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Transcriptional Signatures as a Disease-Specific and Predictive Inflammatory Biomarker for Type 1 Diabetes

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Abstract

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 immunocyte 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

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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.

Keywords

Type 1 diabetes; Cystic Fibrosis; Influenza; Gene expression profiling; Biomarker; Inflammation

INTRODUCTION

Many diseases arise from uncontrolled inflammatory processes and become evident after the progression of significant tissue and organ damage. These diseases include auto-inflammatory 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 colitis[25]), 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 idiopathic arthritis and we have applied it to T1D[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[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 *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[31]. In longitudinal samples collected from progressors to diabetes, this T1D signature was evident as much as 5 years prior to onset and prior to the emergence of auto-antibodies toward islet cell antigens, which are currently considered to be the best predictor of progression to diabetes[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 IL-1. Blocking the IL-1 receptor by treating BioBreeding rats with IL-1 receptor antagonist (IL-1RA) 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[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 blood donors, we found that the plasma samples from a given subject cohort induced relatively homogenous profiles[31] with modest donor cell-specific effects. Commercially available cryopreserved PBMCs are a relatively new option for cell-based assays. These cells are collected via aphaeresis 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 a larger cohort of 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[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[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[31], plasma drawn from 47 RO T1D patients (mean age 9.97 ± 2.89 years, Supplemental Table 1) and 44 unrelated HC subjects (mean age 14.98 ± 4.13 years, Supplemental 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 ($|\log_2 \text{ratio}|>0.263$, 1.2-fold; FDR<0.2; ANOVA $p<0.036$); these represented 622 unique UniGenes (Supplemental Table 2). The relationship of this dataset 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[31] (498 probe sets $|\log_2 \text{ratio}|>0.5$; FDR<0.2) is illustrated in Figure 1A. The Pearson correlation coefficient between the union of these two datasets 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 DAVID to identify regulated Gene Ontology Biological Processes. Representative pathway terms appear in Table 1 (a complete list is provided in Supplemental 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 IL-6 production. These categories included genes encoding immune signaling molecules and receptors including the IL-1 cytokine family members *IL1*, *IL1RN*, *IL1R1*, and *IL1R2*, the IL-10 family member *IL24*, and the chemokines *CXCL1*, *CXCL2*, *CXCL3*, and *CXCL5*, which are involved in neutrophil chemotaxis[38]. RO T1D plasma induced cyclooxygenase-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 *TLR2*, *FCAR*, *LILRA3*, and *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, BMP signaling pathway, negative regulation of T cell proliferation (Table 2 and Supplemental Table 3). Relative to HC plasma, culture with RO T1D plasma led to the under-expression of genes such as *KLF12*, *SKIL* (a TGF-β-induced gene that negatively regulates TGF-β signaling), the histone deacetylase *HDAC3*, *FOXP1* (a transcription factor that is important in maintaining immune quiescence), and *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 pro-inflammatory mediators such as IL-1 and lower levels of anti-inflammatory factors.

Finally, we employed ToppGene[39, 40] to further explore the functional commonality in the originally reported response of multiple fresh PBMC to RO T1D and HC plasma compared to those of cryopreserved PBMC using the two datasets 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 pro-inflammatory 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 to HCs, including IL-1α, which is consistent with the induction of IL-1-mediated gene expression. However, with the exception of TNFα (RO T1D: 9.3 ± 1.0 pg/ml; HC: 6.5 ± 0.6 pg/ml; $p<0.05$), none of these differences reached statistical significance with the number of subjects analyzed. Furthermore, correlations between the intensity of the signature among individual samples (Figure 1B and 1C) and mediator levels, antibody levels, gender, age of onset, or time to post-onset sample collection could not be made.

Analysis of Long-Standing (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 auto-antibody. 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 ($|\log_2 \text{ratio}|>0.263$, 1.2-fold; FDR<0.2) when comparing the LS T1D and HC groups. Consistent with our previous report[31], 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 diagnosed at the age of 21.7 years, and samples were collected at −5.3 years (auto-antibody negative), −3.3 years (+1 auto-antibody), −2.4 years (+3 auto-antibodies), −1.5 years (+3 auto-antibodies), −0.3 years (+3 auto-antibodies), and +0.3 years (+4 auto-antibodies) relative to onset (Supplemental Table 1). The response of cryopreserved PBMCs to the plasma of this longitudinal series was analyzed from the perspective of probe 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[41].

