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Identification of a Molecular Signature in Human Type 1 Diabetes Mellitus Using Serum and Functional Genomics[1]

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Understanding active proinflammatory mechanisms at and before type 1 diabetes mellitus (T1DM) onset is hindered in humans, given that the relevant tissues are inaccessible and pancreatic immune responses are difficult to measure in the periphery by traditional approaches. Therefore, we investigated the use of a sensitive and comprehensive genomics strategy to investigate the presence of proinflammatory factors in serum. The sera of recent onset diabetes patients ($n = 15$, 12 possessing and 3 lacking islet cell autoantibodies), long-standing diabetes patients ($n = 12$), "at risk" siblings of diabetes patients ($n = 9$), and healthy controls ($n = 12$) were used to induce gene expression in unrelated, healthy PBMC. After culture, gene expression was measured with microarrays and normalized expression data were subjected to hierarchical clustering and multidimensional scaling. All recent onset sera induced an expression signature (192 UniGenes; fold change: >1.5, $p < 0.01$; false discovery rate: <0.01) that included IL-1 cytokine family members and chemokines involved in monocyte/macrophage and neutrophil chemotaxis, as well as numerous receptors and signaling molecules. This molecular signature was not induced with the sera of healthy controls or long standing diabetes patients, where longitudinal analysis of "at risk" siblings ($n = 3$) before and after onset support the hypothesis that the signature emerges years before onset. This study supports prior investigations of serum that reflect disease processes associated with progression to T1DM. Identification of unique inflammatory mediators may improve disease prediction beyond current islet autoantibodies. Furthermore, proinflammatory serum markers may be used as inclusion criteria or endpoint measures in clinical trials aimed at preventing T1DM. *The Journal of Immunology,* **2008, 180: 1929–1937.**

Type 1 diabetes mellitus (T1DM)[3] is a β cell-specific autoimmune disease that results in life-long dependency on daily insulin injections. Clinical onset is often preceded by the presence of islet cell autoantibodies that are useful for predicting T1DM (1–3). Risk estimates vary, however; a titer for a single autoantibody among first-degree relatives imparts little risk, whereas high titers for two or three Abs are predictive (>80%) of disease within 5 years (4, 5). T1DM patients have an estimated 80–90% loss in β cell mass at diagnosis, although functioning mass still remains (6). Upon initiation of insulin therapy, 25–100% of newly diagnosed patients experience a transient restoration of remaining β cell function, termed the "honeymoon period", that lasts from months to years (7–10). This immunologically active time is clinically significant because it offers: 1) a window for potential therapeutic intervention aimed at arresting β cell destruction, thus preserving endogenous β cell mass and insulin secretion; and 2) a time to potentially measure processes related to β cell destruction.

The development of T1DM involves both an adaptive T cell response as well as a significant cytokine-based arm that kills pancreatic β cells (11–14). In recent onset T1DM populations, elevated serum levels of IL-1α, IL-1β, IL-6, IL-8, IL-18, TNF-α, CXCL10, and IFN-γ have been reported (15–19); however, sufficiently reliable detection methods to enable potentially mechanistic or prognostic measurement remains challenging. Recently, a genomics approach was used to investigate the presence of inflammatory factors in the sera of juvenile rheumatoid arthritis (JRA) patients (20). Gene expression profiling was conducted on PBMCs isolated from healthy individuals after culture with autologous, patient, or allogeneic control serum. A proinflammatory signature was induced by patient sera and provided a foundation for more directed studies that ultimately led to successful treatment. Because serum cytokine levels in T1DM are often too low to measure directly but potentially sufficient to induce the expression of genes under their influence, we applied this sensitive comprehensive approach to recent onset diabetics, at risk siblings of probands, long-standing diabetics, and healthy controls.

Materials and Methods

Subjects and subject characterization

Recent onset (RO) diabetes patients (after stabilization on exogenous insulin but within 7 mo of diagnosis; $n = 15$), long-standing (LS) diabetes patients (>10 years after diagnosis; $n = 12$) and "at risk" (AR) subjects (Ab-positive siblings of probands; $n = 9$) were recruited through Children's Hospital of Wisconsin. Diabetes was defined according to World Health Organization criteria and included blood glucose levels of >200 mg/dl with symptoms confirmed by a physician (21). Healthy control (HC; $n = 12$) criteria included fasting blood glucose of <100 mg/dl, no familial

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[3] Abbreviations used in this paper: T1DM, type 1 diabetes mellitus; AR, at risk; FDR, false discovery rate; GAD, glutamic acid decarboxylase; HC, healthy control; IA2, protein tyrosine phosphatase-2; JRA, juvenile rheumatoid arthritis; LS, long standing; MDS, multiple dimensional scaling; qRT-PCR, quantitative RT-PCR; RO, recent onset.

