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Immune Profiling by Multiple Gene Expression Analysis in Patients At-risk and with Type 1 Diabetes

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Abstract

There is a need for biomarkers to monitor the development and progression of type 1 DM. We analyzed mRNA expression levels for granzyme B, perforin, fas ligand, TNF- α , IFN- γ , Foxp3, IL-10, TGF- β , IL-4, IL-6, IL-17, Activation-induced cytidine deaminase (AID) and Immunoglobulin G gamma chain (IgGγ)> genes in peripheral blood of at-risk, new-onset and long-term type 1 DM, and healthy controls. The majority of the genes were suppressed in long-term type 1 DM compared to controls and new-onset patients. IFN- γ , IL-4 and IL-10 mRNA levels were significantly higher in new-onset compared to at-risk and long-term groups. There was decreased mRNA expression for AID and IgGγ and up-regulation of IFN- γ with age in controls. Data suggest an overall depressed immunity in long-term type 1 DM. Increased gene expression levels for IFN- γ , IL-4 and IL-10 in new-onset patients from at-risk patients might be used as potential markers for progression of the disease.

Keywords

Age; biomarkers; cytokines; cytotoxic lymphocyte genes; gene expression; type 1 diabetes

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1. Introduction

Type 1 diabetes mellitus (type 1 DM) results from cell-mediated autoimmune destruction of insulin-producing pancreatic beta cells. The activation of autoimmune responses is believed to be initiated by antigen presenting cells [1]. Following activation, CD4 and CD8 T cells, along with B cells, participate in the destruction of beta cells [2;3;4;5]. T cell-mediated beta cell damage involves the release of cytotoxic molecules which include cytokines (e.g. TNF- α , IL-1 β), granzyme B, perforin, and the Fas-Fas ligand interaction [6;7]. Activation of the caspase pathway by these molecules leads to apoptosis of the beta cell. Hence, antigen presenting cells, T cells (CD4+ and CD8+), and cytokines interact to destroy beta cells [8].

Proinflammatory cytokines, such as IFN- γ and TNF- α , play a role in initiation of early inflammatory processes [9] and seem to promote beta-cell destruction [10;11]; while regulatory cytokines, such as IL-10 and TGF- β , seem to regulate beta-cell destruction [7;8;13]. IFN- γ has been observed in islets *in vivo* in patients with new-onset type 1 DM [12]. Studies of the peripheral immune system of patients with new-onset type 1 DM have shown significantly higher levels of IL-1 α , IFN- γ , and TNF- α as compared to healthy controls [13;14]. Th17 cells have been shown to play a crucial role in the induction of autoimmune tissue injury, inflammation, and infection and may be involved in exacerbation of diabetes [15;16]. Increased IL-17 secreting T cells were reported in children with new-onset type 1 DM [17]. B cells have also been implicated in type 1 DM development [3], but the actual role that B cells play is not yet clear. Data from a recent clinical trial demonstrated that targeting B cells in new-onset type 1 DM patients with anti-CD20 (Rituxan) preserved residual insulin secretion for at least one year [18]. A recent study on samples from pancreas from new-onset type 1 DM patients showed that CD20+ cells were present in only small numbers in early insulinitis, but were recruited to islets as beta cell death progressed, suggesting an increasing role for these cells as insulinitis develops [4]. Activation-induced cytidine deaminase (AID) is a molecule selectively expressed *in vivo* and *in vitro* in class switch recombination-induced B cells [19] and was used here because our previous work showed it (and immunoglobulin IgG) to be a good biomarker for human B cell responses [20]. There is reported evidence for linkage and association of type 1 DM with genetic markers located at the immunoglobulin heavy chain gene cluster [21], thereby showing a possible role of immunoglobulin heavy chain in the pathogenesis of type 1 DM.

The cytotoxic lymphocyte gene products granzyme B, perforin, and fas ligand (FasL) have been shown to play an integral part in the development of type 1 DM [22]. We previously reported that mRNA levels of perforin and FasL genes were significantly lower in patients with long-term (>5 years) type 1 DM as compared to healthy controls [23]. There have been other studies reporting similar findings regarding Fas function. Defective expression of the Fas molecule on T and B lymphocytes was reported in patients with both newly diagnosed and long-term diabetes [24]; patients with other autoimmune diseases were also found to have defective Fas function [25]. Detection of insulin gene expression in peripheral blood for monitoring the release of β cells was reported to be effective as a marker of injury to the islet graft after transplantation [26]. We included the insulin gene in this study to find out if β cell injury might be evident in patients in different stages of type 1 DM development.

There have been conflicting reports about the levels of cytokine secretion and cytokine mRNA expression in at-risk, new-onset, and long-term type 1 DM patients [27;28]. These discrepancies could be due to different stages of the disease even within the same group [29]. Some studies hypothesized that an active Th1-like immune response destroys beta cells, followed by presentation of autoantigens during the prediabetic phase [29;30].

Gene expression profiles in peripheral blood or in a specific blood cell population may provide new insights into the pathogenesis of type 1 DM [31;32;33]. Such studies could reveal differences in immune responsiveness between patients with type 1 DM and healthy controls and may identify changes in gene expression that associate with progression of at-risk patients to new-onset type 1 DM. To this end we measured expression levels for fourteen candidate genes, in the peripheral blood of at-risk, new-onset and long-term type 1 DM patients and healthy subjects, including: insulin, the cytotoxic lymphocyte genes granzyme B, perforin, and FasL, and some key T and B cell immune mediators, including TNF- α , IFN- γ , Foxp3, IL-10, TGF- β , IL-4, IL-6, IL-17, AID, and Immunoglobulin G gamma chain (IgG<gamma>).

