ORIGINAL ARTICLE

Transcriptional signatures as a disease-specific and predictive inflammatory biomarker for type 1 diabetes

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The complex milieu of inflammatory mediators associated with many diseases is often too dilute to directly measure in the periphery, necessitating development of more sensitive measurements suitable for mechanistic studies, earlier diagnosis, guiding therapeutic decisions and monitoring interventions. We previously demonstrated that plasma samples from recent-onset type 1 diabetes (RO T1D) patients induce a proinflammatory transcriptional signature in freshly drawn peripheral blood mononuclear cells (PBMCs) relative to that of unrelated healthy controls (HC). Here, using cryopreserved PBMC, we analyzed larger RO T1D and HC cohorts, examined T1D progression in pre-onset samples, and compared the RO T1D signature to those associated with three disorders characterized by airway infection and inflammation. The RO T1D signature, consisting of interleukin-1 cytokine family members, chemokines involved in immune cytokine chemotaxis, immune receptors and signaling molecules, was detected during early pre-diabetes and found to resolve post-onset. The signatures associated with cystic fibrosis patients chronically infected with Pseudomonas aeruginosa, patients with confirmed bacterial pneumonia, and subjects with H1N1 influenza all reflected immunological activation, yet each were distinct from one another and negatively correlated with that of T1D. This study highlights the remarkable capacity of cells to serve as biosensors capable of sensitively and comprehensively differentiating immunological states.

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INTRODUCTION

Many diseases arise from uncontrolled inflammatory processes and become evident after the progression of significant tissue and organ damage. These diseases include autoinflammatory diseases such as type 1 diabetes (T1D), multiple sclerosis and rheumatoid arthritis, as well as bacterial and viral infections. A need remains for discovery and evaluation of biomarkers that can sensitively detect and differentiate inflammatory processes, define immunological mechanisms of action that can guide selection of appropriately targeted therapies and sensitively monitor changes in the inflammatory state associated with disease progression or responses to therapeutic intervention. Fortunately, advances in genomic technologies now offer unprecedented opportunities to not only gain new mechanistic insights into inflammatory processes, but to improve diagnosis and treatment. While many studies have examined transcript levels in tissue biopsies, peripheral blood remains the most accessible human tissue and represents a practical, minimally invasive surrogate biopsy material.

The most common blood-based genomics strategy has been to directly profile transcripts of peripheral blood mononuclear cells (PBMCs) or purified immunocyte subsets of cases and controls. When applied to infectious disease, clinically useful, pathogen-specific transcriptional patterns have been associated with various bacterial, viral, fungal and protozoan infections.1,2 These responses possess species-level or even strain-level specificity and arise because different microbial pathogens exhibit unique pathogen-associated molecular patterns, which interact with specific host pattern recognition receptors (such as the toll-like receptors, TLRs)3,4 to trigger different signal transduction pathways and unique transcriptional programs.5–8 These differential responses have enabled the detection of specific transcriptional signatures in PBMCs from individuals harboring various infections including viruses,9–12 Gram-negative and Gram-positive bacteria,11,13 and eukaryotic parasites such as Plasmodium.14 Transcriptional signatures of patient PBMCs also reflect the inflammatory mechanisms utilized by various autoimmune diseases.15–17 These include systemic lupus erythmatosus, rheumatoid arthritis (where direct profiling of PBMC has been found useful for classifying disease and predicting response to infliximab),18–21 multiple sclerosis,22–24 inflammatory bowel disease (where PBMC profiles have been described that distinguish Crohn’s disease from ulcerative colitis25), psoriasis,26 dermatomyositis,27,28 and systemic onset juvenile idiopathic arthritis.29,30 A less common strategy has been to use patient serum or plasma to induce gene expression in healthy, unrelated third-party PBMCs. This approach, where the cells are used as reporters that sensitively respond to soluble disease-associated factors present in the periphery, has been used by Pascual et al.29 to study the inflammatory state associated with systemic onset juvenile

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idiopathic arthritis and we have applied it to T1D.\textsuperscript{31–33} We previously determined that culturing plasma of recent-onset (RO) T1D patients with freshly drawn unrelated, healthy PBMCs induces a unique innate inflammatory transcriptional signature.\textsuperscript{31} This signature includes many genes regulated by interleukin (IL)1, a cytokine known to co-stimulate T cells and cause pancreatic β-cell death \textit{in vitro}. The RO T1D signature is distinct from that induced by the plasma of unrelated healthy controls (HCs) or long-standing (LS) T1D patients (>10 years post-onset), suggesting that the RO T1D signature is induced by factors related to active autoimmunity.\textsuperscript{31} In longitudinal samples collected from progressed to diabetes, this T1D signature was evident as much as 5 years before onset and before the emergence of autoantibodies toward islet cell antigens, which are currently considered to be the best predictor of progression to diabetes.\textsuperscript{31} Our application of this approach to studies of T1D in the BioBreeding rat disease model also revealed an onset-associated signature partially dependent on IL1. Blocking the IL1 receptor by treating BioBreeding rats with IL1 receptor antagonist (IL1RA) delayed onset and, in part, normalized the disease signature, suggesting that induced transcriptional signatures are not only mechanistically informative, but may possesses utility in monitoring immunotherapeutic intervention.\textsuperscript{32}

A limitation in our past studies, as well as many other studies that have utilized cell-based assays, is the reliance on freshly isolated PBMCs. Despite utilizing PBMCs drawn from multiple healthy donors, we found that the plasma samples from a given subject cohort induced relatively homogenous profiles\textsuperscript{31} with modest cell donor specific effects. Commercially available cryopreserved PBMCs are a relatively new option for cell-based assays. These cells are collected via apheresis from highly characterized blood donors, enabling the harvesting of billions of cells during a single draw. Rapid processing/cryopreservation under optimized and controlled conditions results in high viability and retained functionality. In this report we extend our initial studies by using a cryopreserved PBMC of a single donor to (1) examine differences among pediatric RO T1D patients and unrelated HC, (2) study the development of the signature during the progression of T1D and (3) evaluate the disease-specificity of the technique by examining for the first time transcriptional signatures induced by plasma collected from patients with conditions characterized by pathologic pulmonary inflammation: cystic fibrosis (CF), bacterial pneumonia and H1N1 influenza.

RESULTS

Cross-sectional analysis of RO T1D and unrelated HC samples

At diagnosis of T1D, an estimated 60–90% of β-cell mass is either dysfunctional or destroyed. Upon initiation of insulin therapy, 25–100% of new onset patients experience a transient remission with functional restoration of the residual mass, termed the ‘honeymoon period’, which lasts months to years.\textsuperscript{34–37} This immunologically active time is clinically significant because (1) it offers a window for therapeutic intervention aimed at preserving remaining β-cell mass and (2) as shown by our previous studies,\textsuperscript{31,33} it is a period where it is still possible to measure processes related to β-cell autoimmunity. Therefore, our studies of plasma-induced transcriptional signatures have primarily focused on the first 7 months post-onset.

In order to reproduce and expand the observations of our initial report,\textsuperscript{31} plasma drawn from 47 RO T1D patients (mean age 9.97 ± 2.89 years, Supplementary Table 1) and 44 unrelated HC subjects (mean age 14.98 ± 4.13 years, Supplementary Table 1) were used to induce gene expression in commercially supplied cryopreserved PBMC of a single draw of a single healthy blood donor (UPN727). All RO T1D subjects were positive for ≥1 autoantibody and to avoid inducing gene expression due to factors related to hyperglycemia, samples were collected after stabilization on exogenous insulin from subjects that exhibited histories of good glycemic control.

