blood was collected into Cell Preparation Tubes (Becton Dickinson, Stockholm, Sweden) from children with type 1 diabetes (mean for duration of type 1 diabetes was 20.2 months, range 1–48 months; mean HbA1c [A1C] 6.0%) (n = 15, mean age 11 years; range 6–17 years) and healthy children with (n = 13, mean age 11 years; range 10–18 years) or without (n = 14, mean age 12 years; range 10–16 years) HLA risk genes. The month for collection of blood samples did not differ between the groups. Selected HLA-DQB1 and -DQA1 alleles were typed as described previously (26,27) to detect the presence of type 1 diabetes risk-associated DR3-DQ2 (DQA1*05-DQB1*02) and DR4-DQ8 (DQAB1*0302) haplotypes. The healthy children without HLA risk-associated haplotypes were negative for autoantibodies against GAD, tyrosine phosphatase, and insulin. The healthy children with HLA risk genotype were also negative for autoantibodies, except for two individuals, one with tyrosine phosphatase autoantibodies and the other with insulin autoantibodies.

PBMCs were isolated from the blood samples by centrifugation at 1,500 × g for 20 min, and then the cells were washed three times with RPMI 1640 (Gibco/Life Technologies, Paisley, U.K.) supplemented with 5% inactivated human AB+ serum (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland). The cells were then diluted to 1×10^6 cells/ml with RPMI 1640 supplemented with inactivated human AB+ serum (5%), L-glutamine (2 mmol/l; Invitrogen, Stockholm, Sweden), and gentamicin (25 μg/ml; Sigma-Aldrich, Stockholm, Sweden). The PBMCs were cultured in wells with 2×10^6 cells per well.

The PBMCs were stimulated on day 0 with heat-inactivated (+56°C for 30 min) CVB4 strain J.V.B. (1 μg/ml; obtained from the American *Type Culture* Collection, Manassas, VA). On day 3, IL-2 was added (32 pg/ml; BD PharMingen, Stockholm, Sweden). Supernatants from the cultures were collected on day 7 and stored at −70°C for further analysis of cytokine profiles. On day 7, the cells were analyzed using flow cytometry. Lysed cells for mRNA and cell pellets for Western blot analysis were collected and saved at −70°C and in liquid nitrogen, respectively, for further analysis.

Flow cytometry. The cells were stained with 5 μl/300,000 cells allophycocyanin-anti-CD4, peridinin chlorophyll protein-anti-CD8, phycoerythrin-anti-IL-12R-β_2-chain, phycoerythrin-anti-CCR4, fluorescein isothiocyanate (FITC)-anti-CCR5 as well as isotype-matched controls (BD PharMingen, San José, CA), FITC-anti-CXCR4, FITC-anti-CXCR3, phycoerythrin-anti-IL-18R, FITC-anti-CCR7, phycoerythrin-anti-CCR2, FITC-anti-CCR3, and phycoerythrin-anti-CXCR6 (R&D Systems, Minneapolis, MN) for 30 min. After being washed with PBS (Medicago, Uppsala, Sweden) supplemented with 0.5% BSA (Difco Laboratories, Detroit, MI), the cells were resuspended in 160 μl PBS supplemented with 0.5% BSA. The labeled cells were analyzed with four-color flow cytometry using a FACSCalibur and CellQuest software (Becton Dickinson, San José, CA).

The lymphocytes were gated on forward and side scatter. Cells (15,000) were acquired, and two parameter dot plots were created. For isotype controls, 5,000 cells were acquired. Compensation was performed to adjust for spectrally adjacent dye pairs. The quadrants in the dot plots were placed according to the staining of the unstained cells and isotype controls.

Autoantibodies. Levels of autoantibodies against GAD and thyrosine phosphatase were analyzed in plasma by immunoprecipitation described previously (28). Positivity for GAD and thyrosine phosphatase autoantibodies was determined as antibody levels above the 98th percentile.

Diabetes Autoantibody Standardization Program 2003 was used to set the specificity and sensitivity levels. For GAD autoantibody assay, the specificity was 98% and sensitivity was 78%, and for thyrosine phosphatase autoantibody assay, the specificity was 100% and the sensitivity was 48%.

Autoantibodies against insulin were analyzed by a radio binding assay according to Williams et al. (29). The cutoff for positivity was determined as antibody levels above the 98th percentile. In the Diabetes Autoantibody Standardization Program 2003, we had specificity for autoantibodies against insulin of 100%, whereas the sensitivity was 24%.

Standard curves and interpolated values of samples were performed using GraphPad Prism 4 (GraphPad Software, San Diego, CA).

Enzyme-linked immunosorbent assay for IFN-γ. The concentration of IFN-γ in supernatant samples was studied according to the method described previously (30).

Real-time RT-PCR. For the mRNA expression measurements, the total RNA were isolated from the samples by using Genelute total RNA isolation kit (Sigma-Aldrich, St. Louis, MO). cDNA was prepared using Applied Biosystems TaqMan cDNA transcription kit (Applied Biosystems, Foster City, CA) according to manufacturer's protocol. Elimination of the genomic DNA was done by additional DNAse I (Roche Diagnostics, Mannheim, Germany) treatment before the cDNA synthesis. Random Hexamers (Applied Biosystems) was used to prime the first-strand synthesis, and the reaction was carried out in a total volume of 20 μl with Multiscribe Reverse transcriptase enzyme according to manufacturer's protocol (Applied Biosystems). A total of 1.8 μl cDNA was used for each TaqMan measurement triplicate.

6-carboxyfluorescein–labeled TaqMan Gene Expression assays were used to measure transcription levels of the selected genes (T-bet, Hs00203436_m1; GATA-3, Hs00231122_m1; c-maf, Hs00193519_m1; IL-12Rβ, Hs00234651_m1; IL-4Rβ, Hs00166237_m1; and ribosomal 18s, Hs99999901_s1).

Real-time PCR was carried out according to manufacturer's protocols using TaqMan Universal PCR master mix (Applied Biosystems). ABI Prism 7700 sequence detector instrumentation was used for signal detection. Sequence detector was programmed to an initial step of 2 min at 50°C and 10 min at 95°C, followed by 50 thermal cycles of 15 s at 95°C and 1 min at 60°C.