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 to those identified by STEM analysis of this single longitudinal series (n=1,278 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 ($p<10^{-51}$, X^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 time point $\log_2$ ratio for the longitudinal series) for the union and intersection of these two datasets were 0.55 and 0.78, respectively. The regulated 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 prior to onset of T1D in the longitudinal analysis are distinct from the samples of unrelated HC examined in the cross-sectional analyses (Figure 2B and Supplemental 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[31]. Among the 2,319 probe sets differentially expressed between the analyses of the longitudinal series using fresh versus cryopreserved cells, we identified a shared, significantly nonrandom ($p<10^{-57}$, X^2 test), correlative (Pearson correlation coefficient = 0.63), commonly regulated intersection of 154 probe sets. We again employed ToppGene[39, 40] to examine the functional similarity in the responses of fresh and cryopreserved PBMCs to plasma samples from the longitudinal series. Using the 1,195 probe sets differentially expressed in fresh cells as the training set, we identified 885/1,278 (69.3%) significant ($p<0.01$), functionally related probe sets among those regulated in cryopreserved cells.

As with fresh PBMCs[31], we observed an increase in the robustness and complexity of the signature with disease progression (Figure 2C). Genes such as 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 prior to onset, prior to autoantibody development, and overall the signature of all pre-onset samples was distinct from that of the mean response of the 44 unrelated healthy controls 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 1,365 and lowest 1,365 expressed probe sets). We observed a difference of 0.72 ± 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[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 colonized 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 and 24 age-matched unrelated HCs were used to induce transcription in UPN727 cells at a culture concentration of 20% (Supplemental Table 1). A robust signature consisting of 5,000 differentially expressed probe sets ($|\log_2$ ratio$|>0.263$, 1.2-fold; FDR<0.2; 3,404 unique UniGenes) was distinct from that induced by RO T1D plasma (Figure 3, Supporting Information Table 2). Ontological analysis of the 2,139 probe sets up-regulated by CF plasma vs 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 INF regulatory factor 1 (*IRF1*), *GIMAP1*, *GIMAP5*, *TLR10*, *IL32*, *CCL5*, *CD40*, *IKZF1*, *IKAROS* family zinc finger 1 (*IKZF1*), *IL15*, and *IL16*. Analysis of the 2,861 probe sets down-regulated by CF plasma identified 238 significant Gene Ontology Biological Processes ($p<0.01$; FDR<10%). Many Biological Processes up-regulated by T1D sera were found down regulated by CF sera (Table 1B). Accordingly, many IL-1 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 2B, and the complete list is provided in Supplemental 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. 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=8,121; pneumonia:HC $|\log_2$ ratio$|>0.263$; FDR<0.20; 4,410 up-regulated probe sets and 3,711 down-regulated probe sets; 5,650 unique UniGenes; Supplemental 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 3,034 probe sets that had pneumonia:HC $|\log_2$ ratio$|>0.5$ and FDR<0.20 (2,144 unique UniGenes).

Selected pathway terms appear in Table 1C; a complete list is provided in Supplemental Table 3.

Ontological analysis of the 1,515 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 (*SESN1*), cullin 5 (*CUL5*; an inhibitor of cellular proliferation), tumor protein p53 inducible nuclear protein 1 (*TP53INP1*), junction mediating and regulatory protein p53 cofactor (*JMY*), and others (Figure 3C). Ontological analysis of the down-regulated 1,519 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 (*PANX1*; involved with IL1-β release), *IL6R*, *CCL4*, the endothelial protein C receptor (*PROCR*), protectin (CD59; a complement regulatory protein), coagulation factor V (*F5*), thrombomodulin (*THBD*), and others (Figure 3).

Lastly, samples collected from 5 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 α and γ INF production. Selected pathway terms are tabulated in Table 1D, and the complete list appears in Supplemental Table 3.

Notably, sera collected during H1N1 infection induced transcription of numerous IFN-regulated genes, including INF-induced protein with tetratricopeptide repeats 1 (*IFIT1*), *IFIT5*, *IFITM1*, and *IFITM3*. Members of this gene family are induced in response to type I and type II INF, dsRNA, and endotoxin[42, 43]. Sera of H1N1-infected individuals induced expression of other IFN-regulated genes including *IFI6* and *MX1*, and INF-stimulated exonuclease gene 20kDa (*ISG20*), which encodes an anti-viral ssRNA-specific exonuclease. Also induced by H1N1 sera were 2',5'-oligoadenylate 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, 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 regulated. Correlation 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 to 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 β-cell destruction occur asymptomatically for months to years prior to onset of T1D, providing an excellent opportunity for early diagnosis and intervention. The evaluation of auto-antibody status in combination with HLA genotyping can reliably predict susceptibility to T1D[44]. However, auto-antibodies are thought to appear late during pre-diabetes, their levels can be transient, and not all auto-antibody 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 auto-antibodies are markers of β-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 explored the use of lymphoid and myeloid cell lines as potential PBMC surrogates[33]. 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[31] 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 5 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[31]. We observed >90% post-thaw viability with UPN727 cells based upon trypan blue exclusion, and multi-parameter flow cytometry has shown that the relative abundances of natural killer cells, CD8+ T cells, CD4+ T cells, B cells, and monocytes do not significantly differ from the normal expected ranges for fresh PBMCs (Supplemental 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 5 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 5 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 analysis which possessed a larger number of subjects compared to our initial report[31]. Furthermore, some of the less distinct RO T1D signatures likely arose through post-onset resolution of the autoimmune response similar to that observed the LS T1D cohort. Ongoing studies with this bioassay show that resolution of the immune response occurs during the first year post-onset (data not shown).