We identified 762 probe sets that were differentially regulated when UPN727 cells were co-cultured with plasmas of the RO T1D or HC cohorts (\textit{log}$_2$ ratio > 0.263, 1.2-fold; FDR (false discovery rate) < 0.2; ANOVA (ANalysis Of VAriation) P < 0.036); these represented 622 unique UniGenes (Supplementary Table 2). The relationship of this data set to those genes most significantly regulated following co-culture of plasma collected from 12 RO T1D and 12 HC subjects with fresh PBMCs from six healthy blood donors in our previous report\textsuperscript{31} (498 probe sets \textit{log}$_2$ ratio > 0.5; FDR < 0.10) is illustrated in Figure 1a. The Pearson correlation coefficient between the union of these two data sets is 0.59; however, if the analysis is restricted to the 63 common probe sets, the Pearson correlation coefficient is 0.93. Both principal component analysis (PCA; Figure 1b) and one-way hierarchical clustering (Figure 1c) using the 762 probes differentially expressed in UPN727 cells revealed heterogeneity within the RO T1D and HC cohorts. While the majority of individual samples within the two cohorts induced distinct signatures, a subset of samples did not, resulting in the overlap detected by both clustering methods.

Genes significantly over-expressed (n = 186 probe sets) and under-expressed (n = 576 probe sets) when UPN727 PBMCs were cultured with RO T1D or HC plasma were independently evaluated for biological pathway enrichment using the Database for Annotation, Visualization and Integrated Discovery (DAVID) to identify regulated Gene Ontology Biological Processes. Representative pathway terms appear in Table 1 (a complete list is provided in Supplementary Table 2) and selected probe sets are illustrated in Figure 1d. Ontological analysis of the 186 probe sets up-regulated by RO T1D plasma revealed 41 significant Gene Ontology Biological Processes (P < 0.01; FDR < 0.10), including immune response, chemotaxis, positive regulation of cytokine production, cell surface receptor signal transduction and regulation of IL6 production. These categories included genes encoding immune signaling molecules and receptors including the IL1 cytokine family members \textit{IL1}, \textit{IL1RN}, \textit{IL1R1} and \textit{IL1R2}, the IL10 family member \textit{IL10}, and the chemokines \textit{CXCL1}, \textit{CXCL2}, \textit{CXCL3} and \textit{CXCL5}, which are involved in neutrophil chemotaxis.\textsuperscript{38} RO T1D plasma induced cylooxygenase-2 or COX-2 (PTGS2) and prostaglandin E synthase (PTGES). Genes for several immune receptors were also significantly up-regulated by RO T1D plasma, including \textit{TLR2}, \textit{FCAR}, \textit{LILR3A} and \textit{TREM1}.

Ontological analysis of the 576 probe sets down-regulated by RO T1D plasma identified 27 significant Gene Ontology Biological Processes (P < 0.01; FDR < 10%) that were functionally opposed to the terms enriched in the up-regulated subset, such as negative regulation of mononuclear cell proliferation, negative regulation of transcription, bone morphogenetic protein (BMP) signaling pathway, negative regulation of T-cell proliferation (Table 2; Supplementary Table 3). Relative to HC plasma, culture with RO T1D plasma led to the under-expression of genes such as \textit{KLF2}, \textit{KLF6} and \textit{KLF12} (a TGF (transforming growth factor) (β)-induced gene that negatively regulates TGF-β signaling), the histone deacetylase \textit{HDAC3}, \textit{FOXPI} (a transcription factor that is important in maintaining immune quiescence) and \textit{ZEB2} (a human zinc-finger transcription factor and transcriptional repressor that regulates T-cell activity). Overall, the RO T1D and HC profiles reflect inflammatory versus immune-regulatory processes, respectively, suggesting that RO T1D sera possesses higher levels of proinflammatory mediators such as IL1 and lower levels of anti-inflammatory factors.

Finally, we employed ToppGene\textsuperscript{39,40} to further explore the functional commonality in the originally reported response of multiple fresh PBMC to RO T1D and HC plasma compared with those of cryopreserved PBMC using the two data sets illustrated in Figure 1a. When we used the 498 probe sets differentially
expressed in fresh cells as the training set, we detected 431/762 (56.6%) significant (P < 0.01) functionally related probe sets in cryopreserved cells. Overall, the cross-sectional analysis of the RO T1D and HC samples demonstrates that a proinflammatory signature is induced in cryopreserved PBMCs that is functionally concordant with that previously observed in fresh cells.31

Direct detection of inflammatory mediators in RO T1D and HC plasma samples
In a continuing effort to account for the induced signatures, cytokine levels were measured by multiplex ELISA in a subset of 17 RO T1D and 15 HC subjects examined in the expression studies (Table 2). Higher levels of several cytokines were detected in RO T1D plasma compared with HCs, including IL1α, which is
samples (Figure 2a), LS T1D plasma induced transcription similar differentially expressed in the cross-sectional RO T1D and HC autoantibody. As reflected by PCA of the 762 probe sets onset; 10/11 possessed measurable titers for at least one islet cell

Using cryopreserved UPN727 PBMC as reporter cells, we analyzed

Analysis of LS T1D and pre-diabetes

Consistent with the induction of IL1-mediated gene expression. However, with the exception of TNF-α (tumor necrosis factor-α) (RO T1D: 9.3 ± 1.0 pg ml⁻¹; HC: 6.5 ± 0.6 pg ml⁻¹; P < 0.05), none of these differences reached statistical significance with the number of subjects analyzed. Furthermore, correlations between the intensity of the signature among individual samples (Figures 1b and c) and mediator levels, antibody levels, gender, age of onset, or time to post-onset sample collection could not be made.

Analysis of LS T1D and pre-diabetes

Using cryopreserved UPN727 PBMC as reporter cells, we analyzed sera of 11 LS T1D subjects, all of whom were > 10 years post-onset; 10/11 possessed measurable titers for at least one islet cell autoantibody. As reflected by PCA of the 762 probe sets differentially expressed in the cross-sectional RO T1D and HC samples (Figure 2a), LS T1D plasma induced transcription similar to that induced by HC plasma. Of the 762 probe sets differentially regulated between the RO T1D and HC cohorts, only 84 probe sets (11%) met our thresholds (log₂ ratio > 0.263, 1.2-fold; FDR < 0.02) when comparing the LS T1D and HC groups. Consistent with our previous report, the signature associated with RO T1D is not induced by samples collected from established T1D patients, supporting the hypothesis that the inflammatory signature induced by RO T1D plasma arises from factors related to active autoimmune processes.