Table I. *Subject characteristics*

Sample Identifier	Gender	Antibody Status[a] HgA1c (%)	GAD	IA2	IAA[b]	HLA DQB1	Age at Diagnosis	Sample Collection Relative to Onset
Recent onset T1DM								
RO1	Female	8.4	0.0335	**0.0096**	0.0517	0201–0604	4.0	+2.2 months
RO2	Female	7.0	0.0660	**0.2404**	0.6100	0201–0302	7.1	+2.2 months
RO3	Male	5.9	−0.0004	−0.0046	**7.2202**	0201–0402	5.8	+6.0 months
RO4	Male	7.4	0.0190	**0.6825**	**2.1505**	0201–0302	3.7	+3.6 months
RO5	Female	6.8	0.0083	**0.5944**	**0.2184**	0501–0402	3.5	+7.2 months
RO6	Male	8.3	**0.1062**	0.0047	**0.1083**	0302–0603	4.3	+4.2 months
RO7	Female	8.8	**0.6031**	**0.7622**	**4.5672**	0201–0501	5.4	+5.5 months
RO8	Female	9.8	**0.2988**	0.0002	**1.4891**	0201–0501	5.7	+5.0 months
RO9	Male	8.3	**0.1978**	**0.5656**	**0.7670**	0301–0501	6.4	+3.6 months
RO10	Female	7.4	**0.1396**	**0.4377**	**0.1020**	0201–0501	6.5	+6.0 months
RO11	Female	7.8	0.0495	**0.5027**	**5.2452**	0201–0202	6.8	+3.1 months
RO12	Female	7.0	**0.0800**	**1.0646**	**0.7346**	0301–0303	6.9	+4.7 months
Healthy controls								
HC1	Male	Not tested	−0.0107	−0.0001	−0.0125	0303–0501	12.9[c]	
HC2	Male	Not tested	0.0051	0.0051	0.0020	0301–0602	24.3[c]	
HC3	Female	Not tested	0.0042	−0.0004	−0.0050	Not tested	16.5[c]	
HC4	Female	Not tested	0.0133	−0.0003	0.0069	0602–0603	17.8[c]	
HC5	Female	Not tested	0.0069	0.0004	0	0201–0202	12.9[c]	
HC6	Female	Not tested	0.0001	0.0022	−0.0126	0202–0603	13.1[c]	
HC7	Male	Not tested	−0.0072	0.0017	0.0107	0202–0301	14.9[c]	
HC8	Female	Not tested	0.0090	0.0058	0.0020	0602–0503	25.2[c]	
HC9	Female	Not tested	−0.0100	−0.0007	−0.0041	0301–0602	16.6[c]	
HC10	Male	Not tested	−0.0033	−0.0091	0.0157	0602–0603	19.6[c]	
HC11	Female	Not tested	0.0246	−0.0009	0.0086	0201–0201	21.1[c]	
HC12	Female	Not tested	0.0023	−0.0101	0.0174	0201–0501	21.7[c]	
Long-standing diabetics								
LS1	Male	5.2	0.0120	**0.08250**	**1.5346**	0302–0501	18.4	+17.9 years
LS2	Female	6.5	**0.7123**	0.0018	0.1130	0201–0201	6.6	+29.9 years
LS3	Male	8.2	−0.0097	−0.0026	0.0400	0302–0201	1.6	+13.2 years
LS4	Male	8.5	**0.1193**	**0.0093**	**0.1347**	0201–0302	6.8	+10.6 years
LS5	Female	6.7	0.0527	**0.1227**	**0.0697**	0302–0603	9.0	+10.9 years
LS6	Male	9.9	0.0058	0.0024	**0.9617**	0201–0201	3.6	+13.8 years
LS7	Female	7.7	0.0042	−0.0010	**1.6030**	0201–0302	6.3	+29.7 years
LS8	Female	7.9	**0.0779**	**0.0785**	**1.261**	0201–0201	23.0	+17.9 years
LS9	Male	8.3	0.0024	**0.026**	**1.8221**	0302–0302	14.9	+17.9 years
LS10	Female	8.5	0.0049	**0.0115**	**0.939**	0504–0201	4.5	+10.1 years
LS11	Male	9.1	−0.0037	**0.0103**	**1.0022**	0302–0201	8.5	+19.6 years
LS12	Male	9.0	**0.0813**	**0.2043**	**0.2439**	0302–0302	17.0	+13.1 years
At risk[d]								
AR1a (visit 1)	Male	Not tested	**0.2374**	**0.1123**	**0.1810**	0303–0502	N/A	
AR1b (visit 2)	Male	Not tested	**0.1231**	**0.0852**	**0.0540**	0303–0502	N/A	+3.6 years[e]
AR2a (visit 1)	Male	Not tested	0.0544	0.0047	**0.1241**	0302–0302	N/A	
AR2b (visit 2)	Male	Not tested	**0.1212**	0	0.0031	0302–0302	N/A	+2.9 years[e]
AR3a (visit 1)	Male	Not tested	**1.7759**	**0.0407**	**0.1657**	0402–0501	N/A	
AR3b (visit 2)	Male	Not tested	**0.6668**	**0.3560**	**0.1233**	0402–0501	N/A	+1.2 years[e]
AR4a (visit 1)	Female	Not tested	0.0155	**0.024**	**0.1733**	0201–0302	N/A	
AR4b (visit 2)	Female	Not tested	**0.9720**	**0.1117**	0.0054	0201–0302	N/A	+4.3 years[e]
AR5a (visit 1)	Male	Not tested	**0.6342**	**0.3731**	**0.0689**	0302–0201	N/A	
AR5b (visit 2)	Male	Not tested	**0.8829**	**0.4392**	**0.1425**	0302–0201	N/A	+3.1 years[e]
AR6a (visit 1)	Female	Not tested	0.012	0.0003	**0.1019**	0201–0501	N/A	
AR6b (visit 2)	Female	Not tested	**0.0859**	0.0011	**2.045**	0201–0501	N/A	+1.1 years[e]
RO Ab-negative T1DM								
ROAN1	Male	4.8	0.0100	−0.0002	0.0300	0302–0201	4.3	+7.7 months
ROAN2	Male	6.6	0.0077	−0.0032	0.0258	0302–0502	9.8	+6.4 months
ROAN3	Female	6.5	0.0600	−0.0027	0.0327	0302–0202	13.1	+2.6 months
Sample series[f]								
SS1 (visit 1)	Female	Not tested	0.0590	**0.0100**	0.0175	0301–0502	9.0	−18.0 months
SS1 (visit 2)	Female	Not tested	**0.1518**	**0.2252**	0.0380	0301–0502	9.0	−4.4 months
SS1 (visit 3)	Female	6.6	**0.1026**	**1.0284**	**0.1886**	0301–0502	9.0	+1.7 months
SS2 (visit 1)	Male	Not tested	**1.0189**	**0.6781**	**0.0732**	0302–0603	4.9	−3.7 months
SS2 (visit 2)	Male	6.1	**0.4426**	**0.6483**	**0.0600**	0302–0603	4.9	+1.0 months
SS3 (visit 1)	Male	Not tested	0.0169	−0.0035	−0.0017	0301–0501	21.7	−63.8 months
SS3 (visit 2)	Male	Not tested	0.0659	0.0013	0.0044	0301–0501	21.7	−39.8 months
SS3 (visit 3)	Male	Not tested	**0.1576**	**0.4249**	0.0124	0301–0501	21.7	−29.3 months
SS3 (visit 4)	Male	Not tested	**0.1602**	**1.0131**	0.0052	0301–0501	21.7	−17.6 months
SS3 (visit 5)	Male	Not tested	**0.3064**	**0.8593**	0.007	0301–0501	21.7	−4.0 months
SS3 (visit 6)	Male	5.5	**0.2441**	**1.1301**	**0.8804**	0301–0501	21.7	+3.3 months

[a] Measured as described (22) after the initiation of treatment with exogenous insulin. The sera of healthy controls was used to define 99th percentile threshold cutoffs for positive assays. Threshold cutoffs for GAD, IA2, and IAA were ≥0.07, ≥0.007, and ≥0.05, respectively. Sample values exceeding these respective cutoffs are shown in boldfaced font.

[b] Insulin autoantibody.

[c] Age at sample collection.

[d] Ab-positive siblings of probands; longitudinal sample pairs.

[e] Relative to visit 1.

[f] Preonset to postonset longitudinal sample series.

Table II. *qRT-PCR performance parameters and primer designs*

Gene Name	UniGene	Slope[a]	R2[b]	E[c]	Oligonucleotide Primer Sequences[d]
Interleukin 1, β (IL1B)	Hs.126256	−3.54	0.997	1.92	5′-CGGCCAGGATATAACTGACTTC-3′ 5′-GGGCATTGGTGTAGACAACA-3′
Interleukin 1 receptor, type I (IL1R1)	Hs.557403	−3.15	0.998	2.08	5′-GATTGTGAGCCCAGCTAATGAG-3′ 5′-AGCACTGTGATGAGGGTACTCC-3′
Chemokine (C-X-C motif) ligand 5 (CXCL5)	Hs.89714	−3.73	0.994	1.85	5′-GACACTTGTGAAAAGGCTTG-3′ 5′-GCAGTAGCTTTGTTAATTGCAC-3′
Chemokine (C-X-C motif) ligand 1 (CXCL1)	Hs.789	−3.49	0.99	1.94	5′-GCCAGCCACTGTGATAGA-3′ 5′-TGTCCAAGGGATATTTAGAACA-3′
Chemokine (C-C motif) receptor 1 (CCR1)	Hs.301921	−3.62	0.99	1.89	5′-GCTGGCAGTGGAACTAAGAA-3′ 5′-GTAAATGGAAATGATGAGTCCC-3′
K inwardly-rectifying channel, subfamily J, member 15 (KCNJ15)	Hs.411299	−3.56	0.993	1.91	5′-ACGGACATACAAAATCAATCTT-3′ 5′-CCCTACACCAAGCTCTTAGA-3′
Syndecan 2 (SDC2)	Hs.1501	−3.40	1.00	1.97	5′-CCAGCCGAAGAGGATACAA-3′ 5′-GCAAAGAGAAAGCCAATAACTC-3′
cAMP responsive element modulator (CREM)	Hs.200250	−3.59	0.99	1.90	5′-TTGCCATGTGGACTTGTG-3′ 5′-AAACAGATCAAAGTAAGCTCGG-3′