2. Methods

2.1. Subjects

Peripheral blood from four groups (n, age $\pm$ SD in years, male/female ratio): at-risk (n=18, 14.8 $\pm$ 12.4, 10/8), new-onset (n=29, 15.3 $\pm$ 8.2, 15/14), and long-term (n=62, 39.3 $\pm$ 13.8, 32/30) type 1 DM patients and healthy controls (n=80, 39.0 $\pm$ 10.2, 39/41), were included in the study. At-risk and new-onset patients were selected from those patients undergoing screening for enrollment in Type 1 Diabetes TrialNet studies at the University of Miami. At-risk patients have at least one confirmed positive autoantibody: insulin (IAA), glutamic acid decarboxylase-65 (GAD65), ICA512/IA2 (IA2), and/or ICA. These autoantibodies were measured in the Type 1 Diabetes TrialNet core laboratories. Risk status of the at-risk patients stratified based on the protocol for Type 1 Diabetes TrialNet Natural History Study [34] were low risk (n=2, defined as having one positive autoantibody with a normal oral glucose tolerance test, moderate risk (n=10, defined as having two positive autoantibodies with a normal oral glucose tolerance test), and high risk (n=6, defined as having three or more positive autoantibodies with a normal oral glucose tolerance test or 1-4 positive autoantibodies with an abnormal oral glucose tolerance test). Diagnosis of diabetes was made according to guidelines from the American Diabetes Association [35] and all patients in the new-onset group were diagnosed within a year of sample collection with the mean disease duration of 80 $\pm$ 84 days. Long-term (>5 years duration, mean disease duration = 22.8 $\pm$ 11.5 years, average HbA1c (%) = 7.8 $\pm$ 1.3) type 1 DM patients were identified from those undergoing screening for studies in clinical islet transplant trials at the University of Miami. Control blood samples were obtained from healthy volunteers at the University of Miami (n=60) and at Beckman-Coulter, Inc (Miami, FL) (n=20). No glucose tolerance test was performed, but common autoantibodies (IAA, GAD65, IA2, and/or ICA) were performed in the healthy volunteers and only individuals negative for autoantibodies were selected as healthy controls. Participants were required to be well (afebrile and off antibiotic treatment for at least 2 weeks prior to the visit) and not on steroids or other immunomodulatory therapies for at least one month prior to blood sample collection. All protocols were approved by the Institutional Review Board of the University of Miami. Each patient gave written informed consent.

2.2. Sample collection

Peripheral blood was drawn into a 'Paxgene Blood RNA Tube' (Qiagen, Valencia, CA), stored at -80°C, and processed within three months.

2.3. RNA isolation and reverse transcription

Total RNA was isolated using a Paxgene Blood RNA kit (Qiagen); RNA was then digested with DNase I (Invitrogen, Carlsbad, CA) to remove DNA contamination from the total RNA. First strand cDNA was synthesized using a 'SuperScript III First-Strand Synthesis SuperMix for qRT-PCR' kit (Invitrogen).

2.4. Assessment of Gene Expression Levels

mRNA expression levels for the fourteen target genes were determined with a custom-made TaqMan Low Density Array using the 7900HT fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). Two endogenous control genes, 18S and β -actin, were included in the card. A control sample from a pool of RNA from 22 human tissues (Applied Biosystems) was used for each array to minimize variations between arrays. Relative gene expression levels were calculated as fold change against the common control after normalization with 18S by using the $2^{-\Delta\Delta C_t}$ method [36]. See appendix for further details of the methods, as well as assay IDs for the 16 genes.

Preliminary data indicated that 18S PCR amplification was more consistent and the variation in 18S gene expression level (Ct value) among samples was smaller than the one in β -actin; therefore, 18S was used as the endogenous control gene to normalize individual gene expression. Data was presented in natural logarithmic scale (ln scale).

2.5. Statistical Analysis

General linear models with dummy variables to index the groups under comparison (healthy, at-risk, new-onset, long-term) were constructed for each gene under consideration. A natural logarithmic transformation was applied to the fold change data in order to preserve assumptions of normality. These models were then fit to the transformed data to estimate and compare mean fold changes in gene expression level among comparison groups. The transformed data was additionally analyzed by linear regression to assess the association of the mRNA expression of each gene with age (healthy controls, new-onset and long-term), disease duration (new-onset and long-term) and HbA1c (long-term). False discovery rate (FDR) corrected p-values are used to assess statistical significance, addressing the multiple testing problems arising from simultaneously testing many different genes among groups. A Type I error rate of 0.05 was used to assess all tests of significance.

3. Results

3.1. Gene expression levels in at-risk, new-onset and long-term type 1 DM patients and healthy controls

Relative gene expression levels for individual genes in at-risk, new-onset and long-term type 1 DM patients, and healthy controls are shown in Figures 1-5. Insulin gene expression was not detected in any of the samples analyzed. IL-17 mRNA was undetectable in many samples. The percentages of IL-17 detectable samples for at-risk, new-onset, long-term, and healthy control groups were 33.3% (6/18), 58.6% (17/29), 33.9% (21/62), and 27.5% (22/80), respectively. Gene expression levels for low, medium, and high risk patients in the at-risk groups are illustrated in different colors in Figures 1-5. The sample size in each subgroup is too small to see any patterns.