We also used cryopreserved UPN727 PBMC to evaluate longitudinal samples collected from a prospectively monitored sibling of a proband that progressed to T1D. This subject was a sibling of a proband that progressed to T1D. This subject was

Table 1. Enrichment of gene ontology (GO) terms in differentially expressed probe sets

| GO identifier | Biological process | n² | P-value | FDR (%) |
|---------------|--------------------|----|---------|---------|
| a. Probe sets regulated by RO T1D versus HC plasma | Immune response | 21 | 3.28E - 7 | 5.40E - 4 |
| G00006955 | Chemotaxis | 11 | 7.47E - 7 | 1.22E - 3 |
| G00006955 | Positive regulation of cytokine production | 7 | 1.10E - 4 | 0.18 |
| G00006955 | Response to bacterium | 9 | 1.40E - 4 | 0.23 |
| G00043405 | Regulation of MAP kinase activity | 7 | 1.08E - 3 | 1.76 |
| G0007166 | Cell surface receptor linked signal transduction | 25 | 1.25E - 3 | 2.03 |
| G00032680 | Regulation of TNF production | 4 | 2.29E - 3 | 3.70 |
| G00032675 | Regulation of IL6 production | 4 | 3.53E - 3 | 5.64 |
| G00050672 | Negative regulation of lymphocyte proliferation | 6 | 1.31E - 3 | 2.20 |
| G00016481 | Negative regulation of transcription | 24 | 1.75E - 3 | 2.93 |
| G00030509 | BMP signaling pathway | 6 | 4.47E - 3 | 7.34 |
| G00045892 | Negative regulation of transcription, DNA-dependent | 19 | 4.77E - 3 | 7.81 |
| G00042130 | Negative regulation of T-cell proliferation | 5 | 4.92E - 3 | 8.05 |
| G00016568 | Chromatin modification | 16 | 5.27E - 3 | |
| b. Probe sets regulated by CF versus HC plasma | Immune response | 104 | 3.34E - 12 | 6.06E - 9 |
| G00006955 | T-cell activation | 35 | 1.77E - 10 | 3.22E - 7 |
| G00025212 | Leukocyte differentiation | 34 | 2.83E - 9 | 5.14E - 6 |
| G00042113 | B-cell activation | 17 | 3.33E - 4 | 0.60 |
| G00006955 | Response to bacterium | 9 | 5.02E - 8 | 9.30E - 5 |
| G00006955 | Positive regulation of cytokine production | 24 | 9.46E - 6 | 0.02 |
| G00043405 | Regulation of MAP kinase activity | 28 | 2.48E - 4 | 0.45 |
| G00007166 | Cell surface receptor linked signal transduction | 25 | 1.25E - 3 | 2.03 |
| c. Probe sets regulated by bacterial pneumonia versus HC plasma | Immune response | 74 | 6.72E - 8 | 1.20E - 4 |
| G00006955 | Positive regulation of cell death | 47 | 1.27E - 4 | 0.23 |
| G00034620 | Cellular response to unfolded protein | 8 | 1.64E - 4 | 0.29 |
| G00070500 | Cell-cycle arrest | 16 | 1.36E - 3 | 2.41 |
| G00055832 | Positive regulation of T-cell differentiation | 8 | 2.64E - 3 | 4.62 |
| G00043066 | Negative regulation of apoptosis | 55 | 3.18E - 11 | 5.83E - 8 |
| G00079596 | Blood coagulation | 22 | 2.26E - 7 | 4.14E - 4 |
| G00050715 | Positive regulation of cytokine secretion | 7 | 1.27E - 3 | 2.31 |
| d. Probe sets regulated by pre-H1N1 versus active H1N1 plasma | Response to virus | 24 | 3.91E - 25 | 6.46E - 22 |
| G0009615 | DNA-damage response, signal transduction by p53 class mediator | 5 | 4.03E - 05 | 6.64E - 2 |
| G00030330 | Regulation of interferon-α production | 3 | 2.65E - 3 | 4.29 |
| G00032647 | Regulation of interferon-γ production | 4 | 3.22E - 3 | 5.19 |

Abbreviations: CF, cystic fibrosis; DAVID, Database for Annotation, Visualization, and Integrated Discovery; FDR, false discovery rate; HC, healthy controls; RO T1D, recent-onset type 1 diabetes; TNF, tumor necrosis factor. aAnalysis restricted to GO biological processes with p < 0.01, and FDR < 10%. Arrow denotes up- or down-regulation. Due to the relatively low numbers of genes identified in the H1N1 studies, up- and down-regulated genes were not separated for analysis with DAVID. Total probe sets detected.
sets regulated by RO T1D and HC plasma in the cross-sectional studies, and with short time-series expression miner (STEM), a software tool for the analysis of time-series gene expression data.\textsuperscript{31}

Disease progression was evident in the PCA of the longitudinal plasma samples (Figure 2a). The relationship between the regulated genes identified in the cross-sectional analysis of RO T1D and HC samples (\(n = 762\) probe sets) compared with those identified by STEM analysis of this single longitudinal series (\(n = 1278\) probe sets; STEM profiles with detection \(P < 10^{-25}\) and a minimum 1.68-fold change between any two time points) is illustrated in Figure 2b; the signatures share a significantly nonrandom (\(P < 10^{-51}\), \(\chi^2\) test), commonly regulated intersection of 220 probe sets. The Pearson correlation coefficients (calculated using the RO T1D:HC log\(_2\) ratio for the cross-sectional samples and the last first point time log\(_2\) ratio for the longitudinal series) for the union and intersection of these two data sets were 0.55 and 0.78, respectively. The probe sets uniquely regulated in the longitudinal and cross-sectional analyses expectedly showed lower correlation, as (1) the inflammatory state measured at T1D onset differs from that at points earlier in disease progression and (2) the signature of induced by samples before onset of T1D in the longitudinal analysis are distinct from the samples of unrelated HC examined in the cross-sectional analyses (Figure 2b; Supplementary Table 2).

STEM was also employed to examine the profiles generated when these longitudinally collected plasma samples were previously used to induce transcription in healthy, freshly drawn PBMCs from a single donor.\textsuperscript{31} Among the 2319 probe sets differentially expressed between the analyses of the longitudinal series using fresh versus cryopreserved cells, we identified a shared, significantly nonrandom (\(P < 10^{-57}\), \(\chi^2\) test), correlative (Pearson correlation coefficient = 0.63), commonly regulated intersection of 154 probe sets. We again employed ToppGene\textsuperscript{39,40} to examine the functional similarity in the responses of fresh and cryopreserved PBMCs to plasma samples from the longitudinal series. Using the 1195 probe sets differentially expressed in fresh cells as the training set, we identified 885/1278 (69.3\%) significant (\(P < 0.01\)), functionally related probe sets among those regulated in cryopreserved cells.

As with fresh PBMCs,\textsuperscript{31} we observed an increase in the robustness and complexity of the signature with disease progression (Figure 2b). Genes such as IL1A, IL24, IL10, IL1R1 and PTGS2 exhibited increased induction while others such as CCL24 and SKIL showed lower induction by samples collected closer to onset. Importantly, induction of inflammatory genes was evident in the sample collected 5.3 years before onset, before autoantibody development, and overall the signature of all pre-onset samples was distinct from that of the mean response of the 44 unrelated HCs examined in the cross-sectional analysis. Finally, we examined the mean absolute fold-change for probe sets in the 2.5% tails of the log\(_2\) ratio distribution in the first and last samples of the series (the highest 1365 and lowest 1365 expressed probe sets). We observed a difference of 0.72 ± 0.23 in fresh PBMCs from a single donor (20% plasma) versus 0.67 ± 0.22 in fresh PBMCs from a single donor (40% plasma) and 0.22 in the UPN727 cells (40% plasma) versus 0.67 ± 0.23 in fresh PBMCs from a single donor (20% plasma). The responsiveness of the cryopreserved UPN727 cells under these conditions were on a par with that of the fresh PBMCs originally used to examine this longitudinal series.\textsuperscript{31} The longitudinal studies further highlight the functional concordance of the plasma-induced transcriptional signatures generated in fresh and cryopreserved PBMCs.