[a] Slope of standard curve.
[b] Linear regression of standard curve.
[c] Reaction efficiency, determined as previously described (*27*).
[d] Top sequence is forward primer, bottom is reverse.

history of any autoimmune disorder, and negativity for islet autoantibodies at the 99th percentile (22). All study subjects were free of known infection at the time of sample collection; subject characteristics are shown in Table I. All blood samples were drawn by trained phlebotomists at Children's Hospital of Wisconsin and immediately processed. Under sterile conditions, peripheral blood (acid citrate dextrose solution B, anti-coagulated) was collected and components were separated by Ficoll-Histopaque density gradient centrifugation. PBMCs were viably frozen in RPMI 1640 medium at −80ºC supplemented with 20% FCS and 10% DMSO until DNA or RNA was extracted. Serum was stored at −80°C until use. Autoantibody titers for glutamic acid decarboxylase (GAD), protein tyrosine phosphatase-2 (IA2), and insulin were determined as previously described (22). HLA-DQB1 genotypes were determined with the SeCore DQB1 sequencing kit in accordance with the manufacturer's instructions (Invitrogen Life Technologies). Serum IL-1β levels were determined by ELISA (human IL-1β/IL-1F2 immunoassay, catalog no. DLB50; R&D Systems) in accordance with the manufacturer's instructions. The study was approved by the Institutional Review Board (IRB) of the Children's Hospital of Wisconsin (IRB no. 01-15) and informed consent was obtained from parents/legal guardians.

PBMC cultures, RNA extractions, and GeneChip analysis

Fresh PBMCs of healthy donors for use in cultures were isolated from 20–40 ml of blood by Ficoll-Histopaque density gradient centrifugation and used immediately. As previously described (20), the induction of gene expression was accomplished by culturing the PBMCs of healthy blood donors for 6 h at 37°C in 5% CO_2 with 20% of autologous sera, HC sera, RO sera, LS sera, or AR sera. Cultures were prepared in a Costar 24-well plate (Corning) using 4–6 wells per condition (10^6 cells/well in 800 μl of RPMI 1640 medium plus 200 μl of sera). In this study a total of 62 subject sera were analyzed, requiring 13 different blood donors. A given serum sample was used to induce gene expression in cells of a single donor. The donor cells used in the testing of each serum are shown in supplement B.[4] After culture, the cells distributed over 4–6 wells for analysis of each subject were pooled, and total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies), providing sufficient material for global gene expression analysis and quantitative RT-PCR confirmation. The GeneChip human genome U133 plus 2.0 array interrogates >47,000 transcripts and thus was selected for these studies for its overall comprehensive coverage. Purified RNA (~50 ng) was amplified using an Affymetrix two-cycle cDNA synthesis kit (catalog no. 900432), and cRNA was synthesized, labeled, fragmented, and hybridized to the array in accordance to the Affymetrix GeneChip expression analysis technical manual. RNA from each culture was independently analyzed. After hybridization, arrays were washed, stained with PE-conjugated streptavidin (Molecular Probes), and scanned. Image data were analyzed with Affymetrix GeneChip operating software (GCOS) and normalized with Robust Multichip Analysis (RMA; www.bioconductor.org/) to determine signal log ratios. The statistical significance of differential gene expression was derived through a Student's *t* test and false discovery rates (FDR) were determined with Significance Analysis of Microarrays (SAM) software as described (23). Ontological pathway analysis was performed with OntoExpress (http://vortex.cs.wayne.edu/ontoexpress) and Database for Annotation, Visualization, and Integrated Discovery (DAVID; david.abcc.ncifcrf.gov/) (24, 25). These tools conduct overrepresentation analyses of the functional gene categories detected relative to the total functional gene categories assayed by the array using the Gene Ontology (GO) project databases as references. Hierarchical clustering was conducted with Genesis (26) and multidimensional scaling was performed with MatLab.

Real-time quantitative RT-PCR (qRT-PCR)

Specific oligonucleotide primers for the following selected genes were designed with Oligo 6.66 (Molecular Biology Insights): IL1B, IL1R1, CXCL5, KCNJ15 (potassium inwardly rectifying channel, subfamily J, member 15), SDC2 (syndecan 2), CREM (cAMP-responsive element modulator), CXCL1, and CCR1. Monoplex real-time qRT-PCR was performed using a Rotor-Gene 3000 thermal cycler (Corbett Research), a QuantumRNA 18S internal standards kit (Ambion), locus-specific primers (Sigma Genosys), and QuantiTect SYBR Green PCR Master Mix (Qiagen) according to the manufacturers' instructions. Synthesis of first-strand cDNA from 0.2–0.5 μg unamplified RNA was accomplished with random hexamers (Invitrogen Life Technologies) and SuperScript II (Invitrogen Life Technologies) according to the manufacturer's instructions. Duplicate locus-specific and 18S PCRs were performed for each gene analyzed in 20-μl reactions that included 2 μl of cDNA and 10 μl of 2× SYBR QuantiTect SYBR Green PCR Master Mix (Qiagen) possessing 1.2 μl of locus-specific (10 μM) or 18S-specific competimers (used as a 3:7 ratio of primer/competimer set; each stock is at 5 μM) and 6.8 μl of deionized water. Reactions were typically cycled as follows: stage 1, 95°C for 900 s; stage 2, 50 cycles at 95°C for 30 s, 50–66°C for 30 s (locus specific), 72°C for 30 s, and fluorescence acquisition at 72–82°C for 20 s (locus specific); and stage 3, melt curve at 60–95°C. The 18S reactions were cycled as follows: stage 1, 95°C for 900 s; stage 2, 50 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and 82°C for 15 s; and stage 3, melt curve at 60–95°C. A pooled and concentrated sample of HC or RO cDNA was used for both the

[4] The online version of this article contains supplemental material.