In this study, using cryopreserved cells, we re-examined a single longitudinal series studied in our previous report[31], confirming temporal changes in the inflammatory state preceding T1D onset. Furthermore, a concordant response was induced in the cryopreserved PBMCs (40% plasma) that exhibited an overall fold-change similar to that of freshly drawn PBMCs (20% plasma). The elimination of inter-individual heterogeneity is an advantage of longitudinal analyses compared to cross-sectional studies. This benefit is highlighted by the observation that, in general, for any regulated gene, the fold-change detected in the longitudinal study tended to be larger than that observed between cohorts in the cross-sectional study. For example, at onset a >10-fold change in *IL-6* and *PTGS2* expression was observed in the longitudinal analysis versus a <2-fold mean change observed in the cross-sectional study. The maximal individual fold-changes in *IL-6* and *PTGS2* relative to the mean HC response were 2.25-fold 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 T1D onset. Biobanks and associated databases such as the TrialNet Natural History Study[56] and The Environmental Determinants of Diabetes in the Young (TEDDY)[57] will greatly facilitate such efforts. Importantly, our longitudinal analyses of progressors to T1D studies conducted thus far have identified 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 IL-1 were again over-represented in the transcriptional signatures of cells exposed to RO T1D plasma. These genes included *IL1B*, cyclooxygenase type 2 (*PTGS2*), prostaglandin E synthase (*PTGES*), *IL-1R1*, *CXCL1*, *CXCL2*, *CXCL3*, *CXCL5*, *PLAUR*, *CREM*, and others. This observation is, in general, consistent with the elevated plasma IL-1α 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 IL-1 in T1D pathogenesis[58, 59]. In addition to regulating T-cell activity, IL-1 promotes β-cell dysfunction through the mitogen-activated protein kinase and NFκB pathways, leading to endoplasmic reticulum and mitochondrial stress and cell death[60–62]. Blockade of the IL-1 receptor with IL-1RA can protect β cells from the downstream consequences of IL-1 exposure[63]. The clinical efficacy of blocking IL-1 action has been observed in many inflammatory diseases, including rheumatoid arthritis and Type 2 diabetes mellitus[29, 64]. Furthermore, genetic or pharmacological abrogation of IL-1 action has been reported to delay and/or reduce T1D incidence in rodent models[32, 65, 66]. These findings underlie the rationale for recent efforts to evaluate therapeutic targeting of IL-1 in T1D[67–69], 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 immunocyte subpopulations, we find that plasma-induced transcription offers a mechanistically informative and disease-specific read out. Relative to T1D, the cytokine milieus associated with CF, bacterial pneumonia, and H1N1 drove distinct transcriptional programs. The innate, IL-1 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 peripheral cytokines was not carried out in the samples from CF 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 auto-inflammatory 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[70]. Samples of normoglycemic RO T1D patients (n=47; mean age 9.97 ± 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 HCs (n=44; mean age 14.98 ± 4.13 years) included fasting blood glucose levels <100 mg/dl, no familial history of any autoimmune/autoinflammatory disorder, <25 years of age, and negativity for islet auto-antibodies at the 99th percentile[71]. 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 Ficoll-Histopaque (Sigma Aldrich, St. Louis, MO) density gradient centrifugation, and plasma was stored at −80 °C until use. GAD, IA2, IAA, and ZNT8 autoantibody titers were determined as described[71]. *HLA-DQB1* genotyping in T1D and HC subjects was performed via direct sequencing of the second exon with the SeCore DQB1 Locus Sequencing Lit (Invitrogen Life Technologies, Grand Island, NY) per the manufacturer's instructions. *HLA-DQA1-DQB1* haplotypes were inferred using reported European Caucasian haplotype frequencies[72]. Subject characteristics are shown in Supplemental Table 1A.

The plasma of 20 pediatric CF patients (mean age 14.87 ± 5.54 years) and 24 age-matched, unrelated HCs (mean age 15.17 ± 5.25 years; Supplemental 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/lL). 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 greater than 15% predicted. At the time of sample collection, all patients were screened to identify microbiological flora; all subjects harbored mucoid *Pseudomonas aeruginosa* (Pa), as Pa was cultured from sputum or oropharygneal swab for 6 consecutive months[73, 74]. All subjects were homozygous for the F508del *CFTR* mutation and were pancreatic insufficient, determined by stool elastase level, but none were diabetic (fasting blood glucose <120 mg/dl). Subject characteristics appear in Supplemental Table 1B.