The T1D signature is distinct from signatures associated with CF colonization with Pa, bacterial pneumonia and H1N1 influenza. Next, we focused on the specificity of the signature induced by T1D plasma by evaluating samples from other inflammatory conditions. Plasma samples from 20 non-diabetic, pediatric CF patients chronically colonized with Pa (Pseudomonas aeruginosa) and 24 age-matched unrelated HCs were used to induce transcription in UPN727 cells at a culture concentration of 20% (Supplementary Table 1). A robust signature consisting of 5000 differentially expressed probe sets \(|\log_2\text{ratio}| > 0.263, 1.2\text{-fold}; \\text{FDR} < 0.2; 3404 unique UniGenes) was distinct from that induced by RO T1D plasma (Figure 3, Supporting Information Table 2).

Table 2. Cytokine/chemokine levels in RO T1D (\(n = 17\)) and unrelated HC (\(n = 15\)) plasma samples

| Cytokine | RO T1D (pg ml\(^{-1}\)) | Unrelated HC (pg ml\(^{-1}\)) | Fold RO T1D/HC | Lower detection limit (pg ml\(^{-1}\)) |
|----------|--------------------------|-------------------------------|----------------|-------------------------------------|
| Eotaxin  | 104.2 ± 10.3             | 79.8 ± 25.1                   | 1.3            | >3.2 pg ml\(^{-1}\)                 |
| Granulocyte/macrophage-CSF | 25.9 ± 9.1               | 7.90 ± 4.3                    | 3.3            | >3.2 pg ml\(^{-1}\)                |
| Interferon-\(\gamma\) | 73.9 ± 7.3               | 54.5 ± 7.2                    | 1.4            | >3.2 pg ml\(^{-1}\)                |
| IL1a     | 9.8 ± 4.7                | 5.5 ± 2.6                     | 1.8            | >3.2 pg ml\(^{-1}\)                |
| IL1b     | 59.8 ± 24.1              | 23.4 ± 11.0                   | 2.6            | >3.2 pg ml\(^{-1}\)                |
| IL2      | 7.8 ± 2.4                | 6.2 ± 2.3                     | 1.2            | >3.2 pg ml\(^{-1}\)                |
| IL3      | 2.3 ± 1.2                | 0.8 ± 0.2                     | 2.9            | >3.2 pg ml\(^{-1}\)                |
| IL4      | 0.0 ± 0.0                | 0.2 ± 0.7                     | 0.0            | >3.2 pg ml\(^{-1}\)                |
| IL5      | 9.0 ± 4.0                | 2.1 ± 1.8                     | 4.3            | >3.2 pg ml\(^{-1}\)                |
| IL6      | 1.7 ± 0.6                | 0.5 ± 0.2                     | 3.2            | >3.2 pg ml\(^{-1}\)                |
| IL7      | 8.0 ± 2.9                | 6.2 ± 2.2                     | 1.3            | >3.2 pg ml\(^{-1}\)                |
| IL8      | 14.1 ± 2.7               | 10.0 ± 2.7                    | 1.4            | >3.2 pg ml\(^{-1}\)                |
| IL9      | 14.7 ± 3.4               | 15.4 ± 4.0                    | 1.0            | >3.2 pg ml\(^{-1}\)                |
| IL10     | 11.8 ± 2.9               | 11.6 ± 4.1                    | 1.0            | >3.2 pg ml\(^{-1}\)                |
| IL12p40  | 83.4 ± 22.85             | 58.9 ± 16.6                   | 1.4            | >3.2 pg ml\(^{-1}\)                |
| IL12p70  | 9.5 ± 6.7                | 1.6 ± 0.7                     | 6.0            | >3.2 pg ml\(^{-1}\)                |
| IL13     | 9.5 ± 4.7                | 9.5 ± 4.8                     | 2.5            | >3.2 pg ml\(^{-1}\)                |
| IL15     | 7.4 ± 2.2                | 7.5 ± 2.4                     | 1.0            | >3.2 pg ml\(^{-1}\)                |
| IL17     | 4.8 ± 1.6                | 6.8 ± 3.1                     | 0.7            | >3.2 pg ml\(^{-1}\)                |
| IP-10    | 221.8 ± 26.2             | 242.8 ± 35.4                  | 0.9            | >3.2 pg ml\(^{-1}\)                |
| Monocyte chemoattractant protein-1 | 252.8 ± 14.1 | 214.9 ± 22.3 | 1.2 | >16.0 pg ml\(^{-1}\) |
| Macrophage inflammatory protein-1a | 12.6 ± 4.0 | 13.0 ± 4.5 | 1.0 | >16.0 pg ml\(^{-1}\) |
| Macrophage inflammatory protein-1b | 27.2 ± 6.5 | 13.4 ± 5.3 | 2.0 | >16.0 pg ml\(^{-1}\) |
| TNF-\(\alpha\) | 9.3 ± 1.0 | 6.5 ± 0.6 | 1.4\* | >3.2 pg ml\(^{-1}\) |
| TNF-\(\beta\) | 30.6 ± 12.4 | 10.0 ± 4.5 | 3.1 | >3.2 pg ml\(^{-1}\) |

Abbreviations: HC, healthy controls; IL, interleukin; RO T1D, recent-onset type 1 diabetes; TNF, tumor necrosis factor. Data are mean values ± standard error (s.e., pg ml\(^{-1}\)). Each sample was tested in duplicate using the Millipore BeadLyte cytokine assay kit. *\(P < 0.05\), two-tailed \(t\)-test.
Figure 2. Analysis of T1D progression in longitudinal subject A. (a) PCA using the 762 probe sets regulated by RO T1D (n = 47) versus HC (n = 44) plasma identified in the cross-sectional studies (|log2 ratio| > 0.263; FDR < 0.2). Green spheres, HC; red spheres, RO T1D; gray spheres, LS T1D (> 10 years post-onset); blue cubes, Longitudinal Subject A series (lightest to darkest blue indicates sample order, −5.3, −3.3, −2.4, −1.5, −0.3, +0.3 years relative to onset, respectively). Arrow shows progression to RO T1D. (b) Venn diagram and one-way hierarchical clustering (probe sets only) for each component of the Venn diagram illustrate the relationship between the probe sets identified in the STEM analysis of the Longitudinal Subject A series versus the cross-sectional analyses of the RO T1D and HC samples. The signatures share a significantly nonrandom (P < 10−51, χ2 test), commonly regulated intersection of 220 probe sets (Supplementary Table 2). Relative expression levels are shown for selected, well-annotated genes related to inflammatory processes that were significantly identified by the STEM analyses. Additional well-annotated genes are shown in Figure 3c.
Ontological analysis of the 2139 probe sets up-regulated by CF plasma versus HC plasma revealed 101 significantly enriched Gene Ontology Biological Processes (P < 0.01; FDR < 0.10); many of these annotations were related to immunological activation and T-cell/B-cell activation. CF sera induced transcription of interferon (IFN) regulatory family factor 1 (IRF1), GIMAP1, GIMAP5, TLR10, IL32, CCL5, CD40, IKZF1, IKAROS family zinc finger 1 (IKZF1), IL15 and IL16. Analysis of the 2861 probe sets down-regulated by CF plasma identified 238 significant Gene Ontology Biological Processes (P < 0.01; FDR < 0.10). Many Biological Processes up-regulated by T1D sera were found down-regulated by CF sera (Table 1b). Accordingly, many IL1 regulated genes annotated within these categories representing functions of immune recognition and response were found to be down-regulated by CF sera, including PTGS2, CCL2, IRAK3, IL1B and IL1R1 (Figure 3). Selected pathway terms appear in Table 1b, and the complete list is provided in Supplementary Table 3.