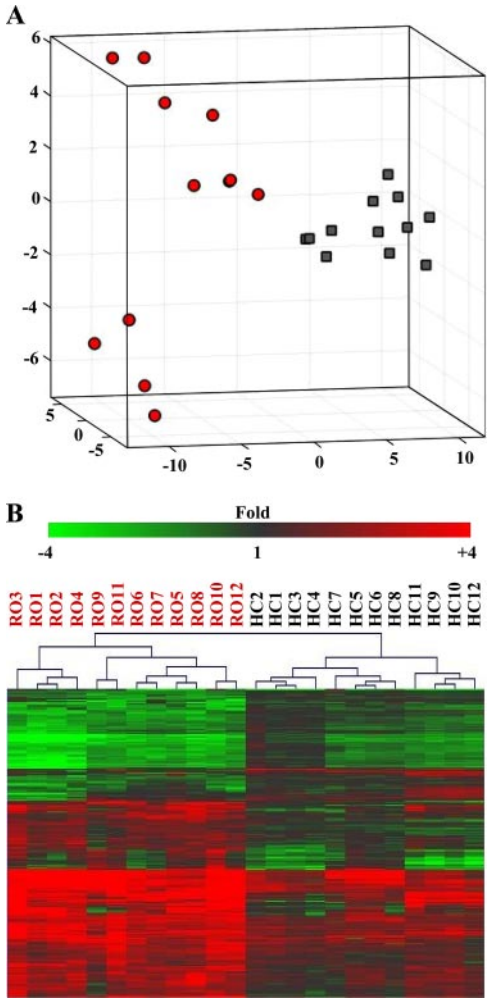


FIGURE 1. Gene expression profiles of 192 unique UniGenes significantly regulated (fold change of >1.5; Student's *t* test, $p < 0.01$) after healthy PBMCs are cultured with RO ($n = 12$) vs HC ($n = 12$) sera. All data were normalized with that of the autologous induction to account for gene expression induced by placing the PBMCs into culture. The relatedness of the 24 profiles was examined by MDS and hierarchical clustering. *A*, In MDS analysis, each sample is plotted in a three-dimensional space where the similar samples are plotted in closer proximity compared with the dissimilar ones. RO samples are shown as red circles and HC samples are shown as black squares. *B*, Hierarchical clustering was conduced with Genesis (26). With either algorithm, the samples cluster to form two distinct groups, each possessing a high degree of intragroup similarity among RO and HC samples. The scale represents fold of change between the serum tested relative to autologous serum (−4-fold to +4-fold). (The supporting data for these analyses are provided in supplement B).

locus-specific and the 18S standard curves at undiluted, 1/5, 1/25, 1/125, and 1/625 concentrations. At least two points from the standard curve were used as positive controls in each assay. Specificity for all qRT-PCR was verified by both melting curve analysis and 1.5% agarose gel detection of a single product of the predicted size. The data were analyzed with the Rotor-Gene 3000 software using the cycle threshold for quantification. Relative gene expression data (fold change) between samples was accomplished using the mathematical model described by Pfaffl (27). Primer designs and reaction performance parameters are provided in Table II.

Results

Analysis of RO diabetes patients and HC

Similarly as in the prediabetic stage, RO T1DM subjects experience active killing of their pancreatic β cells. To determine whether proinflammatory serum factors related to the autoimmune processes of T1DM could be detected through their ability to induce gene expression, healthy PBMCs were cultured in the presence of autologous, allogeneic RO, or allogeneic HC sera. We opted for this strategy vs directly assaying PBMCs of cases and controls because: 1) immune responses are considered to be local events and thus participating cells may not be highly represented in peripheral circulation; and 2) Pascual et al., (20) observed that the gene expression differences between PBMCs incubated with JRA vs control sera were more robust than those observed between the direct gene expression profiling of case and control PBMCs. To avoid inducing gene expression due to factors related to hyperglycemia, samples from RO T1DM patients were collected after stabilization on exogenous insulin (2–7 mo after diagnosis; Table I).

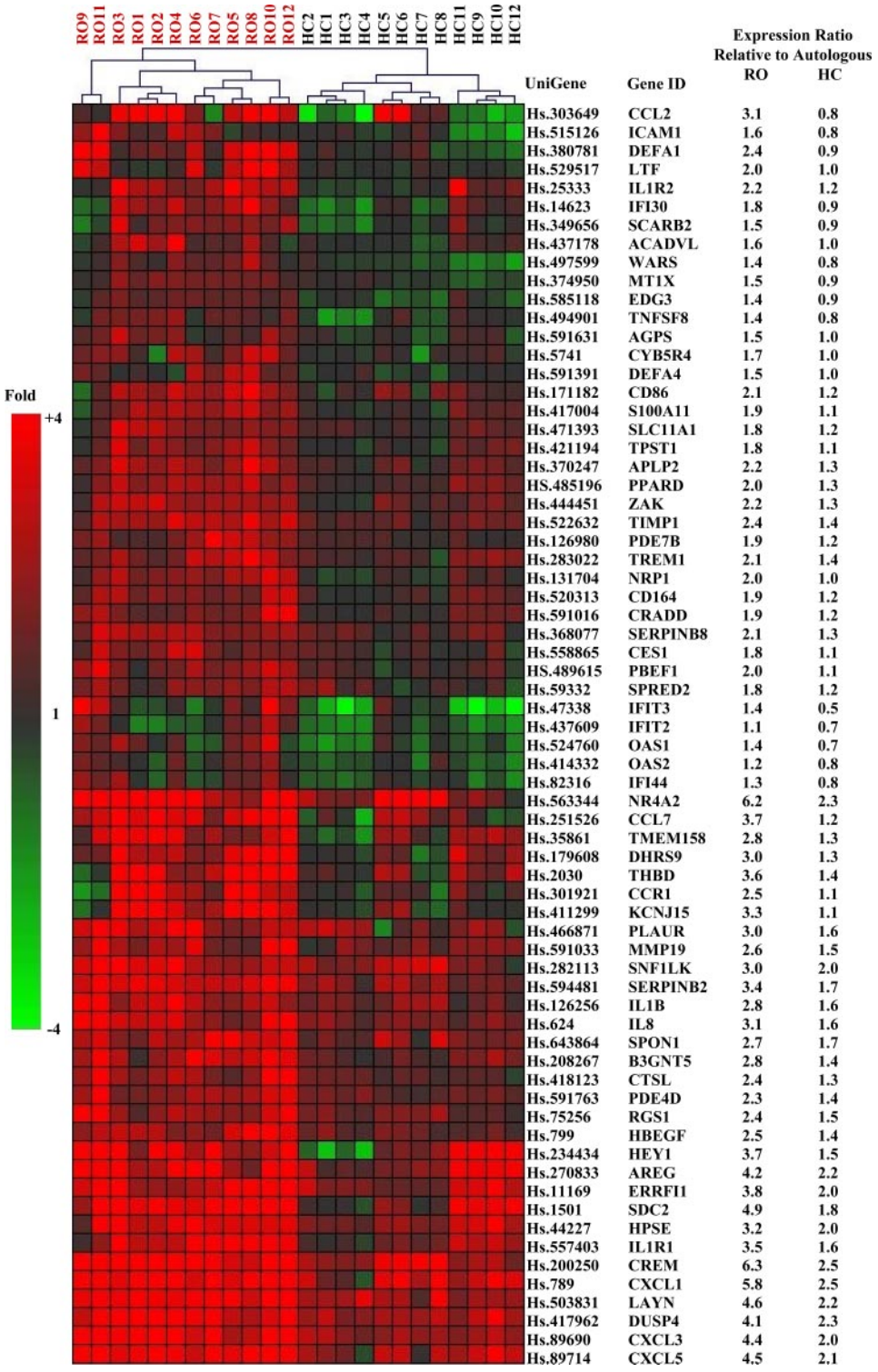
Table III. *Pathway analysis of genes differentially regulated by PBMCs incubated with RO or HC sera*[a]