The plasma of 10 children (mean age 9.37 ± 6.72 years) with community-acquired pneumonia admitted to the Pediatric Intensive Care Unit at the Children's Hospital of Wisconsin was aseptically collected in K+EDTA anticoagulant. Community-acquired pneumonia was defined using previously published guidelines[75] 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 <4,500/μl or >15% immature neutrophils; plus two 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 hours 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 to a cohort of unrelated HCs (mean age 12.92 ± 3.89 years). Subject characteristics are shown in Supplemental 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 prior to 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 Supplemental 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 per the manufacturer's protocol (UPN727, Cellular Technology Ltd., Shaker Heights, OH). Gene expression was accomplished by culturing PBMCs at 37 °C in 5% CO_2 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) using 500,000 cells/well and in RPMI 1640 medium supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin in a total volume of 500 μl. After culture (9 hours), 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). After hybridization, arrays were washed and stained with Affymetrix fluidics protocol FS450_0001 and scanned with a 7G Affymetrix GeneChip Scanner. Image data were analyzed with Affymetrix Expression Console™ 1.1.2 software and normalized with Robust Multichip Analysis (www.bioconductor.org) to determine signal log ratios.

The statistical significance of differential gene expression was determined though ANalysis Of VAriation (ANOVA) and false discovery rates (FDR) using Partek Genomics Suite 6.5. Hierarchical clustering was conducted with Genesis[76]. Pathway analysis was performed with the Database for Annotation, Visualization, and Integrated Discovery (DAVID)[77, 78]. 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)[41]. Datasets were also analyzed with ToppGene[39, 40], 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[79] and have been deposited in the NCBI Gene Expression Omnibus[80], 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) per the manufacturer's protocol and a Bio-Plex Luminex 100 XYP instrument. Concentrations for Eotaxin, GM-CSF, interferon (IFN) α2, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IP-10, MCP-1, MIP-1a, MIP-1b, TNFα, and TNFβ were calculated with the Bio-Plex Manager 4.1 software; a 5-parameter curve-fitting algorithm was applied for standard curve calculations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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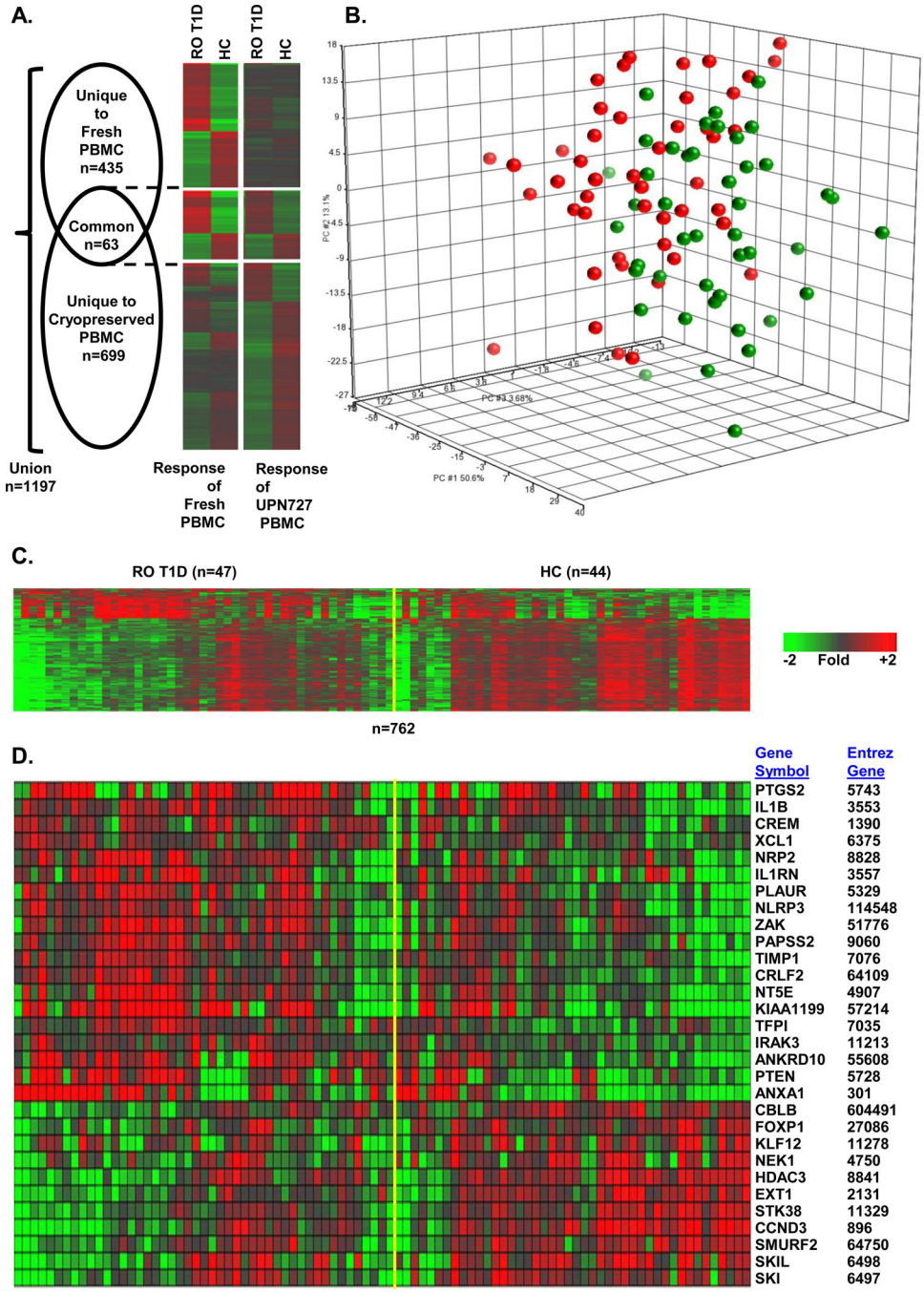