The quantitative value obtained from the TaqMan run is a threshold cycle (Ct), which indicates the number of PCR cycles at which the amount of amplified target molecule exceeds a predefined threshold value. The difference value (ΔCt) is the normalized quantitative value of the expression level of the target gene achieved by subtracting the Ct value of the housekeeping gene (18s) from the Ct value of the target gene.

In vitro-transcribed cDNA pool denoted as a calibrator was considered as an interassay standard and to which all the other samples were compared. Calculations are expressed as follows: $\Delta\Delta Ct(\times 1) = \Delta Ct(\times 1) - \Delta Ct$ (calibrator). The difference in expression level is given by $2^{-\Delta\Delta Ct}$.

Western blot analysis for Tbet and GATA-3. Total protein (5 μg) isolated from CVB4-stimulated PBMCs was run on a 10% SDS-polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA) and transferred onto polyvinylidene difluoride nitrocellulose blotting membrane (Amersham Biosciences UK, Buckinghamshire, U.K.). The membrane was blocked for 1 h in PBS-Tween (Medicago) supplemented with 5% nonfat dried milk (Bio-Rad Laboratories) and 0.2% sodium fluoride (Merck, Damstadt, Germany). The membrane was incubated with anti-T-bet (final dilution 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-GATA-3 (final dilution 1:1,000; Santa Cruz Biotechnology) followed by washes and incubation with horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (final dilution 1:15,000; Bio-Rad Laboratories). The membrane was blocked again as previously described. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (final dilution 1:1,000; Santa Cruz Biotechnology) was then incubated with the membrane followed by washes and incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (final dilution 1:15,000; Bio-Rad Laboratories). The proteins were detected with ECL Plus kit (Amersham Biosciences) and chemiluminiscence films (Amersham Biosciences). The immunoblots were semiquantified by densiometric scanning (Gel-PRO analyzer; Media Cybernetics, Silver Spring, MD).

Micorneutralization test for CVB4 antibodies. CVB4 antibodies were determined by a microneutralization assay in green monkey kidney cells, a continuous cell line from green monkey kidneys. Pretitrated virus (75 μl) corresponding to 30–300 TCID_50 units was mixed with 75 μl fourfold dilutions of plasma specimens on microtiter plates (96-well Nunclon Microtest plates). The mixture was incubated for 1 h at 36°C and then left overnight at 4°C. Then, 30,000 cells were added to each well, and the plates were incubated at 36°C for 6 days before staining with crystal violet. The highest dilution with complete inhibition of virus-induced cytopathic effect was considered the end point titer.

Statistical analysis. For comparison among three groups, Kruskal-Wallis was used as pretest and Mann-Whitney U test was used for comparison of two unrelated groups. For correlation analysis of variables, Spearman's rank order correlation coefficient test was used. P values below 0.05 were considered significant. All statistical analyses were made using GraphPad Prism 4.

Ethical considerations. The study was approved by the Regional Ethics Committee for Human Research at the Faculty of Health Sciences, Linköping University.

RESULTS

Flow cytometry analysis of cytokine- and chemokine-receptor expression on T-cells after CVB4 stimulation of PBMCs. Children with type 1 diabetes showed decreased response to CVB4 when compared with healthy children with or without HLA risk-associated haplotypes as seen in the percentage of CD4 and CD8 T-cells expressing CCR2 (medians for CD4 T-cells 1.50, 5.98, and 7.36, respectively, and for CD8 T-cells 3.56, 7.50, and 13.97, respectively) and CXCR6 (medians for CD4 T-cells 49.53, 67.01, and 67.11, respectively, and medians for CD8 T-cells 49.68, 79.58, and 70.26, respectively) (Fig. 1*A* and *B*). This

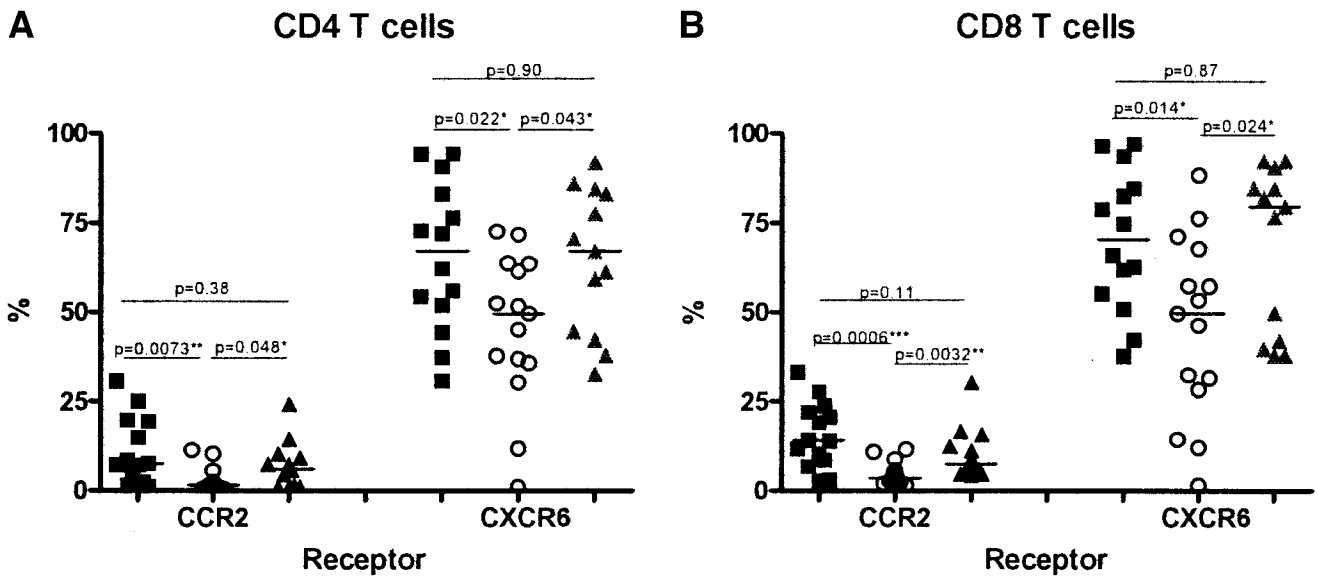

FIG. 1. Percentage CD4 and CD8 T-cells expressing CCR2 and CXCR6. Percentage of CD4 (*A*) and CD8 (*B*) T-cells expressing CCR2 and CXCR6 after CVB4 stimulation of PBMCs. *P* value represents significant differences in percentage of CD4 and CD8 T-cells expressing CCR2 or CXCR6 in healthy children without HLA risk genotype (■), children with type 1 diabetes (○), and healthy children with HLA risk genotype (▲). Horizontal lines indicate median values.