GO Identifier	GO Biological Process Name	A[b]	B[c]	*p* Value
GO:0006935	Chemotaxis	14	103	3.2×10^{-10}
GO:0007267	Cell-cell signaling	20	278	3.2×10^{-10}
GO:0007165	Signal transduction	36	1181	1.2×10^{-8}
GO:0006955	Immune response	16	260	1.3×10^{-8}
GO:0001525	Angiogenesis	7	39	2.4×10^{-6}
GO:0008284	Positive regulation of cell proliferation	9	103	4.2×10^{-6}
GO:0006928	Cell motility	8	110	5.7×10^{-5}
GO:0007155	Cell adhesion	16	425	8.9×10^{-5}
GO:0050896	Response to stimulus	9	260	1.4×10^{-4}
GO:0008283	Cell proliferation	10	239	6.8×10^{-4}
GO:0030154	Cell differentiation	10	248	1.1×10^{-3}
GO:0006629	Lipid metabolism	8	193	1.1×10^{-3}
GO:0007596	Blood coagulation	4	63	3.3×10^{-3}
GO:0007186	G protein-coupled receptor protein signaling pathway	9	415	5.9×10^{-3}

[a] Analysis restricted to Gene Ontology (GO) biological processes where more than three UniGenes were detected per category and possessed FDR-corrected *p* values <0.01.
[b] Unique UniGene total (unique UniGenes detected).
[c] Unique reference UniGene total (unique UniGenes assayed on array).

Gene expression profiling was conducted on healthy PBMCs that were cultured with the allogeneic sera of either RO diabetes patients ($n = 12$) or healthy controls ($n = 12$). The transcriptional responses of healthy PBMCs to either healthy allogeneic serum or recent T1DM onset serum were normalized with those induced by autologous sera to account for gene expression induced by isolating and placing the PBMCs in culture. First, unsupervised hierarchical clustering was conducted. This analysis used any probe set that exhibited a minimum 2-fold change ($\log_2$ ratio of >1.0 relative to the autologous induction; $n = 827$) in any six of the 24 total samples (12 RO and 12 HC). This analysis showed that the RO and HC samples cluster into two groups (supplement A).

To focus the analysis, we first performed a permutation test with SAM (23). We then identified the differentially expressed probe sets ($\log_2$ ratio > 0.58 (1.5-fold); Student's *t* test, $p < 0.01$ of log ratio, FDR < 0.01) between the RO and HC groups ($n = 233$ probe sets, 192 unique UniGenes). These are tabulated in supplement B. Our power, determined through resampling, was calculated to be on average 70% (supplement B). Multiple probe sets targeting the same UniGene were averaged and evaluated by multiple dimensional scaling (MDS) and hierarchical clustering (Fig. 1, *A* and *B*); with either approach the RO and HC groups distinctly clustered. Although the expression profiles induced by RO and HC sera are clearly resolved by MDS and hierarchical clustering,



UniGene	Gene ID	Expression Ratio Relative to Autologous RO	HC
Hs.303649	CCL2	3.1	0.8
Hs.515126	ICAM1	1.6	0.8
Hs.380781	DEFA1	2.4	0.9
Hs.529517	LTF	2.0	1.0
Hs.25333	IL1R2	2.2	1.2
Hs.14623	IFI30	1.8	0.9
Hs.349656	SCARB2	1.5	0.9
Hs.437178	ACADVL	1.6	1.0
Hs.497599	WARS	1.4	0.8
Hs.374950	MT1X	1.5	0.9
Hs.585118	EDG3	1.4	0.9
Hs.494901	TNFSF8	1.4	0.8
Hs.591631	AGPS	1.5	1.0
Hs.5741	CYB5R4	1.7	1.0
Hs.591391	DEFA4	1.5	1.0
Hs.171182	CD86	2.1	1.2
Hs.417004	S100A11	1.9	1.1
Hs.471393	SLC11A1	1.8	1.2
Hs.421194	TPST1	1.8	1.1
Hs.370247	APLP2	2.2	1.3
HS.485196	PPARD	2.0	1.3
Hs.444451	ZAK	2.2	1.3
Hs.522632	TIMP1	2.4	1.4
Hs.126980	PDE7B	1.9	1.2
Hs.283022	TREM1	2.1	1.4
Hs.131704	NRP1	2.0	1.0
Hs.520313	CD164	1.9	1.2
Hs.591016	CRADD	1.9	1.2
Hs.368077	SERPINB8	2.1	1.3
Hs.558865	CES1	1.8	1.1
HS.489615	PBEF1	2.0	1.1
Hs.59332	SPRED2	1.8	1.2
Hs.47338	IFIT3	1.4	0.5
Hs.437609	IFIT2	1.1	0.7
Hs.524760	OAS1	1.4	0.7
Hs.414332	OAS2	1.2	0.8
Hs.82316	IFI44	1.3	0.8
Hs.563344	NR4A2	6.2	2.3
Hs.251526	CCL7	3.7	1.2
Hs.35861	TMEM158	2.8	1.3
Hs.179608	DHRS9	3.0	1.3
Hs.2030	THBD	3.6	1.4
Hs.301921	CCR1	2.5	1.1
Hs.411299	KCNJ15	3.3	1.1
Hs.466871	PLAUR	3.0	1.6
Hs.591033	MMP19	2.6	1.5
Hs.282113	SNF1LK	3.0	2.0
Hs.594481	SERPINB2	3.4	1.7
Hs.126256	IL1B	2.8	1.6
Hs.624	IL8	3.1	1.6
Hs.643864	SPON1	2.7	1.7
Hs.208267	B3GNT5	2.8	1.4
Hs.418123	CTSL	2.4	1.3
Hs.591763	PDE4D	2.3	1.4
Hs.75256	RGS1	2.4	1.5
Hs.799	HBEGF	2.5	1.4
Hs.234434	HEY1	3.7	1.5
Hs.270833	AREG	4.2	2.2
Hs.11169	ERRFI1	3.8	2.0
Hs.1501	SDC2	4.9	1.8
Hs.44227	HPSE	3.2	2.0
Hs.557403	IL1R1	3.5	1.6
Hs.200250	CREM	6.3	2.5
Hs.789	CXCL1	5.8	2.5
Hs.503831	LAYN	4.6	2.2
Hs.417962	DUSP4	4.1	2.3
Hs.89690	CXCL3	4.4	2.0
Hs.89714	CXCL5	4.5	2.1

FIGURE 2. Hierarchical clustering using 68 well-annotated genes up-regulated by RO sera. Expression profiles induced by PBMCs cultured with RO ($n = 12$) and HC ($n = 12$) sera distinctly cluster. Indicated are the UniGene identifier, gene symbol, and mean-induced fold of change induced by incubation with RO and HC (relative to autologous induction) for each gene (the detailed results of samples for the 192 and 68 UniGene subsets are provided in supplement B). The scale represents fold of change between the serum tested relative to autologous serum (−4-fold to + 4-fold).