Figure 1.

Cross-sectional analysis of RO T1D patients identifies an inflammatory signature relative to unrelated HC subjects. (A) Venn diagram and one-way hierarchical clustering (probe sets only) for each component of the Venn diagram illustrate the relationship between the mean expression of probe sets regulated when fresh cells were previously cultured with plasma from 12 RO T1D and 12 HC subjects (498 probe sets $|\log_2 \text{ratio}|>0.5$; FDR<0.2) versus culturing UPN727 cells with plasma from 47 RO T1D and 44 HC subjects (n=762; ($|\log_2 \text{ratio}|>0.263$, 1.2-fold; FDR<0.2; ANOVA p<0.036). (B) PCA using 762 differentially

regulated probe sets RO T1D vs HC) in cross-sectional studies. Green spheres, HCs; red spheres, RO T1D subjects. (C) One-way hierarchical clustering (probe sets only) of the RO T1D and HC expression profiles using the 762 regulated probe sets. (D) Relative expression levels of selected, well-annotated genes reflective of innate immune activity in RO T1D patients relative to HC subjects; additional well-annotated genes appear in Figure 3C. Fold of change is expressed relative to the mean of all samples.

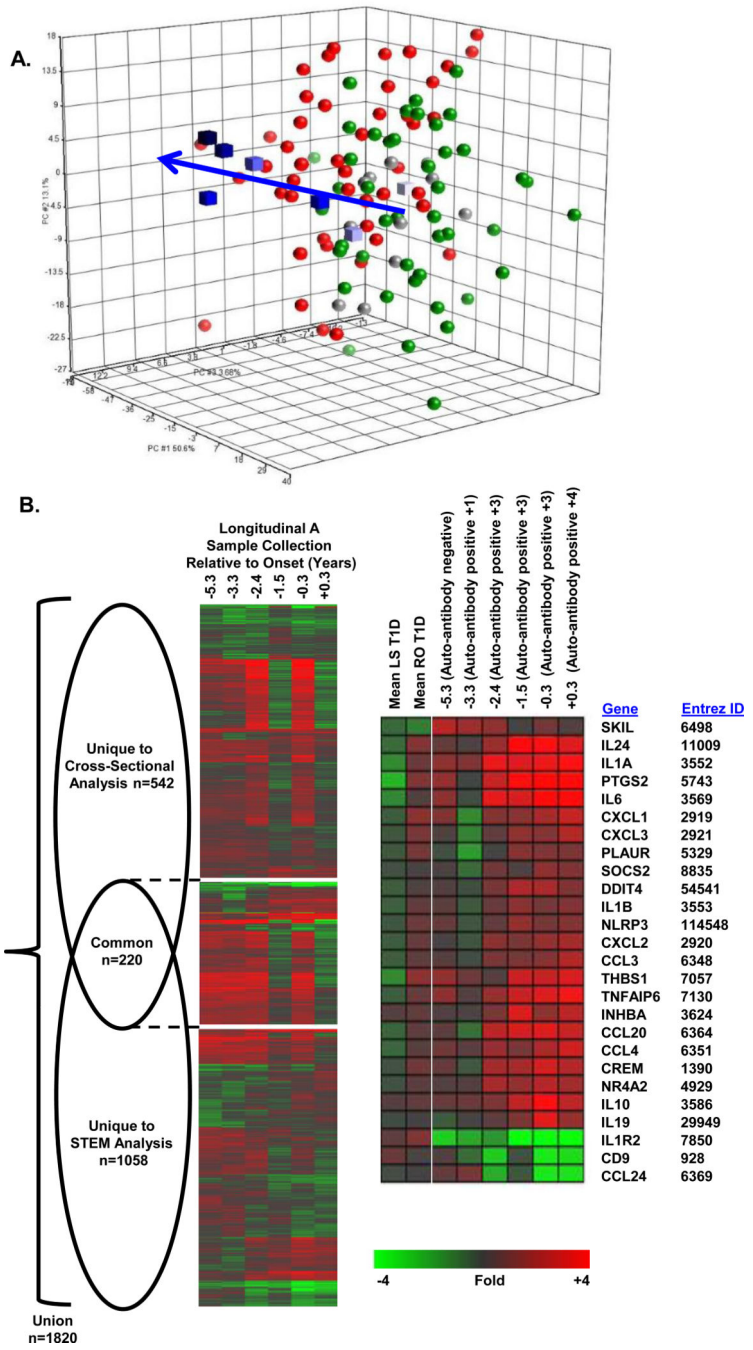

Figure 2.

Analysis of T1D progression in Longitudinal Subject A. (A) PCA using the 762 probe sets regulated by RO T1D (n=47) vs HC (n=44) plasma identified in the cross-sectional studies (|$\log_2$ ratio|>0.263; FDR<0.2). Green spheres, HC; red spheres, RO T1D; grey 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 ($p<10^{-51}$, X^2 test), commonly regulated intersection of 220 probe sets (Supplemental 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.