was also seen in the intensity of CCR2 (medians for CD4 T-cells 3.02, 4.10, and 4.13, respectively, and medians for CD8 T-cells 3.22, 4.22, and 4.55, respectively) and CXCR6 (medians for CD4 T-cells 10.00, 13.70, and 13.78, respectively, and medians for CD8 T-cells 10.00, 20.35, and 16.58, respectively) on CD4 and CD8 T-cells (Fig. 2*A* and *B*).

The percentage of CD4 and CD8 T-cells expressing IL-18R (medians for CD4 T-cells 24.30, 29.31, and 27.29, respectively, and medians for CD8 T-cells 18.18, 24.71, and 27.45, respectively, Fig. 3*A* and *B*) and the intensity of IL-18R (medians for CD4 T-cells 5.42, 6.32, and 6.16, respectively, and medians for CD8 T-cells 4.61, 6.15, and 6.44, respectively; Fig. 4*A* and *B*) on CD4 and CD8 T-cells were also lower in children with type 1 diabetes in comparison with healthy children with or without risk-associated HLA haplotypes. The percentage of CD8 T-cells expressing IL-12Rβ_2-chain and the intensity of IL-12Rβ_2-chain on CD8 T-cells tended to be lower in children with type 1 diabetes in comparison with healthy children with or without HLA risk haplotypes (median percentage 1.07, 2.61, and 2.60, respectively, and median intensity 2.55, 3.16, and 2.84, respectively) (Figs. 3*B* and 4*B*). There were no differences in expression of CXCR3, CXCR4, CCR3, CCR4, CCR5, or CCR7 between the groups.

Cytokine secretion of PBMCs stimulated with CVB4. The secretion of IFN-γ by PBMCs after 7 days of CVB4 stimulation was significantly lower in children with type 1

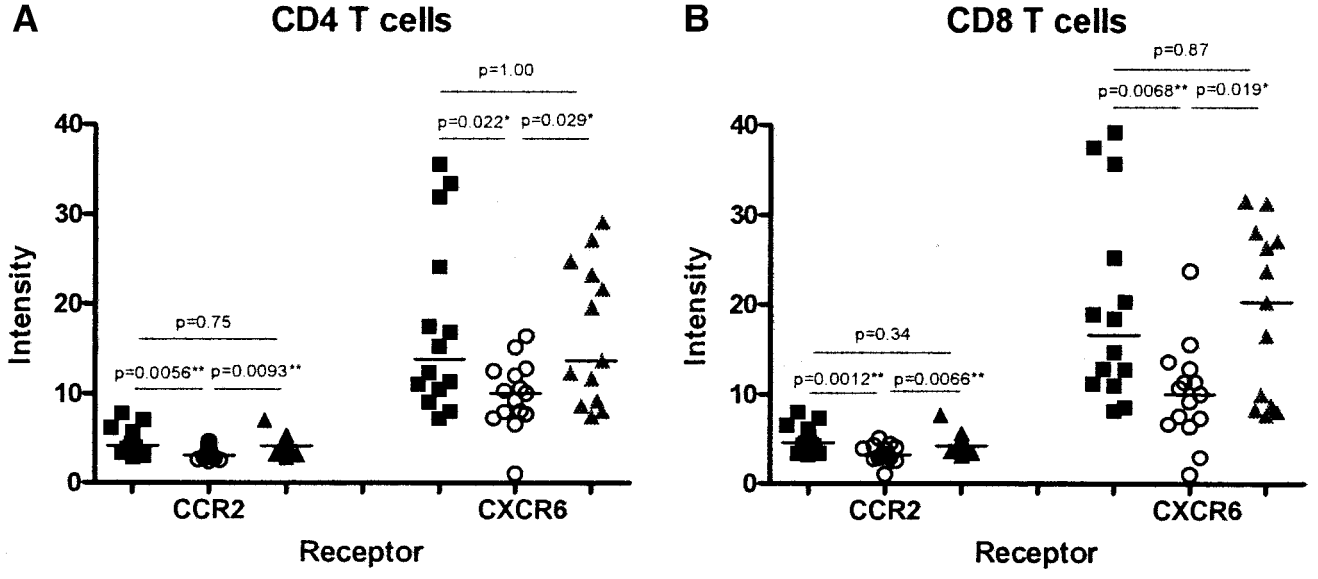

FIG. 2. Intensity of CCR2 and CXCR6 on CD4 and CD8 T-cells. Intensity of CCR2 and CXCR6 on CD4 (*A*) and CD8 (*B*) T-cells after CVB4 stimulation of PBMCs. *P* value represents significant differences in expression of CCR2 and CXCR6 on CD4 and CD8 T-cells in healthy children without HLA risk genotype (■), children with type 1 diabetes (○), and healthy children with HLA risk genotype (▲). Horizontal lines indicate median values.