As shown in Figure 1, gene expression levels for all three cytotoxic lymphocyte molecules were significantly depressed in long-term patients as compared to healthy controls ($p < 0.005$). Granzyme B was significantly lower in at-risk patients as compared to healthy controls ($p < 0.05$). In addition, gene expression levels for granzyme B were higher in new-onset as compared to long-term patients ($p < 0.05$).

mRNA levels for the B cell associated genes AID and IgG<gamma> are shown in Figure 2; expression levels for long-term patients were significantly lower as compared to healthy controls or new-onset patients ($p < 0.05$).

Gene expression levels for IFN- γ were significantly lower in at-risk and long-term as compared to healthy controls ($p < 0.0005$). IFN- γ mRNA levels were also significantly lower in at-risk and long-term patients as compared to new-onset patients ($p < 0.05$) (Figure 3).

The new-onset group had the highest average level for IL-4 mRNA, which was significantly higher than in the at-risk ($p < 0.005$) and long-term ($p < 0.0005$) groups.

IL-10 mRNA expression was significantly lower in the long-term group as compared to healthy controls ($p < 0.05$). Similar to IL-4, the new-onset patients had the highest average level for IL-10 mRNA expression, which was significantly higher, as compared to at-risk ($p < 0.05$) and long-term ($p < 0.005$) groups (Figure 3).

TGF- β , Foxp3, TNF- α , and IL-6 mRNA levels were significantly lower in long-term patients as compared to the healthy controls ($p < 0.05$) and new-onset group ($p < 0.05$) (Figures 4 & 5). TNF- α mRNA expression was also lower in at-risk group as compared to healthy controls ($p < 0.05$).

Gene expression level for each group as compared to healthy controls is summarized in Table 1, and comparison of individual gene level among at-risk, new-onset, and long-term groups is summarized in Table 2. Significantly increased or decreased gene expression is indicated with an up or down arrow, respectively. The data reveals that mRNA expression levels in long-term patients were significantly depressed for 11/12 genes (except IL-4) as compared to healthy controls (Table 1), and depressed for 10/12 genes as compared to new-onset patients (Table 2), suggesting a generalized depression of immunity for long-term patients. None of the gene expression levels in at-risk patients was significantly higher in comparison to the long-term patients. Average expression level of all the studied genes in the at-risk group was lower than the respective gene level in healthy controls, although only granzyme B, IFN- γ , and TNF- α gene expression levels were significantly different between the two groups ($p < 0.05$). However, average expression levels of all the studied genes in new-onset patients were similar to the respective gene level in healthy controls. Compare to at-risk and long-term patients, mRNA levels of IFN- γ , IL-4 and IL-10 were significantly higher in new-onset patients ($p < 0.05$), suggesting that this combination of genes might be useful for determining progression of at-risk patients to new-onset type 1 DM disease.

3.2. Effect of age, disease duration, and HbA1c on gene expression levels

One concern in undertaking these studies is the difficulty in obtaining age matched healthy controls for the younger at-risk and new-onset groups. The long-term patients and healthy controls were similar in age. In order to address potential differences in gene expression that might be related to age, but in the absence of the effects of disease on the immune system, data from the eighty healthy controls was analyzed in relation to age. In addition, we analyzed the effect of age and disease duration on gene expression levels in the new-onset and long-term patient groups. The relationships of HbA1c and the expression levels of the studied genes in long-term patients were also analyzed.

Age was not significantly associated with mRNA expression levels of granzyme B, perforin, FasL, IL-4, IL-17, TGF- β , IL-10, Foxp3, TNF- α , and IL-6 in the peripheral blood of healthy controls (20 – 66 years) ($p > 0.05$); however, a significant decrease in AID and IgG<gamma> gene expression levels (equal to 0.029 and 0.026 in the natural logarithmic scale for each year of age, respectively) was observed. These correspond to a significant exponential decline in AID and IgG<gamma> gene expression level with increasing age, where each year increase in age is associated with an estimated 3% decrease in expression level for both AID ($p = 0.02$) and IgG<gamma> ($p = 0.006$) (Figure 6). These results are

consistent with our previous results showing decreased AID in *in vitro* stimulated B lymphocytes with increased age [20]. Additionally, results indicated a significant increase in IFN- γ gene expression levels (equal to 0.027 in the natural logarithmic scale for each year of age). These correspond to a significant exponential increase in IFN- γ gene expression level with increasing age where each year increase in age is associated with an estimated 3% increase in expression level ($p = 0.02$) (Figure 6).

Age was not significantly associated with mRNA expression levels for 13/13 genes (insulin gene excluded, all undetectable) in long-term (18 – 64 years) and 12/13 genes in new-onset (5 – 34 years) patients ($p > 0.05$). A significant increase in TNF- α gene expression levels (equal to 0.034 in the natural logarithmic scale for each year of age) was observed in new-onset patients, which means that a one year increase in age is associated with an estimated 3.46% increase in expression level for TNF- α ($p=0.02$). In addition, disease duration was not significantly associated with gene expression in both new-onset and long-term patients ($p > 0.05$). There were no significant associations between HbA1c levels and gene expression levels in long-term patients ($p > 0.05$).

4. Discussion

We analyzed gene expression levels in whole blood samples obtained from at-risk, new-onset and long-term type 1 DM patients, as well as healthy controls, for fourteen candidate genes that are related to the disease process. This is the first study to analyze gene expression levels of different types of cytokines as well as cytotoxic lymphocyte and B cell-associated molecules in healthy controls and in patients at different stages of type 1 DM. Our goal was to identify differences in gene expression levels that could potentially be used as biomarkers for identification of at-risk patients and monitoring of disease progression.