We examined 10 previously healthy, pediatric bacterial pneumonia patients with confirmed bacterial infection who had been admitted to the intensive care unit; eight required mechanical ventilation.
ventilation. Of the four inflammatory diseases examined in this investigation, the plasma of these critically ill children (at a culture concentration of 20%) induced the most robust signature (relative to 18 HCs; \( n = 8121 \); pneumonia:HC \( \log_2 \text{ratio} > 0.263 \); FDR < 0.20; 4410 up-regulated probe sets and 3711 down-regulated probe sets; 5650 unique UniGenes; Supplementary Table 2). The transcriptional response of cryopreserved UN727 cells to sera collected from the bacterial pneumonia patients was distinct from that following exposure to T1D or CF sera. The ontological analysis was restricted to the 3034 probe sets that had pneumonia:HC \( \log_2 \text{ratio} > 0.5 \) and FDR < 0.20 (2144 unique UniGenes). Selected pathway terms appear in Table 1c; a complete list is provided in Supplementary Table 3.

Ontological analysis of the 1515 probe sets up-regulated by plasma collected from bacterial pneumonia patients revealed 64 significant Gene Ontology Biological Processes (\( P < 0.01; \) FDR < 0.10), including cellular response to unfolded protein, cell-cycle arrest and positive regulation of T-cell differentiation. Genes uniquely regulated by bacterial pneumonia patient plasma and annotated under these terms included DNA-damage-inducible transcript 3 (DDIT3), selenoprotein S (SELS; involved in the endoplasmic reticulum stress response and mediating inflammation), and the p53 target genes Sestrin1 (Sestrin1), cullin 5 (CUL5; an inhibitor of cellular proliferation), tumor protein p53-inducible nuclear protein 1 (TP53inp1), junction mediating and regulatory protein p53 cofactor (UMY) and others (Figure 3c). Ontological analysis of the down-regulated 1519 probe sets identified 242 significant Gene Ontology Biological Processes (\( P < 0.01; \) FDR < 0.10), many of which were related to immune response modulation, negative regulation of apoptosis, chemotaxis and blood coagulation. Genes annotated within these pathways included pannexin 1 (Pannx1; involved with IL1-β release), IL6R, CCL4, the endothelial protein C receptor (Procr), protecin (CD59); a complement regulatory protein), coagulation factor V (F5), thrombomodulin (THBD) and others (Figure 3).

Lastly, samples collected from five middle-aged subjects (mean age 49.60 ± 2.07 years) before and during H1N1 infection were also analyzed. Analysis of cultures exposed to pre-H1N1 versus symptomatic H1N1 plasma revealed a signature of 271 significantly regulated probe sets representing 182 unique UniGenes (255 over-expressed probe sets; 16 under-expressed probe sets; Figure 3; \( \log_2 \text{ratio} < -0.5 \); paired t-test \( P < 0.05 \)). This signature was distinct from the signatures induced with plasma from RO T1D patients, CF patients colonized with Pa, and previously healthy patients with bacterial pneumonia. We used DAVID to identify 32 significantly enriched annotations (\( P < 0.01; \) FDR < 10%). As anticipated, numerous Biological Processes typically viewed as host responses to infection or immune defense were induced by H1N1 plasma, including response to virus, defense response to virus and regulation of \( \alpha \) and \( \gamma \)INF production. Selected pathway terms are tabulated in Table 1d, and the complete list appears in Supplementary Table 3.

Notably, sera collected during H1N1 infection induced transcription of numerous IFN (interferon)-regulated genes, including INF-induced protein with tetracistic peptide repeats 1 (Ifit1), Ifit5, Ifitm1 and Ifitm3. Members of this gene family are induced in response to type I and type II INF, double-stranded RNA (dsRNA) and endotoxin.\(^{42,43}\) Sera of H1N1-infected individuals induced expression of other IFN-regulated genes including Ifi6 and Mxi1, and INF-stimulated exonuclease gene 20 kDa (isg20), which encodes an anti-viral ssRNA-specific exonuclease. Also induced by H1N1 sera were 2,5'-oligoadenyate synthetase 3 (Oas3) and Oasl, which are INF-induced enzymes that bind dsRNA and polymerize ATP into 2'–5' linked oligomers of adenosine (pppA(2'p5'A)n). H1N1 sera also regulated numerous genes related to pathogen recognition, including Tlr7 and Tlr8, which recognize viral RNA. Interestingly, a several genes differentially expressed in response to H1N1 sera also exhibited significant, opposite induction by T1D sera, including the transcription factor cAMP response element modulator (CREM), Il1a, Ptgs2, Il6, Inhba, and the nuclear receptor subfamily 4 group A member 2 (Nr4a2).

Figure 3 illustrates the uniqueness of each inflammatory state and how the genes induced under the various conditions are correlated. Regulation coefficients between the signatures induced by each of the inflammatory states were calculated using the probe sets significantly regulated by each condition as well as the union of all significantly regulated probe sets of the four conditions (Table 3). The T1D signature was negatively correlated with the signatures of CF patients chronically colonized with Pa, bacterial pneumonia patients, and subjects with H1N1 influenza. The signatures associated with CF colonized with Pa compared with the bacterial pneumonia patients showed the highest, albeit modest, level of overall correlation (0.19), further demonstrating the disease-specificity of the approach.

**DISCUSSION**

Autoimmunity and \( \beta \)-cell destruction occur asymmetrically for months to years before onset of T1D, providing an excellent opportunity for early diagnosis and intervention. The evaluation of autoantibody status in combination with HLA genotyping can reliably predict susceptibility to T1D.\(^{44}\) However, autoantibodies are thought to appear late during pre-diabetes, their levels can be transient, and not all autoantibody positive subjects progress to T1D onset.\(^{45}\) Furthermore, the humoral response is considered secondary to the initiation of autoimmune processes and it is generally accepted that autoantibodies are markers of \( \beta \)-cell destruction but may not directly mediate T1D development.\(^{46-48}\) Thus, a need remains for the discovery and evaluation of minimally invasive biomarkers that are amenable to repeated measurement and capable of detecting early T1D in a mechanistically informative, disease-specific manner.\(^{49}\)

A complex but dilute cytokine milieu is associated with T1D.\(^{50-55}\) While these mediators are difficult to measure directly via present ELISA-based approaches, our studies have shown that they are sufficient to drive transcription in a reporter cell system.\(^{31-33}\) To overcome the inherent limitations associated with cell-based assays that rely on freshly drawn responder PBMCs, we previously

**Table 3.** Pairwise Pearson correlation coefficients for the signatures induced by different inflammatory states

| Condition | n* | T1D | CF with Pa colonization | Bacterial pneumonia | H1N1 |
|-----------|----|-----|------------------------|---------------------|------|
| T1D       | 762 | 1.00 (1.00)b | -0.72 (-0.59) | -0.47 (-0.23) | -0.42 (-0.15) |
| CF/Pa colonization | 5000 | 1.00 (1.00) | 0.23 (0.19) | 1.00 (1.00) | 0.03 (0.03) |
| Bacterial pneumonia | 3034 | 0.50 | 0.39 (0.16) | 1.00 (1.00) |
| H1N1      | 271 | | | |

Abbreviations: CF, cystic fibrosis; FDR, false discovery rate; HC, healthy controls; Pa, Pseudomonas aeruginosa; RO T1D, recent-onset type 1 diabetes. aThe total number of regulated probe sets meeting the following thresholds: RO T1D:HC \( \log_2 \text{ratio} > 0.5 \), FDR < 0.20; CF:HC \( \log_2 \text{ratio} > 0.263 \), FDR < 0.20; pneumonia:HC \( \log_2 \text{ratio} > 0.5 \), FDR < 0.20; H1N1-pre-H1N1 \( \log_2 \text{ratio} > 0.5 \); paired t-test \( P < 0.05 \). bPearson correlation coefficients for genes in the union of the probe sets differentially regulated under all four conditions \( n = 7376 \) are shown in parentheses.
explored the use of lymphoid and myeloid cell lines as potential PBMC surrogates. We found them able to differentially respond to RO T1D versus HC sera; however, the induced profiles of each cell line were distinct from one another and from fresh PBMC, and less biologically informative.