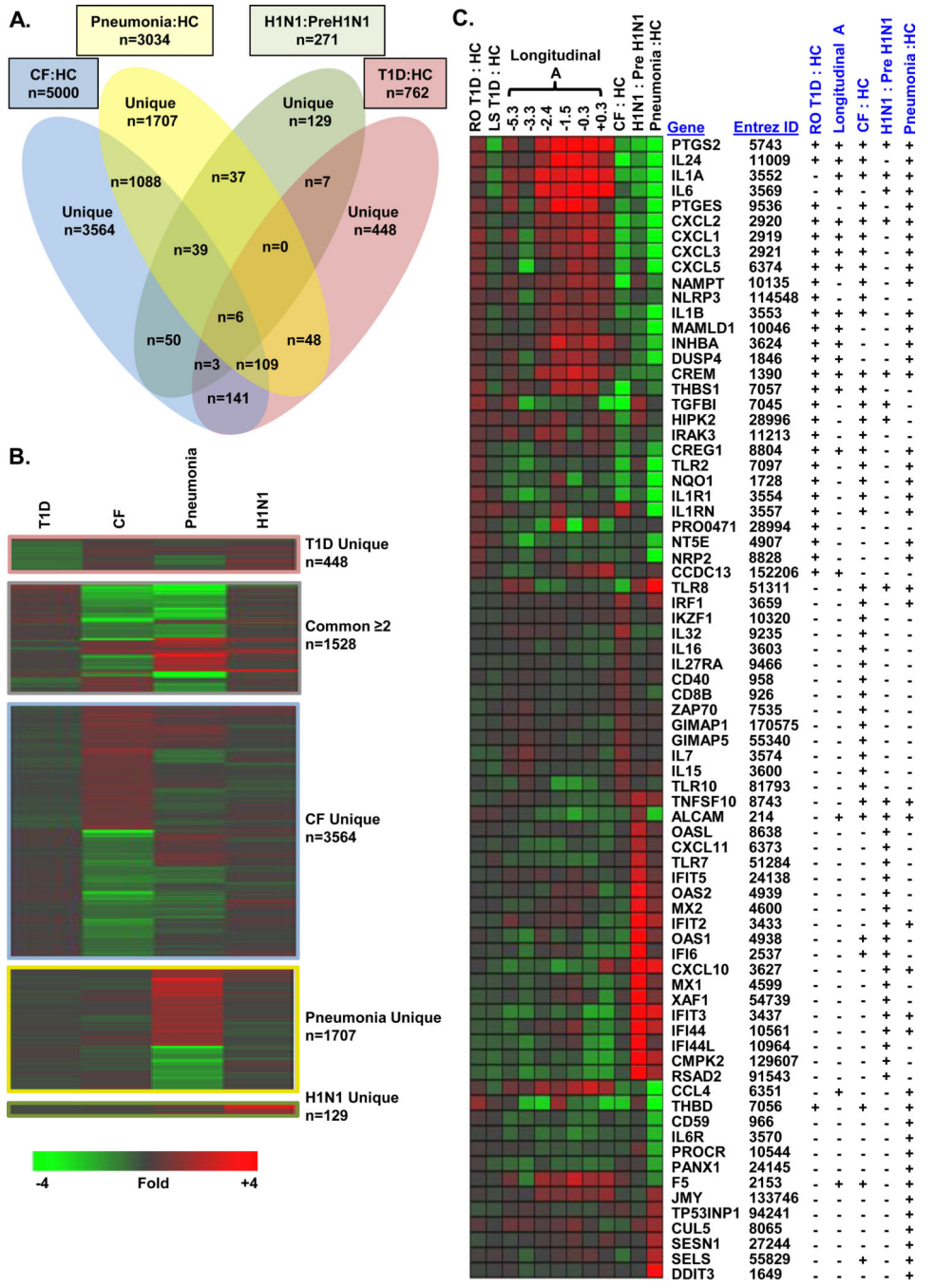

Figure 3.

Distinctiveness of signatures associated with T1D, CF plus Pa colonization, and H1N1. (A) Venn diagram of the probe sets induced in UPN727 cells following exposure to the plasma from T1D patients (n=47) versus age-matched unrelated HCs (n=44; $|\log_2 \text{ratio}|>0.263$; FDR<0.20), CF patients harboring Pa (n=20) versus age-matched HCs (n=24; $|\log_2 \text{ratio}|>0.263$; FDR<0.20), patients with bacterial pneumonia (n=10) versus HCs (n=18; $|\log_2 \text{ratio}|>0.5$; FDR<0.20), and active versus pre-H1N1 infection (five subjects sampled during and before infection; $|\log_2 \text{ratio}|>0.5$; paired *t*-test p<0.05). (B) One-way hierarchical clustering

(probe sets only) was conducted for each component of the Venn diagram using mean expression values for each dataset. (C) Well-annotated, differentially expressed genes related to immunological activation, signal transduction, or transcriptional regulation.

Table 1

Enrichment of gene ontology (GO) terms in differentially expressed probe sets.[a]

GO Identifier[b]	Biological Process	n[c]	p value	FDR (%)
A. Probe sets regulated by RO T1D versus HC plasma				
↑ GO:0006955	Immune response	21	3.28E-7	5.40E-4
↑ GO:0006935	Chemotaxis	11	7.47E-7	1.22E-3
↑ GO:0001819	Positive regulation of cytokine production	7	1.10E-4	0.18
↑ GO:0009617	Response to bacterium	9	1.40E-4	0.23
↑ GO:0043405	Regulation of MAP kinase activity	7	1.08E-3	1.76
↑ GO:0007166	Cell surface receptor linked signal transduction	25	1.25E-3	2.03
↑ GO:0032680	Regulation of TNF production	4	2.29E-3	3.70
↑ GO:0032675	Regulation of IL-6 production	4	3.53E-3	5.64