We observed that 11/12 genes were significantly lower in long-term type 1 DM patients as compared to healthy controls, suggesting an overall depressed immunity in patients with long-term type 1 DM. These patients had been diagnosed with type 1 DM over 5 years, and had an average HbA1c over 7.8%. There were no associations between their HbA1c levels and gene expression levels. Abnormal PBMC function [37] and impaired production of IFN- γ , IL-1, IL-6 and TNF- α in stimulated cultures of PBMC [38] were reported from long-term type 1 DM patients, which suggested a deficiency in mononuclear cell activation and impaired adaptive immune response [38]. This impaired immune response could be a result of “hyperglycemic memory”, where changes in gene expression and biological reactions are “imprinted” on cells due to hyperglycemia [38]. Induced expression of IL-1 β -regulated gene signature was absent in PBMCs cultured with sera from long-term type 1 DM patients; this was attributed to the significantly reduced or no β cell mass and the absence of active autoimmunity in long-term type 1 DM [39].

We identified three genes, IFN- γ , IL-4 and IL-10 for which expression was significantly higher in new-onset patients as compared to the at-risk and long-term groups. Gene expression in long-term was generally suppressed, as discussed above. Average expression level of all the studied genes in the at-risk group was lower than the respective gene level in healthy controls. On the other hand, although statistically not different from healthy controls, expression levels of all of the studied genes were increased in new-onset patients as compared to the at-risk groups, and the increase was significant for IFN- γ , IL-4 and IL-10 genes. Therefore, changes in gene expression levels for IFN- γ , IL-4 and IL-10 in new-onset patients from at-risk patients could be used as a potential marker for the progression of the disease. IFN- γ plays a major role in immune-mediated inflammation [9] and is considered a mediator of islet β -cell destruction [27;40]. There have been conflicting reports about the levels of IFN- γ secretion and mRNA expression in at-risk and new-onset type 1 DM patients

[27;41]. Differences may be attributable to time from onset and collection of sample or to patient numbers. IL-4 has been suggested to have protective effects against insulinitis and diabetes, possibly by suppressing IFN- γ producing T cells [40]. The higher levels of IL-4 mRNA expression observed in new-onset type 1 DM suggests that the regulatory and effector arms of the immune system are battling against each other. IL-10 is an immunoregulatory cytokine that has a critical function in inducing T reg cells both in vitro and in vivo [42]; however, under certain conditions, IL-10 was reported to have proinflammatory effects through increased IFN- γ production [43;44]. Higher mRNA expression of IFN- γ , IL-4 and IL-10 in new-onset patients as compared to at-risk groups might suggest that the immune system has up-regulated regulatory mechanisms in an attempt to counteract autoimmune destruction [45].

Autoimmune diseases may involve direct cell-specific damage mediated by cytotoxic T lymphocytes [46]. CD8+ T cells play an important role in the pathogenesis of type 1 DM [47]. We have previously reported that mRNA expression for perforin and FasL were significantly lower in long-term type 1 DM patients as compared to healthy controls [23]. Our current study confirmed these findings. The decreased expression of perforin and FasL in patients with long-term type 1 DM might affect the capacity to regulate immune responses and to maintain normal levels of peripheral tolerance, which are essential for protection from autoimmune disease [24;48;49]. This may contribute to higher frequency of other autoimmune diseases in type 1 DM. Gene expression levels for GB in at-risk and long-term T1D patients were significantly lower compared to healthy controls. Lower expression of GB in long-term T1D patients compared to controls was not observed in our previous study [23]. The smaller sample size of healthy controls in the previous study (n = 29) might not have allowed for enough power to detect the differences in GB expression between long-term T1D patients and healthy controls.

The role of different B cell subsets in the etiology of type 1 DM is not clear [47]. For the first time, we reported here that gene expression levels of two key B cell markers, AID and IgGγ, were significantly higher in new-onset and healthy control groups as compared to the long-term group and these differences do not appear to be age-related (see below for discussion of age). AID deficiency has been associated with other autoimmune and inflammatory disorders and with an increased risk of acquiring severe infections [50]. There is reported evidence for linkage and association of type 1 DM with genetic markers located at the IGH gene cluster [21].

IL-17 mRNA was undetectable in many samples, with the lowest undetectable rate in the new-onset group. IL-17 mRNA levels were significantly higher in new-onset patients as compared to at-risk and long-term patients and healthy controls ($p < 0.05$) (data not shown); however, not all new-onset patients had detectable levels of IL17 mRNA. There is increasing evidence that IL-17 producing Th17 cells are pathogenic in the induction and propagation of autoimmunity in animal models [51]. Our finding of higher IL17 gene expression in new-onset type 1 DM patients as compared to at-risk and long-term type 1 DM patients and healthy controls, may imply that IL17 is actively involved in type 1 DM pathogenesis.