Here, we expanded the observations of our initial report using commercially supplied cryopreserved PBMCs from a single donor as the responder cell population. Such cells are collected in high numbers through aphaeresis and are rapidly processed to maximize post-thaw viability. The use of these cells allows for control of HLA type, gender and immune reactivities, reduces data heterogeneity introduced by using responder PBMCs from multiple donors or draws of the same individual, eliminates the need of repeated recruitment of volunteer blood donors and simplifies laboratory workflow by obviating the need for monocyte/lymphocyte isolation from whole blood for each assay. As a prelude to this study, we compared the response of cryopreserved PBMC drawn from five different donors to RO T1D and HC plasma (data not shown). While PBMCs of all the donors tested were able to differentially respond to RO T1D and HC plasma, UPN727 PBMC were selected as they most closely mimicked the mean response of freshly isolated PBMC of multiple donors used previously and the highest number of cryopreserved cells were available. We observed >90% post-thaw viability with UPN727 cells based upon trypan blue exclusion, and multiparameter flow cytometry has shown that the relative abundance of natural killer cells, T cells, CD4+ and CD8+ T cells, B cells and monocytes do not significantly differ from the normal expected ranges for fresh PBMCs (Supplementary Table 4).

When analyzing the transcription induced in UPN727 cells by plasma of 47 RO T1D and 44 HC subjects, we identified 762 significantly regulated probe sets. The analysis of these samples required multiple assays where good inter and intra assay reproducibility were observed. Specifically, in the duplicate analysis of five independent RO T1D and HC samples, we observed low mean intra-assay coefficients of variation (0.029 ± 0.030 for the entire array and 0.034 ± 0.036 for the 762 probe set RO T1D:HC signature). When analyzing the same sample in five independent assays, we observed low mean inter-assay CVs (0.051 ± 0.041 for the entire array and 0.095 ± 0.062 for the 762 probe set RO T1D:HC signature). In these analyses, the intra- and inter-assay correlation coefficients for the 762 probe set RO T1D:HC signature was 0.94 and 0.71, respectively.

PCA and hierarchical clustering revealed heterogeneity within the RO T1D and HC cohorts (Figure 2). We attribute this to the possibility that greater inter-subject variability was captured in this cross-sectional study. For example, at onset a maximal individual fold-changes in IL6 and PTGS2 relative to the mean HC response were 2.25- and 2.97-fold, respectively. Thus, a baseline sample collected before disease initiation is likely the best control sample for a given individual in the study of disease progression through T1D onset. Since significantly regulated genes identified in cross-sectional studies of samples collected at onset may not necessarily be the most informative early biomarkers of T1D, longitudinal studies of pre-onset samples are needed to identify the earliest indices of immunological activation and/or loss of immune regulation. The transcripts most consistently regulated from disease initiation through onset in most/all subjects will reflect the most common pathways associated with diabetogenesis and will represent the most reliable biomarkers for predicting RO T1D onset.

We found them able to differentially expressed genes not identified in cross-sectional studies, and vice versa, clearly indicating that both analysis strategies are necessary.

In our larger study sample, genes known to be influenced by IL1 were again over-represented in the transcriptional signatures of cells exposed to RO T1D plasma. These genes included IL1β, cyclooxygenase type 2 (PTGS2), prostaglandin E synthase (PTGES), IL1R1, CXCL1, CXCL2, CXCL3, CXCL5, PLAUR, CREM and others. This observation is, in general agreement with findings that IL1 expression in rodent models of T1D has been shown to be elevated. The IL1α levels detected in the RO T1D cohort by multiplex cytokine analysis (Table 2) as well as other functional genomics-based investigations of T1D that have implicated innate immunity and IL1 in T1D pathogenesis. In addition to regulating T-cell activity, IL1 promotes β-cell dysfunction through the mitogen-activated protein kinase and nuclear factor kappa B (NFκB) pathways, leading to endoplasmic reticulum and mitochondrial stress and cell death. Blockade of the IL1 receptor with IL1RA can protect β cells from the downstream consequences of IL1 exposure. The clinical efficacy of blocking IL1 action has been observed in many inflammatory diseases, including rheumatoid arthritis and type 2 diabetes mellitus. Furthermore, genetic or pharmacological abrogation of IL1 activity has been reported to delay and/or reduce T1D incidence in rodent models.32,60-62 These findings underlie the rationale for recent efforts to evaluate therapeutic targeting of IL1 in T1D, where we believe this approach may hold utility in monitoring such interventions and yielding additional insight.

Similar to studies that have identified disease-specific signatures from the transcriptomes of PBMC or immune subpopulations, we find that plasma-induced transcription offers a mechanistically informative and disease-specific read out. Relative to T1D, the cytokine milieu associated with CF, bacterial pneumonia and H1N1 drove distinct transcriptional programs. The innate, IL1 related components of the T1D signature were oppositely regulated in these three inflammatory states and the overall signatures associated with these disorders negatively correlated with the T1D signature. While multiplex analysis of primary cytokines was not carried out in this series from patients with chronic Pa colonization, bacterial pneumonia or H1N1, the number of regulated transcripts and fold-change in induced gene expression are consistent with these conditions being more robust inflammatory pathologies of larger tissues. It is important to note that although the genes differentially regulated among the other conditions have varying roles in pathologic pulmonary inflammation, as a group these genes participate in immune recognition and response, phagocytosis and matrix degradation.

Overall, these studies validate the remarkable capacity of cryopreserved cells to serve as disease-specific biosensors that are capable of sensitively and comprehensively capturing the plasticity of the immune system and differentiating the diverse
range of inflammatory processes that underlie human disease. Ongoing studies include analyses of our methodology to other autoinflammatory disorders that are mechanistically more related to T1D; we anticipate that these investigations will more rigorously test the disease-specificity of our approach within the context of autoimmunity.