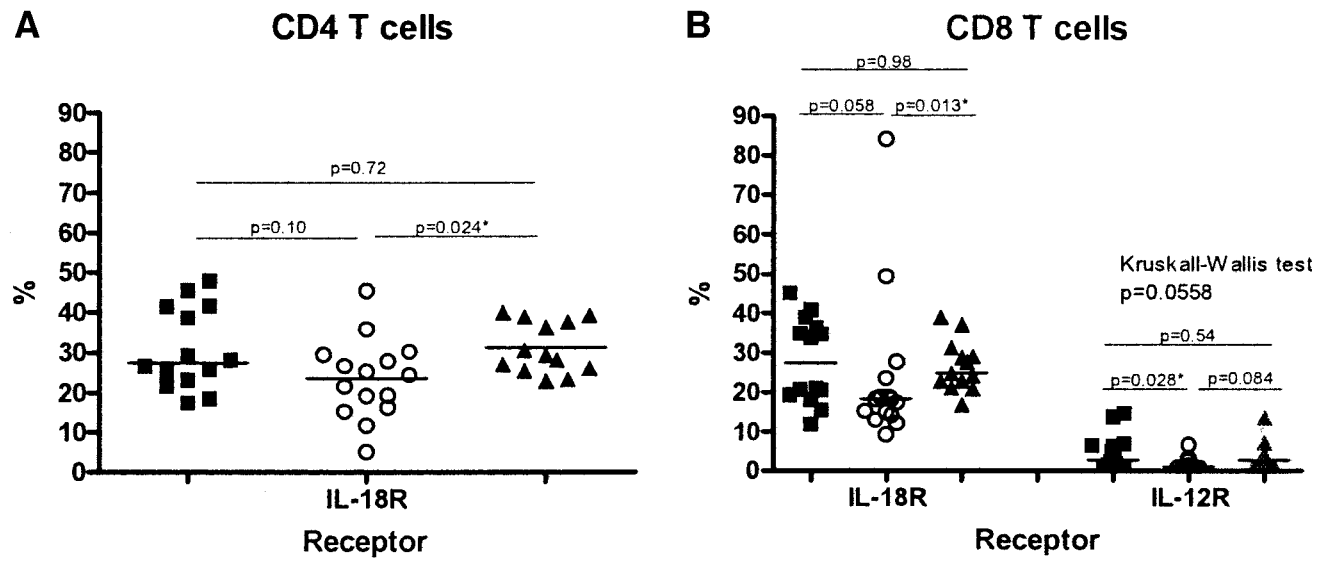

FIG. 3. Percentage of CD4 T-cells expressing IL-18R and percentage of CD4 and CD8 T-cells expressing IL-18R and IL-12Rβ_2-chain. Percentage CD4 (*A*) expressing IL-18R and CD4 and CD8 (*B*) T-cells expressing IL-18R and IL-12Rβ_2-chain after CVB4 stimulation of PBMCs. *P* value represents significant differences in percentage CD4 and CD8 T-cells expressing IL-18R or IL-12Rβ_2-chain in healthy children without HLA risk genotype (■), children with type 1 diabetes (○), and healthy children with HLA risk genotype (▲). Horizontal lines indicate median values.

diabetes in comparison with healthy children with or without HLA risk haplotypes (medians 10,441, 36,680, and 79,080 pg/ml, respectively) (Fig. 5).

Real-time RT-PCR analysis of Tbet, GATA-3, c-maf, IL-12R, and IL-4R in CVB4-stimulated PBMCs. The CBV4-induced mRNA levels of Tbet were significantly lower in children with type 1 diabetes in comparison with both healthy children with and without type 1 diabetes–associated risk haplotypes (medians 16.89, 36.52, and 36.39, respectively) (Fig. 6*A*). There were no differences in mRNA levels of GATA-3, c-maf, IL-12R, and IL-4R between the three groups.

Western blot analysis of Tbet and GATA-3 in CVB4-stimulated PBMCs. A tendency of lower ratio of Tbet to GAPDH measured as optical density was observed in children with type 1 diabetes in comparison with healthy children without HLA-associated risk genotype (medians 0.83 and 1.12, respectively) (Fig. 6*B*). There were no differences in the ratio of GATA-3 to GAPDH measured as optical density between the groups.

Neutralizing antibodies against CVB4. There were no differences in the titer of neutralizing antibodies against CVB4 between the three groups.

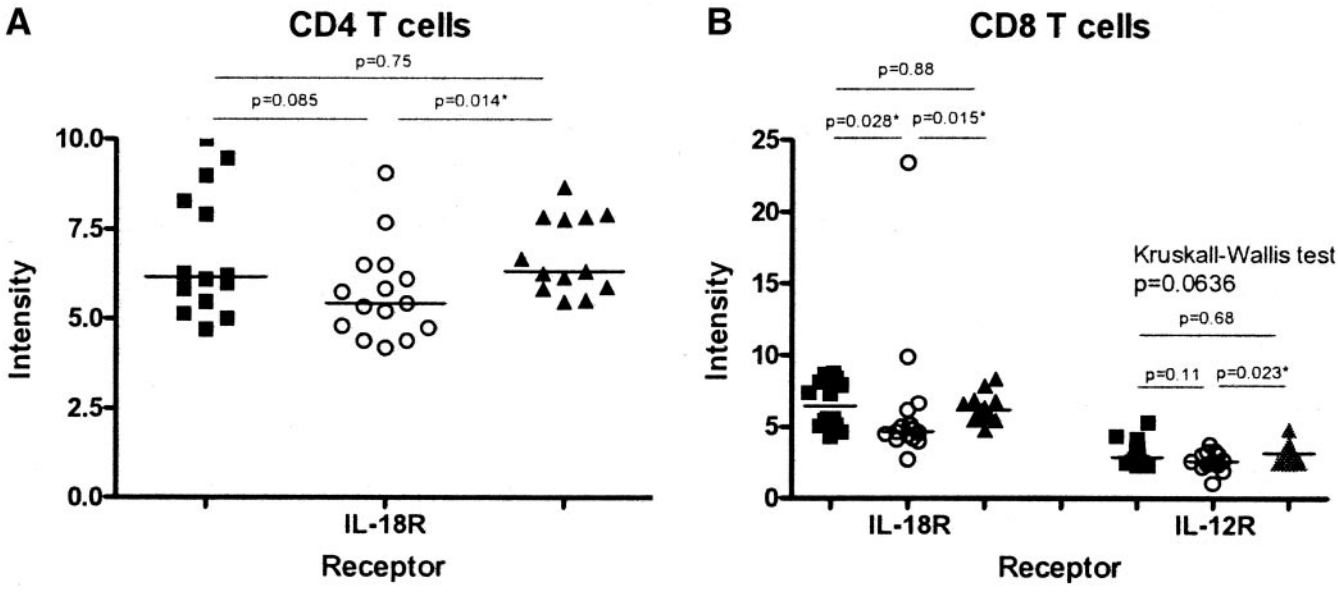

FIG. 4. Expression of IL-18R on CD4 T-cells and of IL-18R and IL-12Rβ_2-chain on CD8 T-cells. Expression of IL-18R on CD4 (*A*) and expression of IL-18R and IL-12Rβ_2-chain on CD8 (*B*) T-cells after CVB4 stimulation of PBMCs. *P* value represents significant differences in expression of IL-18R and IL-12Rβ_2-chain on CD4 and CD8 T-cells in healthy children without HLA risk genotype (black squares), children with type 1 diabetes (white circles), and healthy children with HLA risk genotype (▲). Horizontal lines indicate median values.