the molecular signature induced by RO sera is not an all or none response as is reflected by Fig. 1*B*, where it is clear that a given gene within a cohort is not always up- or down-regulated to the same degree. Ontological analysis of this subset of 192 genes identified 14 significant Gene Ontology biological processes ($p < 0.01$, after FDR correction) possessing at least four unique UniGenes (Table III). These Gene Ontology biological processes included chemotaxis, cell-cell signaling, signal transduction, immune response, positive regulation of cell proliferation, cell adhesion, and G protein-coupled receptor protein signaling. Annotated within these 14 categories were 68 UniGenes up-regulated by RO sera related to proinflammatory processes (Fig. 2). RO sera induced transcription of numerous immune signaling molecules and receptors including: 1) the IL-1 cytokine family members IL-1β, IL-1R1, and IL-1R2; and 2) the chemokines CCL2 and CCL7, involved in monocyte/macrophage chemotaxis, as well as IL-8, CXCL1, CXCL3, and CXCL5, involved in neutrophil chemotaxis (28). CCR1, a receptor associated with a number of autoinflammatory diseases, was also induced by RO sera (29, 30). Several innate immunity receptors were also significantly up-regulated by RO sera, including HEBGF (heparin-binding EGF-like growth factor) and the TREM1 (triggering receptor on myeloid cells 1). Not

Table IV. *qRT-PCR of selected genes on cultured PBMCs vs PBMCs directly collected from subjects*

	Gene expression induced in cultured PBMCs incubated with RO, HC, and autologous (Auto) serum				Gene expression measured directly in PBMCs of RO and HC subjects	
Gene Symbol	RO vs Auto Fold Change	HC vs Auto Fold Change	RO vs HC Fold Change	RO vs HC Student's *t* test[a]	RO vs HC Fold Change	RO vs HC Student's *t* test[a]
IL1B	8.6 ± 15.3	2.6 ± 1.5	3.3 ± 1.9	$p < 0.0003$	0.5 ± 1.5	$p > 0.8$
IL1R1	15.7 ± 24.4	1.8 ± 1.1	9.0 ± 1.7	$p < 0.0009$	1.0 ± 2.1	$p > 0.3$
CXCL5	18.9 ± 33.6	5.0 ± 4.8	3.8 ± 2.0	$p < 0.00001$	0.6 ± 1.7	$p > 0.6$
CXCL1	69.5 ± 169.1	18.2 ± 28.6	3.8 ± 2.9	$p < 0.0005$	0.2 ± 2.9	$p > 0.8$
CCR1	6.9 ± 7.8	1.4 ± 0.6	4.9 ± 1.2	$p < 0.005$	2.5 ± 1.0	$p = 0.0415$
KCNJ15	4.9 ± 6.3	2.9 ± 2.5	1.7 ± 1.6	$p < 0.005$	1.7 ± 1.5	$p > 0.8$
SDC2	25.6 ± 24.9	7.6 ± 4.6	4.6 ± 3.4	$p < 0.00001$	0.5 ± 1.6	$p > 0.1$
CREM	11.7 ± 13.5	3.2 ± 2.3	3.6 ± 1.4	$p < 0.00001$	1.6 ± 1.4	$p > 0.09$

[a] Student's *t* test performed on log ratio.

listed are TLR2 and TLR4 (>1.6-fold) that were detected at a significance of $p < 0.05$. Genes involved with cell adhesion and/or cell motility were up-regulated, including ICAM1, SDC2, and endolyn (CD164). Genes associated with G protein-coupled receptor signaling, such as EDG3 (endothelial differentiation, sphingolipid G protein-coupled receptor 3) and RGS1 (regulator of G protein signaling 1) were also up-regulated by RO sera.

Follow-up qRT-PCR and ELISA studies

In the array analysis, many of the genes differentially regulated when healthy PBMCs are cultured with either RO or HC sera are known to be functionally related to and/or influenced by IL-1β. Confirmatory qRT-PCR was performed for eight such loci (Table IV). The IL-1 family members IL-1β and IL-1R1 were determined to be 3.3 ± 1.9- and 9.0 ± 1.7 -fold increased, respectively, when comparing PBMCs incubated with RO vs HC sera. CXCL5, CXCL1, and CCR1 were respectively determined to be 3.8 ± 2.0-, 3.8 ± 2.9-, and 4.9 ± 1.2 -fold increased when comparing PBMCs incubated with RO vs HC sera. Lastly, KCNJ15, SDC2, and CREM were determined to be 1.7 ± 1.6-, 4.6 ± 3.4-, and 3.6 ± 1.4-fold increased, respectively, in cultures incubated with RO vs HC sera. Consistent with the array results, qRT-PCR analysis confirmed statistically significant overrepresentation of each of these eight transcripts in PBMCs cultured with 20% RO sera.

The serum levels of IL-1β of RO and HC subjects were measured by a commercial ELISA possessing a sensitivity of detection of ~1 pg/ml. Among the 12 RO and 12 HC serum samples analyzed in the microarray studies, sufficient serum was available for 10 of the RO subjects and all 12 HC subjects. Serum samples from an additional 42 RO T1DM subjects ($n = 52$ total) and an additional 41 HC subjects ($n = 53$ total) were added to this analysis. These additional subjects met the RO and HC criteria defined in the methods section. Differences in serum IL-1β levels were not observed between these two groups (RO, 0.93 ± 1.1 pg/ml; HC, 0.77 ± 1.0 pg/ml; $p > 0.5$, Student's *t* test).

Using qRT-PCR, we investigated whether PBMCs drawn directly from RO T1DM patients overexpressed these eight transcripts relative to PBMCs collected from healthy controls. These studies used PBMCs isolated (by density gradient centrifugation) from the same blood draws (2–6 mo after diagnosis) that were used in the serum culture experiments described above. Aside from finding a marginally significant increase in the abundance of CCR1 transcript (2.5 ± 1.0-fold increase in RO PBMCs relative to HC PBMCs; $p = 0.0415$), we did not find statistically significant differential expression (Table IV).

Overall, the qRT-PCRs on PBMCs cultured with RO and HC sera confirm the differential expression of transcripts regulated by or related to IL-1β that were detected in the array analysis. The ELISA results fail to show differences between RO and HC subjects in serum IL-1β levels, highlighting the difficulty measuring events related to T1DM in the periphery and suggesting that if IL-1β is present in the sera of the RO cohort, it is below the ELISA detection limit. Alternatively, other cytokine(s) may be responsible for the molecular signature induced by RO sera. The qRT-PCR analysis of the RO and HC PBMCs indicate that IL-1β is not being produced by leukocytes in the periphery.

Analysis of other subject groups

To better understand the nature of the RO signature, other subject groups were investigated. Twelve LS subjects were analyzed, all were >10 years postonset and 11/12 possessed a measurable titer for at least one islet cell autoantibody (Table I). LS sera induced expression signatures that clustered with those induced by HC sera (Fig. 3*A* and supplement B). This is consistent with long-standing T1DM not being immunologically active and supports the hypothesis that the molecular signature expressed by PBMCs cultured with RO serum arises from factors related to active autoimmune processes.