**MATERIALS AND METHODS**

Subjects and subject characterization

T1D patients were recruited through the Diabetes Clinic at Children's Hospital of Wisconsin and diabetes was defined according to World Health Organization criteria. Samples of normoglycemic RO T1D patients (n = 47; mean age 9.27 ± 2.89 years; mean glycosylated hemoglobin (HgA1c) fraction 7.47 ± 1.20%) were collected after stabilization on exogenous insulin 2–7 months after diagnosis. Samples from LS T1D patients (n = 11; mean age 28.20 ± 9.29 years, mean HgA1c fraction 7.91 ± 1.34%) were collected from well-controlled normoglycemic subjects that were > 10 years post-onset. The recruitment criteria for unrelated Caucasian HC (n = 44; mean age 14.98 ± 4.13 years) included fasting blood glucose levels < 100 mg dL-1, no familial history of any autoimmune/autoinflammatory disorder, <25 years of age and negativity for islet autoantibodies at the 99th percentile. All HC and T1D study subjects were free of known infection at the time of sample collection and had peripheral blood drawn aseptically and collected in acid citrate dextrose solution A or K + EDTA anti-coagulant at Children's Hospital of Wisconsin by trained phlebotomists. Blood components were immediately separated by Ficol-Histopaque (Sigma Aldrich, St Louis, MO, USA) density gradient centrifugation, and plasma was stored at –80 °C until use. GAD, IA2, IAA and ZNT8 autoantibody titers were determined as described. HLA-DQB1 genotyping in T1D and HC subjects was performed via direct sequencing of the second exon with the SeqCore DQB1 Loops Sequencing Kit (Invitrogen Life Technologies, Grand Island, NY, USA) as per the manufacturer's instructions. HLA-DQA1–DQB1 haplotypes were inferred using reported European Caucasian haplotype frequencies. Subject characteristics are shown in Supplementary Table 1A.

The plasma of 20 pediatric CF patients (mean age 14.87 ± 5.54 years) and 24 age-matched, unrelated HC (mean age 15.17 ± 5.25 years; Supplementary Table 1B) was aseptically collected in acid citrate dextrose solution A or K + EDTA anti-coagulant. The diagnosis of CF was documented in the medical record based on the results of the pilocarpine iontophoresis sweat test (sweat chloride > 60 meq L-1). Clinical serum marker levels and lung function measurements confirmed that the values were performed at baseline and not during a pulmonary exacerbation defined as a forced expired volume change of > 15% predicted. At the time of sample collection, all patients were screened to identify microbiological flora; all subjects harbored mucoid Pa, as Pa was cultured from sputum or oropharyngeal swab for 6 consecutive months. All subjects were homozygous for the F508del mutation and were compatible with pneumonia including one of the following three criteria: tachypnea (respiratory rate > 2 standard deviations from the mean for age), dyspnea or hypoxemia (pulse oximetry > 94% on room air on initial evaluation without a known mixing heart lesion). Plasma was collected within 24 h of admission to the Pediatric Intensive Care Unit at the Children's Hospital of Wisconsin was aseptically collected in K + EDTA anti-coagulant. Community-acquired pneumonia was defined using previously published guidelines and included: (1) acute illness (< 14 days of symptoms), (2) the presence of a new chest radiographic infiltrate or consolidation confirmed by a radiologist and (3) clinical features compatible with pneumonia including one of the following three features: fever > 37.8 °C, hypothermia < 36 °C, peripheral white blood count > 10,000 µL or < 4500 µL or > 15% immature neutrophils; plus two of the following three criteria: tachycardia (respiratory rate > 2 standard deviations from the mean for age), dyspnea or hypoxemia (pulse oximetry > 94% on room air on initial evaluation without a known mixing heart lesion). Plasma was collected within 24 h of admission to the Pediatric Intensive Care Unit and analyzed as described below. These children all had confirmed bacterial pneumonia; 8/10 required mechanical ventilation for acute respiratory failure. These subjects were compared with a cohort of unrelated HC (mean age 12.92 ± 3.89 years). Subject characteristics are shown in Supplementary Table 1C.

We also examined plasma from five healthy middle-aged (mean age 49.60 ± 2.07 years), HLA-A2.1 blood donors. Samples were collected before influenza infection, and at the onset of flu-like symptoms, subjects were re-drawn and H1N1 status was ascertained by PCR-based testing of a nasal swab. The blood samples were collected as part of a protocol approved by the Institutional Review Board of the Blood Center of Wisconsin. The characteristics of these subjects appear in Supplementary Table 1D.

This study was approved by the Institutional Review Board of the Children's Hospital of Wisconsin and informed consent was obtained from subjects or parents/legal guardians.

**PBMC cultures and gene expression analysis**

Commercial cryopreserved PBMCs from a Caucasian HLA-A2 male donor were thawed and washed as per the manufacturer's protocol (UPN727, Cellular Technology Ltd., Shaker Heights, OH, USA). Gene expression was accomplished by culturing PBMCs at 37 °C in 5% CO2 with either 20 or 40% of autologous, HC, RO T1D, LS T1D, longitudinal pre-T1D, CF, pneumonia or H1N1 plasma. Cultures were prepared in a Costar 24-well plate (Corning, Corning, NY, USA) using 500,000 cells per well and in RPMI 1640 medium supplemented with 100 U ml-1 penicillin and 100 µg ml-1 streptomycin in a total volume of 500 µL. After culture (9 h), total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies). Using purified total RNA (100 ng), cRNA was synthesized and amplified/labeled using the Affymetrix Express Kit, then fragmented and hybridized to the GeneChip Human Genome U133 plus 2.0 array in accordance with the Affymetrix GeneChip expression analysis technical manual (Affymetrix, Santa Clara, CA, USA). After hybridization, arrays were washed and stained with Affymetrix fluids protocol FS5450_0001 and scanned with a 7G Affymetrix GeneChip Scanner. Image data were analyzed with Affymetrix Expression Console 1.1.2 software (Affymetrix) and normalized with Robust Multichip Analysis (www.biocductor.org) to determine signal log ratios.

The statistical significance of differential gene expression was determined though ANOVA and false discovery rates (FDR) using Partek Genomics Suite 6.5 (Partek Inc., St Louis, MO, USA). Hierarchical clustering was conducted with Genesis. Pathway analysis was performed with the DAVID7,9,10 Longitudinal samples collected from progressors to T1D were analyzed with Short Time-series Expression Miner version 1.3.8 (STEM), software for the analysis of time-series gene expression data (3–8 time points). Data sets were also analyzed with ToppGene, which prioritizes genes based on functional similarity using information on gene expression, protein domains and interactions, transcription factor binding sites, miRNAs, ontologies, human disease, and mouse phenotypes, drug–gene associations, and literature co-citation. The data generated in this investigation are MIAME compliant and have been deposited in the NCBI Gene Expression Omnibus, accessible through GEO Series accession numbers GSE35725 (T1D data), GSE35713 (CF data), GSE35716 (pneumonia data) and GSE35712 (H1N1 data).

Direct detection of inflammatory mediators

Plasma from RO T1D and HC subjects were assayed with the BeadLyte cytokine assay kit (Millipore, Billerica, MA, USA) as per the manufacturer's protocol and a Bio-Plex Lumineq 100 XFP instrument. Concentrations for Eotaxin, granulocyte-macrophage colony stimulating factor, IFNγ, IL1α, IL-1β, IL2, IL3, IL4, IL5, IL7, IL8, IL10, IL12p40, IL12p70, IL13, IL15, IL17, IP-10, monocyte chemotactic protein-1, MIP-1α, MIP-1β, TNF-α and TNF-β were calculated with the Bio-Plex Manager 4.1 software; a five-parameter curve-fitting algorithm was applied for standard curve calculations.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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REFERENCES

1 Jenner RG, Young RA. Insights into host responses against pathogens from transcriptional profiling. Nat Rev Microbiol 2005; 3: 281–294.