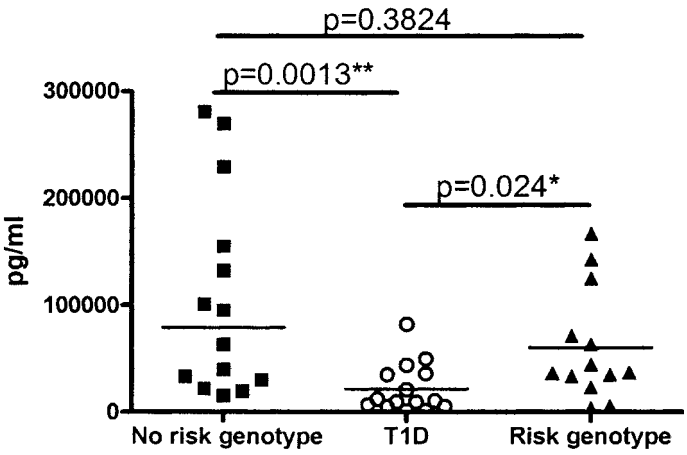

FIG. 5. Secretion of IFN-γ from PBMCs stimulated with CVB4. Secretion of IFN-γ in picograms/milliliter from PBMCs stimulated with CVB4 for 7 days in healthy children without HLA risk genotype (■), children with type 1 diabetes (T1D) (○), and healthy children with HLA risk genotype (▲). Horizontal lines indicate median values.

Correlation between A1C values and immune response parameters. There were no correlations between the A1C values in the children with type 1 diabetes and the expression of CCR2, CXCR6, IL-18R, IL-12Rβ_2-chain, or Tbet or the secretion of IFN-γ (Table 1).

TABLE 1
Correlation between A1C and different immunological parameters in children with type 1 diabetes

Parameter	Spearman's r	P value
CD4 T-cells expressing CCR2	0.059	0.83
CD8 T-cells expressing CCR2	−0.065	0.82
CD4 T-cells expressing CXCR6	0.086	0.76
CD8 T-cells expressing CXCR6	−0.084	0.77
CD4 T-cells expressing IL-18R	−0.26	0.35
CD8 T-cells expressing IL-18R	−0.33	0.23
CD4 T-cells expressing IL-12Rβ_2-chain	0.22	0.42
CD8 T-cells expressing IL-12Rβ_2-chain	−0.057	0.84
IFN-γ secretion	−0.37	0.17
Tbet mRNA	−0.19	0.51
Tbet protein	0.26	0.34

DISCUSSION

In children with type 1 diabetes, a decreased number of CD4 and CD8 T-cells expressing CCR2, CXCR6, and IL-18R and a lower intensity of these receptors on CD4 and CD8 T-cells after stimulation of PBMCs with CVB4 was found in comparison with healthy children with or without HLA risk haplotype. Furthermore, also the number of CD8 T-cells expressing IL-12Rβ_2-chain and the intensity of this receptor on CD8 T-cells tended to be lower in children with type 1 diabetes compared with both groups of healthy children after CVB4 stimulation. Type 1 immune response is associated with expression of the receptors CCR2 (23), CXCR6 (25), IL-18R, and IL-12Rβ_2-chain (24); the cytokine IFN-γ (31); and the transcription factor Tbet (24). Thus, our results suggest that the induction of type 1 immune response against CVB4 is impaired in children with type 1 diabetes. This interpretation was further supported by the findings of decreased secretion of IFN-γ and decreased mRNA levels of the transcription factor Tbet in CVB4-stimulated PBMCs in children with type 1 diabetes. A similar trend was also seen in the protein levels of Tbet. Impaired development of type 1 immune response to CVB4 may cause a delay in the elimination of CVB4 followed by systemic spread of the virus and infection of the β-cells. Enteroviruses are able to infect human β-cells in vitro (32). Also, enterovirus-infected β-cells have been reported in vivo in human pancreata (33). The appearance of β-cell autoimmunity has been reported to be associated with enterovirus infections diagnosed by antibody increases and RNA in the serum (2,34).

In our study, we have used inactivated CVB4, but we still observed activation of both CD4 and CD8 cells. CD8 cell activation in PBMCs was thus not dependent on the presentation of intracellular viral proteins produced in infected cells. It has been previously shown that inactivated virus can be cross-presented by dendritic cells and induce a cytotoxic T-cell response in in vitro cell cultures (35).

For an efficient elimination of viruses, the induction of Th1 cell responses and effector Tc1 cells play a major role. Interaction between chemokines and their receptors allows migration of effector Tc1 cells to inflammation sites, leading to an efficient elimination of the virus.

In our study, we found that the percentage of CD4 and CD8 T-cells expressing CCR2 in response to CVB4 is