↓ GO:0050672	Negative regulation of lymphocyte proliferation	6	1.31E-3	2.20
↓ GO:0016481	Negative regulation of transcription	24	1.75E-3	2.93
↓ GO:0030509	BMP signaling pathway	6	4.47E-3	7.34
↓ GO:0045892	Negative regulation of transcription, DNA-dependent	19	4.77E-3	7.81
↓ GO:0042130	Negative regulation of T cell Proliferation	5	4.92E-3	8.05
↓ GO:0016568	Chromatin modification	16	5.27E-3	8.60
B. Probe sets regulated by CF versus HC plasma				
GO Identifier[b]	Biological Process	n[c]	p value	FDR (%)
↑ GO:0006955	Immune response	104	3.34E-12	6.06E-9
↑ GO:0042110	T cell activation	35	1.77E-10	3.22E-7
↑ GO:0002521	Leukocyte differentiation	34	2.83E-9	5.14E-6
↑ GO:0042113	B cell activation	17	3.33E-4	0.60
↓ GO:0009617	Response to bacterium	39	5.02E-8	9.30E-5
↓ GO:0001819	Positive regulation of cytokine production	24	9.46E-6	0.02
↓ GO:0043405	Regulation of MAP kinase activity	28	2.48E-4	0.45
↓ GO:0007166	Cell surface receptor linked signal transduction	25	1.25E-3	2.03
C. Probe sets regulated by bacterial pneumonia versus HC plasma.				
GO Identifier[b]	Biological Process	n[c]	p value	FDR (%)
↑ GO:0006955	Immune response	74	6.72E-8	1.20E-4
↑ GO:0010942	Positive regulation of cell death	47	1.27E-4	0.23
↑ GO:0034620	Cellular response to unfolded protein	8	1.64E-4	0.29
↑ GO:0007050	Cell cycle arrest	16	1.36E-3	2.41
↑ GO:0045582	Positive regulation of T cell differentiation	8	2.64E-3	4.62
↓ GO:0043066	Negative regulation of apoptosis	55	3.18E-11	5.83E-8
↓ GO:0007596	Blood coagulation	22	2.26E-7	4.14E-4
↓ GO:0050715	Positive regulation of cytokine secretion	7	1.27E-3	2.31
D. Probe sets regulated by pre-H1N1 versus active H1N1 plasma				
GO Identifier[b]	Biological Process	n[c]	p value	FDR (%)