As is commonly recognized in the field, it was difficult for us to obtain healthy, age matched controls for the younger at-risk and new-onset groups; however, the average age for healthy controls was matched with the long-term group, and at-risk and new-onset groups were age matched. In order to assess the effect of age on gene expression, in the absence of disease, we analyzed the data from healthy subjects (20-66 years old, n=80 patients) and found no effect on gene expression levels, with the exception of the B cell molecules AID and IgGγ (significantly decreased with age) and IFN- γ (significantly increased with

age). To our knowledge, this is the first time an effect of age on the gene expression levels of AID, IgG<gamma>, and IFN- γ has been observed in unstimulated blood samples. Decreased gene expression of AID is consistent with our previous data [20], in which there was an age related decrease in AID mRNA expression in anti-CD40/IL4-stimulated CD19+ B cells isolated from human peripheral blood (18-86 years old). IFN- γ has been indicated as a strong inducer of cell-mediated immune responses and investigators have hypothesized that age-related changes in IFN- γ may play a role in the decline of cell-mediated immune responses with age [52].

Gene expression levels in the whole blood, instead of PBMC, were measured in this study. Whole blood assay requires less manipulation, and contains cell populations that may be missed in the PBMC isolation process. In addition, whole blood used in this study was collected into a 'PAXgene Blood RNA' Tube, which contains RNA stabilizing reagents to protect RNA molecules from degradation and minimize induction of gene expression changes after blood collection. The pathogenesis of type 1 DM has proved difficult to study in humans because of ethical and practical problems that limit access to appropriate samples, such as pancreatic tissue. Study in peripheral blood has the advantage of easy sampling and minimum body invasion as compared to tissue samples; however, the correlation of the gene expression levels in peripheral blood with what is taking place in the pancreas at different stages of type 1 DM need to be considered and need to be further studied.

While we are able to obtain relatively large numbers of samples from long-term type 1 DM patients and healthy controls, it is relatively difficult to obtain well-characterized at-risk and new-onset individuals. Our findings need to be corroborated in larger and longitudinal studies of these two subject groups. We have tried to see if there are any trends for the expression of any genes in the low, medium, and high risk patients, but the sample size in each subgroup was too small to make any conclusions. Future analysis of additional samples from low, moderate and high risk type 1 DM patients will allow us to more clearly monitor gene expression changes in disease development. In addition, in future studies, we will investigate the in vitro activation of T and B cells with distinct stimuli in the four groups of subjects to further elucidate the mechanism for the impaired gene expression.

In conclusion, our data demonstrate an overall depressed immunity in patients with long-term type 1 DM. In addition, increased gene expression levels for IFN- γ , IL-4 and IL-10 in new-onset patients from at-risk patients might be used as a potential marker for the progression of the disease. Prospective studies will allow us to determine whether specific gene signatures may aid in prediction of disease progression and in detection of recurrent autoimmunity in transplanted type 1 DM patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Jansen A, Homo-Delarche F, Hooijkaas H, Leenen PJ, Dardenne M, Drexhage HA. Immunohistochemical characterization of monocytes-macrophages and dendritic cells involved in the initiation of the insulinitis and beta-cell destruction in NOD mice. *Diabetes*. 1994; 43:667–75. [PubMed: 8168644]
2. Anderson MS, Bluestone JA. The NOD mouse: a model of immune dysregulation. *Annu Rev Immunol*. 2005; 23:447–85. [PubMed: 15771578]
3. Silveira PA, Grey ST. B cells in the spotlight: innocent bystanders or major players in the pathogenesis of type 1 diabetes. *Trends Endocrinol Metab*. 2006; 17:128–35. [PubMed: 16580840]
4. Willcox A, Richardson SJ, Bone AJ, Foulis AK, Morgan NG. Analysis of islet inflammation in human type 1 diabetes. *Clin Exp Immunol*. 2009; 155:173–81. [PubMed: 19128359]
5. Pinkse GG, Tysma OH, Bergen CA, Kester MG, Ossendorp F, van Veelen PA, Keymeulen B, Pipeleers D, Drijfhout JW, Roep BO. Autoreactive CD8 T cells associated with beta cell destruction in type 1 diabetes. *Proc Natl Acad Sci U S A*. 2005; 102:18425–30. [PubMed: 16339897]
6. Kawasaki E, Abiru N, Eguchi K. Prevention of type 1 diabetes: from the view point of beta cell damage. *Diabetes Research and Clinical Practice*. 2004; 66:S27–S32. [PubMed: 15563975]
7. Kim EY, Moudgil KD. Regulation of autoimmune inflammation by pro-inflammatory cytokines. *Immunol Lett*. 2008; 120:1–5. [PubMed: 18694783]
8. Yoon JW, Jun HS. Autoimmune destruction of pancreatic beta cells. *Am J Ther*. 2005; 12:580–91. [PubMed: 16280652]
9. Germolec DR, Frawley RP, Evans E. Markers of inflammation. *Methods Mol Biol*. 2010; 598:53–73. [PubMed: 19967506]
10. Almawi WY, Tamim H, Azar ST. Clinical review 103: T helper type 1 and 2 cytokines mediate the onset and progression of type I (insulin-dependent) diabetes. *J Clin Endocrinol Metab*. 1999; 84:1497–502. [PubMed: 10323367]
11. Rabinovitch A, Suarez-Pinzon WL. Role of cytokines in the pathogenesis of autoimmune diabetes mellitus. *Rev Endocr Metab Disord*. 2003; 4:291–9. [PubMed: 14501180]
12. Foulis AK, McGill M, Farquharson MA. Insulinitis in type 1 (insulin-dependent) diabetes mellitus in man--macrophages, lymphocytes, and interferon-gamma containing cells. *J Pathol*. 1991; 165:97–103. [PubMed: 1744803]
13. Hussain MJ, Peakman M, Gallati H, Lo SS, Hawa M, Viberti GC, Watkins PJ, Leslie RD, Vergani D. Elevated serum levels of macrophage-derived cytokines precede and accompany the onset of IDDM. *Diabetologia*. 1996; 39:60–9. [PubMed: 8720604]
14. Kallmann BA, Huther M, Tubes M, Feldkamp J, Bertrams J, Gries FA, Lampeter EF, Kolb H. Systemic bias of cytokine production toward cell-mediated immune regulation in IDDM and toward humoral immunity in Graves' disease. *Diabetes*. 1997; 46:237–43. [PubMed: 9000700]
15. Cooke A. Th17 cells in inflammatory conditions. *Rev Diabet Stud*. 2006; 3:72–5. [PubMed: 17487329]
16. Honkanen J, Nieminen JK, Gao R, Luopajarvi K, Salo HM, Ilonen J, Knip M, Otonkoski T, Vaarala O. IL-17 immunity in human type 1 diabetes. *J Immunol*. 2010; 185:1959–67. [PubMed: 20592279]
17. Marwaha AK, Crome SQ, Panagiotopoulos C, Berg KB, Qin H, Ouyang Q, Xu L, Priatel JJ, Levings MK, Tan R. Cutting edge: Increased IL-17-secreting T cells in children with new-onset type 1 diabetes. *J Immunol*. 2010; 185:3814–8. [PubMed: 20810982]
18. Pescovitz MD, Greenbaum CJ, Krause-Steinrauf H, Becker DJ, Gitelman SE, Golland R, Gottlieb PA, Marks JB, McGee PF, Moran AM, Raskin P, Rodriguez H, Schatz DA, Wherrett D, Wilson DM, Lachin JM, Skyler JS. Rituximab, B-lymphocyte depletion, and preservation of beta-cell function. *N Engl J Med*. 2009; 361:2143–52. [PubMed: 19940299]
19. Muramatsu M, Sankaranand VS, Anant S, Sugai M, Kinoshita K, Davidson NO, Honjo T. Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. *J Biol Chem*. 1999; 274:18470–6. [PubMed: 10373455]