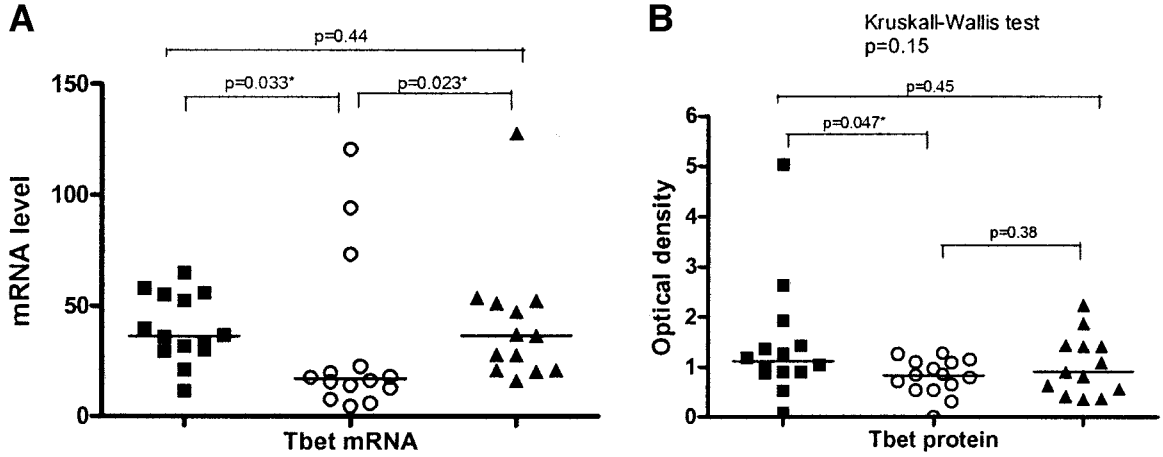

FIG. 6. Levels of Tbet mRNA and protein in PBMCs stimulated with CVB4. Relative level of Tbet mRNA (*A*) and ratio of Tbet to GAPDH optical density (*B*) from PBMCs stimulated with CVB4 for 7 days in healthy children without HLA risk genotype (■), children with type 1 diabetes (○), and healthy children with HLA risk genotype (▲). Horizontal lines indicate median values.

decreased in children with type 1 diabetes. Previous reports have reported that viruses induce CCR2 expression on CD8 T-cells in vivo (36). Several studies have shown that CCR2-deficient mice have a severe Th1 defect seen as a reduced IFN-γ production in response to purified protein derivative immunization and defects in elimination of intracellular pathogens (37,38).

We found impaired response to CVB4 stimulation in several of the parameters, which characterize type 1 immune response. Discrepant results showing increased IFN-γ production in response to CVB4 antigens in young adults with newly diagnosed type 1 diabetes have been reported (19). However, the choice of CVB4 antigens and the age of the subjects differ significantly between the studies. Our results are limited to peripheral blood, and this response may not mirror the local response in the pancreas.

Our measures of type 1 response included IFN-γ secretion, which is known to induce IL-12R and is secreted by CXCR6-expressing type 1 cells and by NK cells, macrophages, and dendritic cells. We also studied the expression of Tbet, a transcription factor that activates the IFN-γ gene (39), leading to increased IFN-γ secretion. Tbet further induces IL-12Rβ_2-chain (40), supporting via IL-12/STAT-4 signaling IFN-γ production. IFN-γ production is induced synergistically by IL-12 and IL-18 through the IL-12R and IL-18R, respectively (41). In CD8 T-cells, Tbet has been reported to control both cytokine production and effector function (42). In T-bet$^{-/-}$ OT-1 TCR^{+} mice, a reduced cytotoxicity was observed, and T-bet$^{-/-}$ mice were also reported to be more susceptible to lymphocytic choriomeningitis virus, which implies an impaired elimination of viruses. CVB4 has been shown to induce production of IFN-γ, IL-1β, TNF-α, IL-2, and IL-10 in PBMCs from healthy donors (43), and proper signaling of IFNs is important in protecting the β-cell from destruction by the CVB4 infection (44).

Because we measured IFN-γ in PBMC population we are not able to identify the origin of IFN-γ responses. The blood volume taken from the children is limited and did not allow us to study the response in isolated cell populations. A reduced secretion of IFN-γ of CVB4-stimulated NK cells, macrophages, and dendritic cells in the PBMC cultures may contribute to decreased T-cell responses detected as lower amounts of Tbet on both mRNA and protein levels. Reduced levels of Tbet may further be responsible for the reduced number of T-cells expressing IL-12Rβ_2-chain and IL-18R. On the other hand, reduced IFN-γ secretion in the PBMC cultures may also reflect poor IFN-γ production from the T-cells.

We and others have previously reported that children with type 1 diabetes show a generally impaired type 1 immune response in vitro (30,45–47). A previous study in patients with newly diagnosed and longstanding type 1 diabetes reported reduced levels of intracellular IFN-γ in CD4 T-cells from PMA+I-stimulated PBMCs (45). Another study reported lower secretion of IFN-γ and IL-10 from phytohemagglutinin-stimulated PBMCs at diagnosis of type 1 diabetes in children and young adults (46). Lohmann et al. (47) have reported a reduced secretion of IFN-γ and TNF-α from fresh PBMCs in children with newly diagnosed type 1 diabetes, but this was normalized 6 months after diagnosis.

Immune responses against CVB4 or CVB4 proteins in individuals with type 1 diabetes have also been investigated. Patients with type 1 diabetes tended to respond less frequently with IFN-γ production and proliferation of PBMCs stimulated with a fusion protein between maltose binding protein and the nonstructural CVB4 protein P2C (MBP-P2C) compared with healthy controls (18). It has also been reported that the T-cell proliferation response to purified CVB4 (20) or different CVB4 proteins (19) is lower at onset of type 1 diabetes and increases with duration of type 1 diabetes. In contrast, we did not see any relation between the IFN-γ secretion or expression of cytokine and chemokine receptors on T-cells and the duration of type 1 diabetes in the present study (data not shown). In addition, there were no correlations between the actual A1C values of the children with type 1 diabetes and the immune response parameters. This suggests that the immune response against CVB4 is not affected by the metabolic balance in individuals with type 1 diabetes. We also studied the effect of in vitro glucose concentration on the upregulation of CCR2, CXCR6, and IL-12R after CVB4 stimulation in PBMCs from four healthy individuals. Preincubation of the PBMCs at hyperglycaemic conditions before CVB4 stimulation did not result in decreased immune response (data not shown). Although we did not see a direct effect of high glucose concentrations in vitro or in vivo (A1C) on immune response to CVB4, we are not able to exclude the possibility that hyperglycemia may modulate the cell-mediated response to CVB4. If this is the case, decreased elimination of viruses may then be an accelerating contributor to β-cell destruction in slightly hyperglycemic pre-diabetic individuals. We demonstrate here a poor induction of type 1 immune response against CVB4, and suggest that this could explain the more frequent occurrence of enterovirus infections in children who develop type 1 diabetes.

In our study, the impaired immune response against CVB4 could not be explained by HLA risk haplotypes of type 1 diabetes. Juhela et al. (20) have previously shown that the T-cell proliferation against purified CVB4 was lower in children with type 1 diabetes compared with HLA-matched controls, which is in accordance with our findings. It has also been reported that the proliferation and cytokine response against MBP-P2C in individuals with HLA risk haplotypes is not different from individuals without HLA risk haplotypes (18). However, antibody response to CVB4 is modulated by HLA genotype (21).