We then investigated how the emergence of the molecular signature correlates with islet cell autoantibody status and disease progression in 12 individuals. Six currently healthy autoantibody-positive AR siblings of diabetics were each monitored on two occasions with a minimum interval between visits of 1 year (Table I and Fig. 3*B*). Sera of subjects AR1, AR4, AR5, and AR6 induced profiles similar to those induced with HC sera when analyzing the 192 UniGenes differentially regulated between inductions with HC and RO sera or the focused subset of 68 genes (Fig. 3*B*). The sera of subjects AR2 and AR3 induced a signature more similar to and clustered with the RO inductions. Sample pairs from a given subject were always concordant for the presence or absence of the proinflammatory signature. Among these subjects, no correlation could be made between the induced expression profile and the number of autoantibodies, Ab specificity, or HLA-DQB1 genotype. Given the current sample size, the power to detect such associations is limited. The serum of three recent onset T1DM patients that lacked measurable titer for GAD, IA2, or insulin autoantibodies were analyzed and found to induce expression profiles that clustered with those induced by RO sera when using the complete set of 192 regulated UniGenes or the focused set of 68 UniGenes (Fig. 3*C*), further associating the molecular signature with active inflammatory processes vs autoantibody status. However, the presence of autoantibodies targeting other β cell proteins cannot be excluded in these subjects. Lastly, three longitudinal subject series collected from prospectively monitored siblings of diabetics that developed T1DM were also evaluated. The first subject

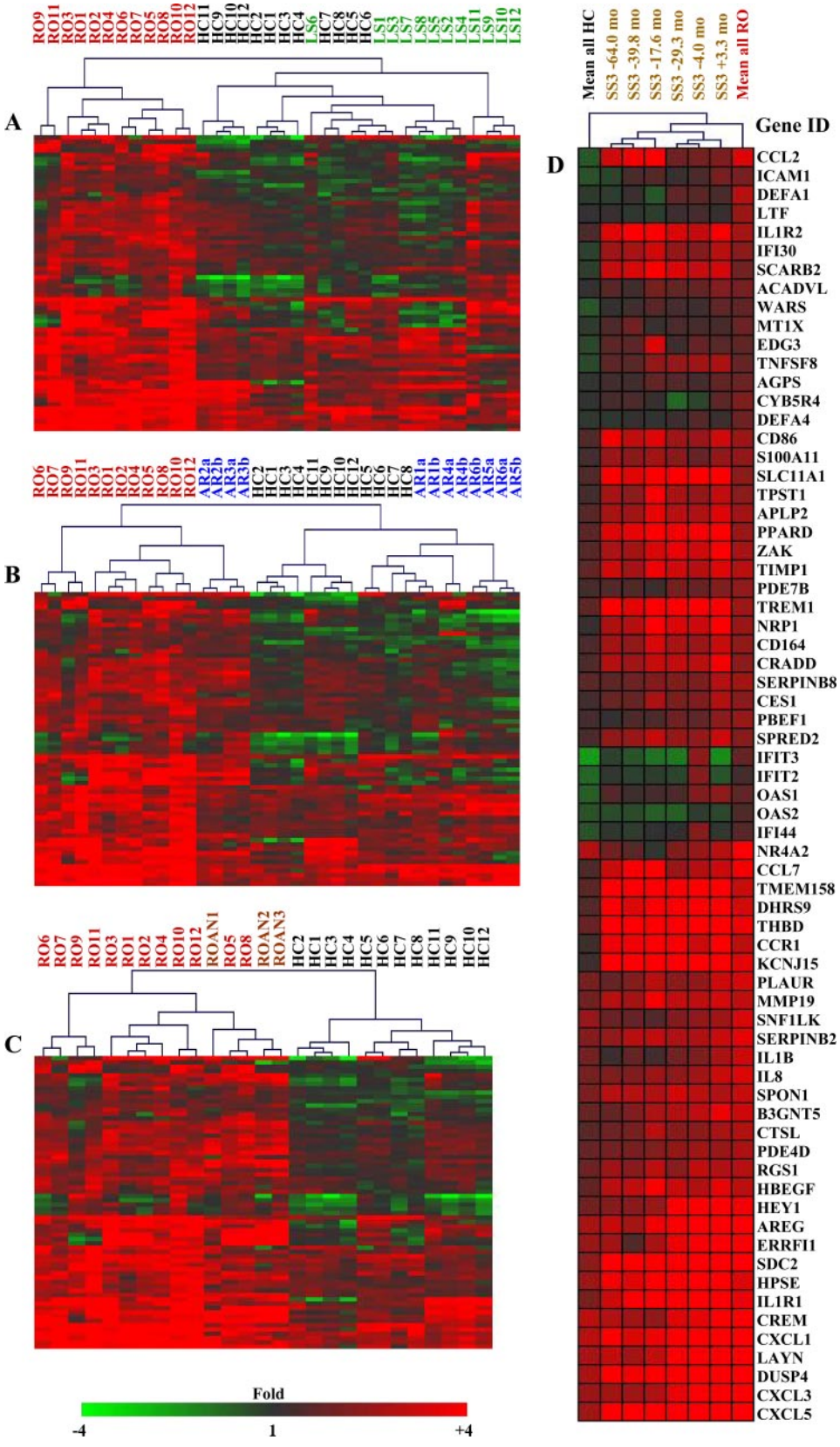


FIGURE 3. Expression profiles of PBMCs incubated with the sera of additional subject groups using the 68 annotated genes up-regulated by RO sera. *A*, Hierarchical clustering of expression profiles induced by RO ($n = 12$), HC ($n = 12$), and LS ($n = 12$) sera. Profiles of PBMCs incubated with sera collected from LS diabetics (>10 years postonset) cluster with those incubated with HC sera, consistent with an absence of active autoimmunity. *B*, The sera of AR autoantibody-positive siblings of diabetics ($n = 6$) were analyzed at two longitudinal time points each (a vs b; Table I). The sera of two subjects (AR2 and AR3 at both time points analyzed) induced proinflammatory expression profiles similar to those induced by RO sera. *C*, The sera of three RO Ab-negative T1DM patients (ROAN; lacking titers for GAD, IA2, and insulin autoantibodies) were analyzed and found to induce signatures similar to those of autoantibody-positive T1DM serum. *D*, Longitudinal sample series 3 (SS3) is from an AR sibling of a diabetic that developed T1DM. Samples were collected at −63.8 mo (autoantibody negative), −39.8 mo (autoantibody negative), −29.3 mo (+2 autoantibodies), −17.6 mo (+2 autoantibodies), −4.0 mo (+2 autoantibodies), and + 3.3 mo (+2 autoantibodies) relative to onset. Hierarchical clustering was conducted using an average of the RO ($n = 12$) and HC ($n = 12$) expression values to better view the development of the proinflammatory profile. All preonset sample profiles cluster with the recent onset sera profile. The scale represents fold of change between the serum tested relative to autologous serum (−4-fold to + 4-fold).