20. Frasca D, Landin AM, Lechner SC, Ryan JG, Schwartz R, Riley RL, Blomberg BB. Aging down-regulates the transcription factor E2A, activation-induced cytidine deaminase, and Ig class switch in human B cells. *J Immunol.* 2008; 180:5283–90. [PubMed: 18390709]
21. Field LL, Larsen Z, Pociot F, Nerup J, Tobias R, Bonnevie-Nielsen V. Evidence for a locus (IDDM16) in the immunoglobulin heavy chain region on chromosome 14q32.3 producing susceptibility to type 1 diabetes. *Genes Immun.* 2002; 3:338–44. [PubMed: 12209360]
22. Kreuwel HTC, Morgan DJ, Krahl T, Ko A, Sarvetnick N, Sherman LA. Comparing the relative role of perforin/granzyme versus Fas/Fas ligand cytotoxic pathways in CD8(+) T cell-mediated insulin-dependent diabetes mellitus. *Journal of Immunology.* 1999; 163:4335–4341.
23. Han DM, Leith J, Alejandro R, Bolton W, Ricordi C, Kenyon NS. Peripheral blood cytotoxic lymphocyte gene transcript levels differ in patients with long-term type 1 diabetes compared to normal controls. *Cell Transplantation.* 2005; 14:403–409. [PubMed: 16180659]
24. Giordano C, De Maria R, Stassi G, Todaro M, Richiusa P, Giordano M, Testi R, Galluzzo A. Defective expression of the apoptosis-inducing CD95 (Fas/APO-1) molecule on T and B cells in IDDM. *Diabetologia.* 1995; 38:1449–54. [PubMed: 8786019]
25. DeFranco S, Bonisconi S, Cerutti F, Bona G, Bottarel F, Cadario F, Brusco A, Loffredo G, Rabbone I, Corrias A, Pignata C, Ramenghi U, Dianzani A. Defective function of Fas in patients with type 1 diabetes associated with other autoimmune diseases. *Diabetes.* 2001; 50:483–488. [PubMed: 11246866]
26. Ritz-Laser B, Oberholzer J, Toso C, Brulhart MC, Zakrzewska K, Ris F, Bucher P, Morel P, Philippe J. Molecular detection of circulating beta-cells after islet transplantation. *Diabetes.* 2002; 51:557–61. [PubMed: 11872650]
27. Nicoletti F, Conget I, Di Mauro M, Di Marco R, Mazzarino MC, Bendtzen K, Messina A, Gomis R. Serum concentrations of the interferon-gamma-inducible chemokine IP-10/CXCL10 are augmented in both newly diagnosed Type I diabetes mellitus patients and subjects at risk of developing the disease. *Diabetologia.* 2002; 45:1107–10. [PubMed: 12189440]
28. Halminen M, Simell O, Knip M, Ilonen J. Cytokine expression in unstimulated PBMC of children with type 1 diabetes and subjects positive for diabetes-associated autoantibodies. *Scand J Immunol.* 2001; 53:510–3. [PubMed: 11309160]
29. Karlsson Faresjo MG, Ernerudh J, Ludvigsson J. Cytokine profile in children during the first 3 months after the diagnosis of type 1 diabetes. *Scand J Immunol.* 2004; 59:517–26. [PubMed: 15140063]
30. Hedman M, Ludvigsson J, Faresjo MK. Nicotinamide reduces high secretion of IFN-gamma in high-risk relatives even though it does not prevent type 1 diabetes. *Journal of Interferon and Cytokine Research.* 2006; 26:207–213. [PubMed: 16704296]
31. Beyan H, Drexhage RC, van der Heul Nieuwenhuijsen L, de Wit H, Padmos RC, Schloot NC, Drexhage HA, Leslie RD. Monocyte gene-expression profiles associated with childhood-onset type 1 diabetes and disease risk: a study of identical twins. *Diabetes.* 2010; 59:1751–5. [PubMed: 20393150]
32. Reynier F, Pachot A, Paye M, Xu Q, Turrel-Davin F, Petit F, Hot A, Auffray C, Bendelac N, Nicolino M, Mouglin B, Thivolet C. Specific gene expression signature associated with development of autoimmune type-I diabetes using whole-blood microarray analysis. *Genes Immun.* 2010; 11:269–79. [PubMed: 20090770]
33. Planas R, Pujol-Borrell R, Vives-Pi M. Global gene expression changes in type 1 diabetes: insights into autoimmune response in the target organ and in the periphery. *Immunol Lett.* 2010; 133:55–61. [PubMed: 20708640]
34. Mahon JL, Sosenko JM, Rafkin-Mervis L, Krause-Steinrauf H, Lachin JM, Thompson C, Bingley PJ, Bonifacio E, Palmer JP, Eisenbarth GS, Wolfsdorf J, Skyler JS. The TrialNet Natural History Study of the Development of Type 1 Diabetes: objectives, design, and initial results. *Pediatr Diabetes.* 2009; 10:97–104. [PubMed: 18823409]
35. Diagnosis and classification of diabetes mellitus. *Diabetes Care.* 2010; 33 1:S62–9. [PubMed: 20042775]
36. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-(Delta Delta C(T))} Method. *Methods.* 2001; 25:402–8. [PubMed: 11846609]