In conclusion, children with type 1 diabetes seem to have an impaired type 1 immune response against CVB4, which was not explained by disease-associated HLA genotypes. The impaired type 1 immune response in children with type 1 diabetes may explain the more frequent CVB4 infections and systemic spread of the infection, which could then cause β-cell damage ultimately leading to the development of type 1 diabetes.

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REFERENCES

1. Onkamo P, Vaananen S, Karvonen M, Tuomilehto J: Worldwide increase in incidence of type I diabetes: the analysis of the data on published incidence trends. *Diabetologia* 42:1395–1403, 1999
2. Lonnrot M, Korpela K, Knip M, Ilonen J, Simell O, Korhonen S, Savola K, Muona P, Simell T, Koskela P, Hyoty H: Enterovirus infection as a risk factor for β-cell autoimmunity in a prospectively observed birth cohort: the Finnish Diabetes Prediction and Prevention Study. *Diabetes* 49:1314–1318, 2000
3. Lonnrot M, Knip M, Roivainen M, Koskela P, Akerblom HK, Hyoty H: Onset of type 1 diabetes mellitus in infancy after enterovirus infections. *Diabet Med* 15:431–434, 1998
4. Hiltunen M, Hyoty H, Knip M, Ilonen J, Reijonen H, Vahasalo P, Roivainen M, Lonnrot M, Leinikki P, Hovi T, Akerblom HK, Childhood Diabetes in Finland (DiMe) Study Group: Islet cell antibody seroconversion in children is temporally associated with enterovirus infections. *J Infect Dis* 175:554–560, 1997
5. Hyoty H, Hiltunen M, Knip M, Laakkonen M, Vahasalo P, Karjalainen J, Koskela P, Roivainen M, Leinikki P, Hovi T, Childhood Diabetes in Finland (DiMe) Study Group, et al.: A prospective study of the role of coxsackie B and other enterovirus infections in the pathogenesis of IDDM. *Diabetes* 44:652–657, 1995
6. Dahlquist GG, Ivarsson S, Lindberg B, Forsgren M: Maternal enteroviral infection during pregnancy as a risk factor for childhood IDDM: a population-based case-control study. *Diabetes* 44:408–413, 1995
7. Dahlquist G, Frisk G, Ivarsson SA, Svanberg L, Forsgren M, Diderholm H: Indications that maternal coxsackie B virus infection during pregnancy is a risk factor for childhood-onset IDDM. *Diabetologia* 38:1371–1373, 1995
8. Viskari HR, Roivainen M, Reunanen A, Pitkaniemi J, Sadeharju K, Koskela P, Hovi T, Leinikki P, Vilja P, Tuomilehto J, Hyoty H: Maternal first-trimester enterovirus infection and future risk of type 1 diabetes in the exposed fetus. *Diabetes* 51:2568–2571, 2002
9. Fuchtenbusch M, Irnstetter A, Jager G, Ziegler AG: No evidence for an association of coxsackie virus infections during pregnancy and early childhood with development of islet autoantibodies in offspring of mothers or fathers with type 1 diabetes. *J Autoimmun* 17:333–340, 2001
10. Gamble DR, Kinsley ML, FitzGerald MG, Bolton R, Taylor KW: Viral antibodies in diabetes mellitus. *Br Med J* 3:627–630, 1969
11. Frisk G, Friman G, Tuvemo T, Fohlman J, Diderholm H: Coxsackie B virus IgM in children at onset of type 1 (insulin-dependent) diabetes mellitus: evidence for IgM induction by a recent or current infection. *Diabetologia* 35:249–253, 1992
12. Helfand RF, Gary HE Jr, Freeman CY, Anderson LJ, Pallansch MA, Pittsburgh Diabetes Research Group: Serologic evidence of an association between enteroviruses and the onset of type 1 diabetes mellitus. *J Infect Dis* 172:1206–1211, 1995
13. King ML, Shaikh A, Bidwell D, Voller A, Banatvala JE: Coxsackie-B-virus-specific IgM responses in children with insulin-dependent (juvenile-onset; type I) diabetes mellitus. *Lancet* 1:1397–1399, 1983
14. Clements GB, Galbraith DN, Taylor KW: Coxsackie B virus infection and onset of childhood diabetes. *Lancet* 346:221–223, 1995
15. Andreoletti L, Hober D, Hober-Vandenberghe C, Belaich S, Vantyghem MC, Lefebvre J, Wattre P: Detection of coxsackie B virus RNA sequences in whole blood samples from adult patients at the onset of type I diabetes mellitus. *J Med Virol* 52:121–127, 1997
16. Nairn C, Galbraith DN, Taylor KW, Clements GB: Enterovirus variants in the serum of children at the onset of type 1 diabetes mellitus. *Diabet Med* 16:509–513, 1999
17. Yin H, Berg AK, Tuvemo T, Frisk G: Enterovirus RNA is found in peripheral blood mononuclear cells in a majority of type 1 diabetic children at onset. *Diabetes* 51:1964–1971, 2002
18. Varela-Calvino R, Sgarbi G, Arif S, Peakman M: T-cell reactivity to the P2C nonstructural protein of a diabetogenic strain of coxsackievirus B4. *Virology* 274:56–64, 2000
19. Varela-Calvino R, Ellis R, Sgarbi G, Dayan CM, Peakman M: Characterization of the T-cell response to coxsackievirus B4: evidence that effector memory cells predominate in patients with type 1 diabetes. *Diabetes* 51:1745–1753, 2002
20. Juhela S, Hyoty H, Roivainen M, Harkonen T, Putto-Laurila A, Simell O, Ilonen J: T-cell responses to enterovirus antigens in children with type 1 diabetes. *Diabetes* 49:1308–1313, 2000
21. Sadeharju K, Knip M, Hiltunen M, Akerblom HK, Hyoty H: The HLA-DR phenotype modulates the humoral immune response to enterovirus antigens. *Diabetologia* 46:1100–1105, 2003