GO:0009615	Response to virus	24	3.91E-25	6.46E-22
GO:0051607	Defense response to virus	5	4.03E-05	6.64E-2
GO:0030330	DNA damage response, signal transduction by p53 class mediator	4	2.13E-3	3.45
GO:0032647	Regulation of interferon-α production	3	2.65E-3	4.29
GO:0032649	Regulation of interferon-γ production	4	3.22E-3	5.19

[a] Analysis restricted to GO biological processes with ≥3 UniGenes were detected per category, $p<0.01$,and FDR<10%.

[b] 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.

[c] Total probe sets detected.

Table 2

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

Cytokine	RO T1D (pg/ml)	Unrelated HC pg/ml	Fold RO T1D/HC	Lower detection limit (pg/ml)
Eotaxin	104.2 ± 10.3	79.8 ± 25.1	1.3	>3.2 pg/ml
Granulocyte/macrophage CSF	25.9 ± 9.1	7.90 ± 4.3	3.3	>3.2 pg/ml
Interferon-α2	73.9 ± 7.3	54.5 ± 7.2	1.4	>3.2 pg/ml
Interferon-γ	9.8 ± 4.7	5.5 ± 2.6	1.8	>3.2 pg/ml
IL-1a	59.8 ± 24.1	23.4 ± 11.0	2.6	>3.2 pg/ml
IL-1b	7.8 ± 2.4	6.4 ± 2.3	1.2	>3.2 pg/ml
IL-2	2.3 ± 1.2	0.8 ± 0.2	2.9	>3.2 pg/ml
IL-3	0.0 ± 0.0	0.2 ± 0.7	0.0	>3.2 pg/ml
IL-4	9.0 ± 4.0	2.1 ± 1.8	4.3	>3.2 pg/ml
IL-5	1.7 ± 0.6	0.5 ± 0.2	3.2	>3.2 pg/ml
IL-6	8.0 ± 2.9	6.2 ± 2.2	1.3	>3.2 pg/ml
IL-7	14.1 ± 2.7	10.0 ± 2.7	1.4	>3.2 pg/ml
IL-8	14.7 ± 3.4	15.4 ± 4.0	1.0	>3.2 pg/ml
IL-10	11.8 ± 2.9	11.6 ± 4.1	1.0	>3.2 pg/ml
IL-12p40	83.4 ± 22.85	58.9 ± 16.6	1.4	>3.2 pg/ml
IL-12p70	9.5 ± 6.7	1.6 ± 0.7	6.0	>3.2 pg/ml
IL-13	9.5 ± 4.7	9.5 ± 4.8	2.5	>3.2 pg/ml
IL-15	7.4 ± 2.2	7.5 ± 2.4	1.0	>3.2 pg/ml
IL-17	4.8 ± 1.6	6.8 ± 3.1	0.7	>3.2 pg/ml
IP-10	221.8 ± 26.2	242.8 ± 35.4	0.9	>3.2 pg/ml
Monocyte chemoattractant protein -1	252.8 ± 14.1	214.9 ± 22.3	1.2	>16.0 pg/ml
Macrophage inflammatory protein-1a	12.6 ± 4.0	13.0 ± 4.5	1.0	>16.0 pg/ml
Macrophage inflammatory protein-1b	27.2 ± 6.5	13.4 ± 5.3	2.0	>16.0 pg/ml
TNFa	9.3 ± 1.0	6.5 ± 0.6	1.4*	>3.2 pg/ml
TNFb	30.6 ± 12.4	10.0 ± 4.5	3.1	>3.2 pg/ml

Data are means ± standard error (SE, pg/ml). Each sample was tested in duplicate using the Millipore BeadLyte cytokine assay kit.

*p<0.05, two-tailed *t*-test.

Table 3

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

	n=[a]	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)	−0.03 (−0.03)
Bacterial pneumonia	3034			1.00 (1.00)	0.39 (0.16)
H1N1	271				1.00 (1.00)

[a] The total number of regulated probe sets meeting the following thresholds: RO T1D:HC |$\log_2$ ratio|>0.5, FDR<0.20; CF:HC |$\log_2$ ratio|>0.263, FDR<0.20; pneumonia:HC |$\log_2$ ratio|>0.5, FDR<0.20; H1N1:pre-H1N1 |$\log_2$ ratio|>0.5; paired *t*-test p<0.05).

[b] Pearson correlation coefficients for genes in the union of the probe sets differentially regulated under all four conditions (n=7,376) are shown in parentheses.