37. Alexandraki KI, Piperi C, Ziakas PD, Apostolopoulos NV, Makrilakis K, Syriou V, Diamanti-Kandarakis E, Kaltsas G, Kalofoutis A. Cytokine secretion in long-standing diabetes mellitus type 1 and 2: associations with low-grade systemic inflammation. *J Clin Immunol.* 2008; 28:314–21. [PubMed: 18224429]
38. Foss NT, Foss-Freitas MC, Ferreira MA, Cardili RN, Barbosa CM, Foss MC. Impaired cytokine production by peripheral blood mononuclear cells in type 1 diabetic patients. *Diabetes Metab.* 2007; 33:439–43. [PubMed: 17997340]
39. Wang X, Jia S, Geoffrey R, Alemzadeh R, Ghosh S, Hessner MJ. Identification of a molecular signature in human type 1 diabetes mellitus using serum and functional genomics. *J Immunol.* 2008; 180:1929–37. [PubMed: 18209091]
40. Rabinovitch A, Suarez-Pinzon WL. Cytokines and their roles in pancreatic islet beta-cell destruction and insulin-dependent diabetes mellitus. *Biochem Pharmacol.* 1998; 55:1139–49. [PubMed: 9719467]
41. Halminen M, Simell O, Knip M, Ilonen J. Cytokine expression in unstimulated PBMC of children with type 1 diabetes and subjects positive for diabetes-associated autoantibodies. *Scandinavian Journal of Immunology.* 2001; 53:510–513. [PubMed: 11309160]
42. Battaglia M, Stabilini A, Draghici E, Migliavacca B, Gregori S, Bonifacio E, Roncarolo MG. Induction of tolerance in type 1 diabetes via both CD4+CD25+ T regulatory cells and T regulatory type 1 cells. *Diabetes.* 2006; 55:1571–80. [PubMed: 16731819]
43. Mocellin S, Panelli MC, Wang E, Nagorsen D, Marincola FM. The dual role of IL-10. *Trends Immunol.* 2003; 24:36–43. [PubMed: 12495723]
44. Lauw FN, Pajkrt D, Hack CE, Kurimoto M, van Deventer SJ, van der Poll T. Proinflammatory effects of IL-10 during human endotoxemia. *J Immunol.* 2000; 165:2783–9. [PubMed: 10946310]
45. Ryden A, Stechova K, Durilova M, Faresjo M. Switch from a dominant Th1-associated immune profile during the pre-diabetic phase in favour of a temporary increase of a Th3-associated and inflammatory immune profile at the onset of type 1 diabetes. *Diabetes Metab Res Rev.* 2009; 25:335–43. [PubMed: 19382103]
46. Andersen MH, Schrama D, Thor Straten P, Becker JC. Cytotoxic T cells. *J Invest Dermatol.* 2006; 126:32–41. [PubMed: 16417215]
47. Marino E, Grey ST. A new role for an old player: do B cells unleash the self-reactive CD8+ T cell storm necessary for the development of type 1 diabetes? *J Autoimmun.* 2008; 31:301–5. [PubMed: 18809297]
48. Gronska MA, Weinem M. Death pathways in T cell homeostasis and their role in autoimmune diabetes. *Rev Diabet Stud.* 2006; 3:88–95. [PubMed: 17487332]
49. Peng SL, Moslehi J, Robert ME, Craft J. Perforin protects against autoimmunity in lupus-prone mice. *J Immunol.* 1998; 160:652–60. [PubMed: 9551899]
50. Quartier P, Bustamante J, Sanal O, Plebani A, Debre M, Deville A, Litzman J, Levy J, Ferman J, Lane P, Horneff G, Aksu G, Yalcin I, Davies G, Tezcan I, Ersoy F, Catalan N, Imai K, Fischer A, Durandy A. Clinical, immunologic and genetic analysis of 29 patients with autosomal recessive hyper-IgM syndrome due to Activation-Induced Cytidine Deaminase deficiency. *Clin Immunol.* 2004; 110:22–9. [PubMed: 14962793]
51. Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, Sudo K, Iwakura Y. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol.* 2006; 177:566–73. [PubMed: 16785554]
52. Gardner EM, Murasko DM. Age-related changes in Type 1 and Type 2 cytokine production in humans. *Biogerontology.* 2002; 3:271–90. [PubMed: 12237564]