(SS1), diagnosed at the age of 9.0 years, was evaluated at −18.0 mo (+1 autoantibody), −4.4 mo (+2 autoantibodies), and + 1.7 mo (+3 autoantibodies) relative to onset. The second subject (SS2), diagnosed at the age of 4.9 years, was evaluated at −3.7 mo (+3 autoantibodies) and + 1.0 mo (+3 autoantibodies) relative to onset. In subjects 1 and 2, sera from all preonset time points induced the proinflammatory signature and clustered with the recent onset expression profile when using the complete set of 192 regulated UniGenes or the focused set of 68 UniGenes. The third subject (SS3), diagnosed at the age of 21.7 years, was the most comprehensively studied. Samples were collected at −63.8 mo (autoantibody negative), −39.8 mo (autoantibody negative),

−29.3 mo (+2 autoantibodies), −17.6 mo (+2 autoantibodies), −4.0 mo (+2 autoantibodies), and +3.3 mo (+2 autoantibodies) relative to onset. In SS3, the proinflammatory signature was again observed in all preonset samples analyzed before the emergence of islet cell autoantibodies (Fig. 3*D*).

Discussion

In this article, we define a molecular signature that is induced by factors present in the sera of RO diabetes patients. The sera of HC and LS diabetes patients fail to induce the molecular signature. These observations are consistent with an absence of inflammatory factors in HC sera and an overall absence of autoimmunity. Likewise, in LS sera these factors are absent due to significantly reduced or no β cell mass and an absence of active autoimmunity. Together, these results support the hypothesis that induction of the signature by RO sera is reflective of active autoimmune processes.

Three longitudinal sample series were analyzed. Although the number of cases is small, the proinflammatory signature was observed before the clinical onset of T1DM in all cases. In SS3, the signature was present 63.8 mo (>5 years) before onset and before the emergence of islet cell autoantibodies, implying that this indirect yet sensitive approach may be useful in predicting onset in AR subjects. Among the six autoantibody positive subjects studied the signature was induced by the sera of only two, raising two questions. First, is onset pending in signature-positive/Ab-positive individuals? Second, do signature-negative/Ab-positive individuals represent those Ab-positive individuals that never progress to T1DM? The prospective study of these and additional subjects will clarify potential prognostic value of these strategies. It is not likely that these studies have merely measured a general inflammatory response, as the sera of individuals undergoing an atopic asthmatic response to *Parietaria judaica* pollen induce a completely unique profile consisting of different proinflammatory transcripts/pathways (M. J. Hessner, unpublished results).

Previous studies have clearly established that there exists a complex cytokine milieu in T1DM patients (15–19); however, many of the 68 UniGenes of the proinflammatory signature are known to be influenced by IL-1 through either increased transcription or increased mRNA half-life. These include IL-1β, IL-1R2, IL-8, CCR1, CCL2, CCL7, CXCL1, CXCL3, CXCL5, SDC2, ICAM1, TIMP1, MT1X, CTSL, APLP2, PLAUR, heparin-binding EGF-like growth factor (HBEGF), CD86, SLC11A1, plasminogen activator inhibitor 2 (SERPINB2), and CREM. Furthermore, KCNJ15, which has been linked to the lysosome-mediated control of IL-1β secretion (31), is also up-regulated by RO sera. Although not reaching our significance threshold, RO sera increased the expression of cyclooxygenase type 2 (PTGS2) and platelet activating factor (PAFAH1B1), consistent with the presence of IL-1. The expression profiles induced by T1DM and JRA serum (20) exhibit some parallels. Pascual et al. (20) reported on 46 genes up-regulated by JRA serum, and 22 of these are detected among the 192 UniGenes significantly induced when comparing RO to HC serum. It must be emphasized that these studies have evaluated the induction of gene expression after culturing healthy PBMCs with RO sera; the degree to which the transcription of these genes will correlate to detection of their respective translated protein products remains to be determined.

Recently, Kaizer et al. (32) reported the direct expression profiling of PBMCs isolated from healthy controls, T1DM patients at diagnosis, T1DM patients at 1 mo postdiagnosis, and T1DM patients at 4 mo postdiagnosis. In patient PBMCs at diagnosis, they identified an expression signature that included IL1β, CCR1, CXCL1, and TREM1, which are in common with the focused 68 UniGene signature induced in healthy PBMCs by RO serum. The PBMC signature observed in PBMCs collected at diagnosis was reported to resolve by 4 mo postonset despite the fact that pancreatic β cell destruction is ongoing during this time, prompting the authors to hypothesize that many of their observations may be a direct or indirect consequence of hyperglycemia (32). Although there is a mixed body of evidence showing that hyperglycemia can affect IL-1β secretion by pancreatic β cells and other cell types (33–37), the molecular signature defined here is likely not a consequence of hyperglycemia because it was detected years before T1DM onset in the longitudinal case studies and all of the RO samples evaluated were collected after stabilization on exogenous insulin, were normoglycemic, and possessed histories of good glycemic control based upon hemoglobin A1c values (group average 7.7 $\pm$ 1.1% SD). In contrast, the long-standing diabetes patients had a slightly poorer history of glycemic control (average 8.0 $\pm$ 1.3 SD), yet the profiles induced by LS sera cluster with those of induce by HC sera. The molecular signature induced by RO serum also resolves sometime between 7 mo (maximum collection time of our recent onset cohort) and 10 years (minimum time of our LS cohort). Defining when the molecular signature resolves and how this coincides with the end of the honeymoon period is the focus of ongoing studies. In this study, we performed confirmatory qRT-PCR for eight genes (IL-1β, IL-1R1, CXCL5, CXCL1, CCR1, KCNJ15, SDC2, and CREM) that were significantly up-regulated in healthy PBMCs cultured with RO sera. Parallel studies directly examining PBMCs isolated from peripheral blood in general failed to show overexpression of these same genes in RO vs HC PBMCs, although the CCR1 transcript was 2.5-fold more abundant in RO than in the HC PBMCs ($p = 0.045$). These results, generated from PBMCs collected 2–6 mo postdiagnosis, are consistent with those of Kaizer et al., (32) in that our measurements were taken at a time when differential gene expression in T1DM PBMCs is reported to be largely diminished to levels similar to those of healthy controls.

Given that the IL-1 receptor antagonist has been identified as a successful therapy for JRA (20, 38), other autoinflammatory diseases (39), and most recently type II diabetes (40), questions are raised as to whether such treatment of recent onset diabetics may prolong the honeymoon period and whether treatment of Ab-positive/profile-positive AR subjects may delay or prevent T1DM onset. As a first step to address this possibility and dissect active inflammatory pathways, experiments to impair induction of the proinflammatory signature in vitro by blocking the action of IL-1β (and IL-1α) with IL-1RA are ongoing. Because cytokine milieu associated with T1DM is complex (15–19), we are also exploring the effect of immunodepletion of multiple cytokines in an effort to define the major contributors to the signature induced in healthy PBMCs by RO serum.

We have determined that sera of preonset and RO diabetics possess factors that induce a unique proinflammatory signature in PBMCs that is reflective of active autoimmunity. Although the overall sample size is modest, we observe a dramatic result that is supported through the comprehensive testing of appropriate additional subject cohorts. This study lays the foundation for urgent further work to define the utility of this approach in predicting onset in AR subjects and as a measure in primary and secondary prevention trials.

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Disclosures

The authors have no financial conflict of interest.

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