2 Lewis CC, Yang JY, Huang X, Banerjee SK, Blackburn MR, Baluk P et al. Disease-specific gene expression profiling in multiple models of lung disease. Am J Respir Crit Care Med 2006; 177: 376–387.

3 Medzhitov R, Janeway Jr C. Innate immune recognition: mechanisms and pathways. Immunol Rev 2000; 173: 89–97.

4 Elsa G, Dunn-Siegrist I, Daubeuf B, Pugin J. Contribution of Toll-like receptors to the innate immune response to Gram-negative and Gram-positive bacteria. Blood 2007; 109: 1574–1583.

5 Underhill DM, Ozinsky A. Toll-like receptors: key mediators of microbe detection. Curr Opin Immunol 2002; 14: 103–110.

6 Nau GJ, Schlesinger A, Richmond JF, Young RA. Cumulative Toll-like receptor activation in human macrophages treated with whole bacteria. J Immunol 2003; 170: 5203–5209.

7 Toshchakov V, Jones BW, Perera PY, Thomas K, Cady MJ, Zhang S et al. TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. Nat Immunol 2002; 3: 392–398.

8 Huang Q, Liu D, Majewski P, Schulte LC, Korn JM, Young RA et al. The plasticity of dendritic cell responses to pathogens and their components. Science 2001; 294: 870–875.

9 Lempicki RA, Polis MA, Yang J, McLaughlin M, Koratich C, Huang DW et al. Gene expression profiles in hepatitis C virus (HCV) and HIV coinfection: class prediction analyses before treatment predict the outcome of anti-HCV therapy among HIV-coinfected persons. J Infect Dis 2006; 193: 1172–1177.

10 Thach DC, Agan BK, Olsen C, Diao J, Lin B, Gomez J et al. Surveillance of transcriptionomes in basic military trainees with normal, febrile respiratory illness, and convalescent phenotypes. Genes Immun 2005; 6: 588–595.

11 Ramilo O, Allman W, Chung W, Mejias A, Ardura M, Glaser C et al. An interferon signature in the peripheral blood of patients undergoing beta-interferon therapy. J Mol Diagn 2006; 8: 51–61.

12 Stoeckman AK, Baechler EC, Ortmann WA, Behrens TW, Michet CJ, Peterson EJ. A distinct inflammatory gene expression profile in patients with psoriatic arthritis. Genes Immun 2006; 7: 583–591.

13 Greenberg SA, Pinkus JL, Pinkus GS, Burleson T, Sanoudou D, Tawell R et al. Interferon-alpha/beta-mediated innate immune mechanisms in dermatomyositis. Ann Neurol 2005; 57: 664–678.

14 Baechler EC, Bauer JW, Slattery CA, Ortmann WA, Esp KJ, Nowitzke I et al. An interferon signature in the peripheral blood of dermatomyositis patients is associated with disease activity. Mol Med 2007; 13: 59–68.

15 Pascual V, Allantaz F, Arce E, Munaro P, Banchereau J. Role of interleukin-1 (IL-1) in the pathogenesis of systemic onset juvenile idiopathic arthritis and clinical response to IL-1 blockade. J Exp Med 2005; 201: 1479–1486.

16 Allantaz F, Chassabel D, Stichweh D, Bennett L, Allman W, Mejias A et al. Blood leukocyte microarrays to diagnose systemic onset juvenile idiopathic arthritis and follow the response to IL-1 blockade. J Exp Med 2007; 204: 2131–2144.

17 Wang X, Sal, Geoffrey R, Alemazedi 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: 1929–1937.

18 Kaldunski M, Jia S, Geoffrey R, Basken J, Prosser S, Kansra S et al. Identification of a serum-induced transcriptional signature associated with type 1 diabetes in the BioBreding rat diabetes. Diabetologia 2010; 59: 2375–2385.

19 Jenner RG, Young RA. The ‘honeymoon phase’ response in children with type 1 diabetes mellitus: frequency, duration, and influential factors. Pediatr Diabetes 2006; 7: 101–107.

20 Bonfanti R, Bazzizaluppi E, Calori G, Riva MC, Viscardi M, Bognetti E et al. Parameters associated with residual insulin secretion during the first year of disease in children and adolescents with type 1 diabetes mellitus. Diabet Med 1998; 15: 844–850.

21 Bonfanti R, Bognetti E, Meschi F, Brnelli A, Riva MC, Pastore MR et al. Residual beta-cell function and spontaneous clinical remission in type 1 diabetes mellitus: the role of puberty. Acta Diabetol 1999; 35: 91–95.

22 Mooser B, Wolf M, Walz A, Loetscher P. Chemokines: multiple levels of leukocyte migration control. Trends Immunol 2004; 25: 75–84.

23 Chen J, Xu H, Aronov BJ, Jegga AGA. Improved human disease candidate gene prioritization using mouse phenotype. BMC Bioinformatics 2007; 8: 392.

24 Chen J, Bardees EE, Aronov BJ, Jegga AGA. ToppGene Suite for gene list enrichment analysis and candidate gene prioritization. Nucleic Acids Res 2009; 37(Web Server issue): W305–W311.

25 Ernst J, Bar-Joseph Z. STEM: a tool for the analysis of short time series gene expression data. BMC Bioinformatics 2006; 7: 191.

26 Terenzi F, White C, Pal S, Williams BR, Sen GC. Tissue-specific and inducer-specific differential induction of ISG56 and ISG54 in mice. J Virol 2007; 81: 8656–8665.

27 Haendley SA, Due PH, Madsen OL. Histamine signaling through the H2R receptor of the Peyer’s patch is important for controlling Yersinia enterocolitica infection. Proc Natl Acad Sci USA 2006; 103: 9268–9273.

28 Ziegler AG, Nepom GT. Prediction and pathogenesis in type 1 diabetes. Immunity 2010; 32: 468–478.

29 Yu J, Yu L, Bugawan TL, Erlich HA, Harriga K, Hoffmann M et al. Transient antilistet autoantibodies: insufficient occurrence and lack of association with ‘genetic’ risk factors. J Clin Endocrinol Metab 2005; 90: 2421–2428.

30 Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. Lancet 2001; 358: 221–229.

31 Devendra D, Liu E, Eisenbarth GS. Type 1 diabetes: recent developments. BMJ 2004; 328: 750–754.

32 Skyler JS. Prediction and prevention of type 1 diabetes: progress, problems, and prospects. Clin Pharmacol Ther 2007; 81: 768–771.

33 Roep BO, Peukman M. Surrogate end points in the design of immunotherapy trials: emerging lessons from type 1 diabetes. Nat Rev Immunol 2010; 10: 145–152.

34 Hussain MJ, Peukman M, Gallant H, Lo SS, Hava M, Viberti GC et al. Elevated serum levels of macrophage-derived cytokines precede and accompany the onset of IDDM. Diabetologia 1996; 39: 60–69.

35 Perez F, Oyarzun A, Carasco E, Angel B, Albala C, Santos JL. Plasma levels of interleukin-1beta, interleukin-2, and interleukin-4 in recently diagnosed type 1 diabetic children and their association with beta-pancreatic autoreactivities. Rev Med Chile 2004; 132: 413–420.

36 Embagaci AB, Taracicil M, Coskun Y, Sivasi E, Biberd Namiduru E. Mediators of inflammation in children with type 1 diabetes mellitus: cytokines in type 1 diabetic children. Clin Biochem 2001; 34: 645–650.
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