Abbreviations

AID	Activation-induced cytidine deaminase
FasL	Fas Ligand
FDR	False discovery rate

IgG<gamma>	Immunoglobulin G gamma chain
NOD	Non-obese diabetic
Type 1 DM	Type 1 diabetes mellitus

Figure 1.

Relative gene expression levels for granzyme B, perforin and fas ligand in at-risk, new-onset, and long-term type 1 DM patients and healthy controls. Data was calculated as fold change against a common control and is represented in natural logarithmic scale (ln scale). The line indicates the average value for each group. Low, medium, and high risk patients in at-risk groups are illustrated as green, black, and red dots, respectively.

Figure 2. Relative gene expression levels for activation-induced cytidine deaminase (AID), and Immunoglobulin G gamma chain (IgG<gamma>) in at-risk, new-onset, and long-term type 1 DM patients and healthy controls. Data was calculated as fold change against a common control and is represented in natural logarithmic scale (ln scale). The line indicates the average value for each group. Low, medium, and high risk patients in at-risk groups are illustrated as green, black, and red dots, respectively.

Figure 3.

Relative gene expression levels for IFN- γ , IL-4 and IL10 in at-risk, new-onset, and long-term type 1 DM patients and healthy controls. Data was calculated as fold change against a common control and is represented in natural logarithmic scale (ln scale). The line indicates the average value for each group. One sample for IL-4 at-risk group was undetectable and was not shown in the figure. Low, medium, and high risk patients in at-risk groups are illustrated as green, black, and red dots, respectively.

Figure 4.

Relative gene expression levels for TGF- β and Foxp3 in at-risk, new-onset, and long-term type 1 DM patients and healthy controls. Data was calculated as fold change against a common control and is represented in natural logarithmic scale (ln scale). The line indicates the average value for each group. Low, medium, and high risk patients in at-risk groups are illustrated as green, black, and red dots, respectively.

Figure 5.

Relative gene expression levels for TNF- α and IL-6 in at-risk, new-onset, and long-term type 1 DM patients and healthy controls. Data was calculated as fold change against a common control and is represented in natural logarithmic scale (ln scale). The line indicates the average value for each group. Low, medium, and high risk patients in at-risk groups are illustrated as green, black, and red dots, respectively.

Figure 6.

Relative gene expression levels for AID, Immunoglobulin G gamma chain (IgG<gamma>), and IFN-γ in healthy controls with different age. Data was calculated as fold change against a common control and is represented in natural logarithmic scale (ln scale). Aging decreases mRNA expression of AID and IgG<gamma> and increases mRNA expression of IFN-γ in human peripheral blood. Pearson's r values for the linear curves for the correlation between age and immune variables and the significance of these analyses are the followings: AID: $r = -0.26$, $p = 0.02$; IGHG: $r = -0.31$, $p = 0.006$; and IFN-γ: $r = 0.26$, $p = 0.02$.

Table 1

Relative gene expression pattern in at-risk, new-onset and long-term type 1 DM patients as compared to healthy controls. Up or down arrow means significantly different between the two indicated groups ($p < 0.05$).

Gene	At-risk (AR)	New onset (NO)	Long-term (LT)	Healthy control (HC)
Granzyme B	↓ vs HC		↓ vs HC	↑ vs AR ↑ vs LT
Perforin			↓ vs HC	↑ vs LT
Fas Ligand			↓ vs HC	↑ vs LT
AID			↓ vs HC	↑ vs LT
IgG<gamma>			↓ vs HC	↑ vs LT
IFN- γ	↓ vs HC		↓ vs HC	↑ vs AR ↑ vs LT
IL-4				
IL-10			↓ vs HC	↑ vs LT
TGF- β			↓ vs HC	↑ vs LT
Foxp3			↓ vs HC	↑ vs LT
TNF- α	↓ vs HC		↓ vs HC	↑ vs AR ↑ vs LT
IL-6			↓ vs HC	↑ vs LT

Table 2

Relative gene expression pattern in at-risk, new-onset and long-term type 1 DM patients. Up or down arrow means significantly different between the two indicated groups ($p < 0.05$).

Gene	At-risk (AR)	New onset (NO)	Long-term (LT)
Granzyme B		↑ vs LT	↓ vs NO
Perforin			
Fas Ligand			
AID		↑ vs LT	↓ vs NO
IgG<gamma>		↑ vs LT	↓ vs NO
IFN- γ	↓ vs NO	↑ vs AR ↑ vs LT	↓ vs NO
IL-4	↓ vs NO	↑ vs AR ↑ vs LT	↓ vs NO
IL-10	↓ vs NO	↑ vs AR ↑ vs LT	↓ vs NO
TGF- β		↑ vs LT	↓ vs NO
Foxp3		↑ vs LT	↓ vs NO
TNF- α		↑ vs LT	↓ vs NO
IL-6		↑ vs LT	↓ vs NO