22. Green J, Casabonne D, Newton R: Coxsackie B virus serology and type 1 diabetes mellitus: a systematic review of published case-control studies. *Diabet Med* 21:507–514, 2004
23. Sallusto F, Lanzavecchia A, Mackay CR: Chemokines and chemokine receptors in T-cell priming and Th1/Th2-mediated responses. *Immunol Today* 19:568–574, 1998
24. Agnello D, Lankford CS, Bream J, Morinobu A, Gadina M, O'Shea JJ, Frucht DM: Cytokines and transcription factors that regulate T helper cell differentiation: new players and new insights. *J Clin Immunol* 23:147–161, 2003
25. Kim CH, Kunkel EJ, Boisvert J, Johnston B, Campbell JJ, Genovese MC, Greenberg HB, Butcher EC: Bonzo/CXCR6 expression defines type 1-polarized T-cell subsets with extralymphoid tissue homing potential. *J Clin Invest* 107:595–601, 2001
26. Sjoroos M, Iitia A, Ilonen J, Reijonen H, Lovgren T: Triple-label hybridization assay for type-1 diabetes-related HLA alleles. *Biotechniques* 18:870–877, 1995
27. Laaksonen M, Pastinen T, Sjoroos M, Kuokkanen S, Ruutiainen J, Sumelahti ML, Reijonen H, Salonen R, Wikstrom J, Panelius M, Partanen J, Tienari PJ, Ilonen J: HLA class II associated risk and protection against multiple sclerosis: a Finnish family study. *J Neuroimmunol* 122:140–145, 2002
28. Savola K, Laara E, Vahasalo P, Kulmala P, Akerblom HK, Knip M: Dynamic pattern of disease-associated autoantibodies in siblings of children with type 1 diabetes: a population-based study. *Diabetes* 50:2625–2632, 2001
29. Williams AJ, Bingley PJ, Bonifacio E, Palmer JP, Gale EA: A novel micro-assay for insulin autoantibodies. *J Autoimmun* 10:473–478, 1997
30. Skarsvik S, Ludvigsson J, Vaarala O: Aberrant regulation of interleukin-12 receptor beta chain on type 1 cytokine-stimulated T lymphocytes in type 1 diabetes. *Immunology* 114:287–293, 2005
31. Mosmann TR, Coffman RL: TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 7:145–173, 1989
32. Roivainen M, Rasilainen S, Ylipaasto P, Nissinen R, Ustinov J, Bouwens L, Eizirik DL, Hovi T, Otonkoski T: Mechanisms of coxsackievirus-induced damage to human pancreatic beta-cells. *J Clin Endocrinol Metab* 85:432–440, 2000
33. Ylipaasto P, Klingel K, Lindberg AM, Otonkoski T, Kandolf R, Hovi T, Roivainen M: Enterovirus infection in human pancreatic islet cells, islet tropism in vivo and receptor involvement in cultured islet beta cells. *Diabetologia* 47:225–239, 2004
34. Salminen K, Sadeharju K, Lonnrot M, Vahasalo P, Kupila A, Korhonen S, Ilonen J, Simell O, Knip M, Hyoty H: Enterovirus infections are associated with the induction of beta-cell autoimmunity in a prospective birth cohort study. *J Med Virol* 69:91–98, 2003
35. Bender A, Bui LK, Feldman MA, Larsson M, Bhardwaj N: Inactivated influenza virus, when presented on dendritic cells, elicits human CD8+ cytolytic T cell responses. *J Exp Med* 182:1663–1671, 1995
36. Nansen A, Marker O, Bartholdy C, Thomsen AR: CCR2+ and CCR5+ CD8+ T cells increase during viral infection and migrate to sites of infection. *Eur J Immunol* 30:1797–1806, 2000
37. Boring L, Gosling J, Chensue SW, Kunkel SL, Farese RV Jr, Broxmeyer HE, Charo IF: Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. *J Clin Invest* 100:2552–2561, 1997
38. Kurihara T, Warr G, Loy J, Bravo R: Defects in macrophage recruitment and host defense in mice lacking the CCR2 chemokine receptor. *J Exp Med* 186:1757–1762, 1997
39. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH: A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100:655–669, 2000
40. Afkarian M, Sedy JR, Yang J, Jacobson NG, Cereb N, Yang SY, Murphy TL, Murphy KM: T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nat Immunol* 3:549–557, 2002
41. Yoshimoto T, Takeda K, Tanaka T, Ohkusu K, Okamura H, Akira S, Nakanishi K: IL-12 up-regulates IL-18 receptor expression on T cells, Th1 cells, and B cells: synergism with IL-18 for IFN-γ production. *J Immunol* 161:3400–3407, 1998
42. Sullivan BM, Juedes A, Szabo SJ, von Herrath M, Glimcher LH: Antigen-driven effector CD8 T cell function regulated by T-bet. *Proc Natl Acad Sci U S A* 100:15818–15823, 2003
43. Vreugdenhil GR, Wijnands PG, Netea MG, van der Meer JW, Melchers WJ, Galama JM: Enterovirus-induced production of pro-inflammatory and T-helper cytokines by human leukocytes. *Cytokine* 12:1793–1796, 2000

44. Flodstrom M, Maday A, Balakrishna D, Cleary MM, Yoshimura A, Sarvetnick N: Target cell defense prevents the development of diabetes after viral infection. *Nat Immunol* 3:373–382, 2002
45. Kukreja A, Cost G, Marker J, Zhang C, Sun Z, Lin-Su K, Ten S, Sanz M, Exley M, Wilson B, Porcelli S, Maclaren N: Multiple immuno-regulatory defects in type-1 diabetes. *J Clin Invest* 109:131–140, 2001
46. Mayer A, Rharbaoui F, Thivolet C, Orgiazzi J, Madec AM: The relationship between peripheral T cell reactivity to insulin, clinical remissions and cytokine production in type 1 (insulin-dependent) diabetes mellitus. *J Clin Endocrinol Metab* 84:2419–2424, 1999
47. Lohmann T, Laue S, Nietzschmann U, Kapellen TM, Lehmann I, Schroeder S, Paschke R, Kiess W: Reduced expression of Th1-associated chemokine receptors on peripheral blood lymphocytes at diagnosis of type 1 diabetes. *Diabetes* 51:2474–2480, 2002