Monocyte Gene-Expression Profiles Associated With Childhood-Onset Type 1 Diabetes and Disease Risk: A Study of Identical Twins

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OBJECTIVE—Monocytes in childhood-onset type 1 diabetes show distinct gene expression. We hypothesize that monocyte activation in monozygotic (MZ) twin pairs discordant for childhood-onset type 1 diabetes could reflect distinct stages of the disease process including diabetes susceptibility (differences between twins, both diabetic and nondiabetic, and control subjects) and/or disease progression (differences between diabetic and nondiabetic twins).

RESEARCH DESIGN AND METHODS—We studied patterns of inflammatory gene expression in peripheral blood monocytes of MZ twin pairs ($n = 10$ pairs) discordant for childhood-onset type 1 diabetes, normal control twin pairs ($n = 10$ pairs), and healthy control subjects ($n = 51$) using quantitative-PCR (Q-PCR). We tested the 24 genes previously observed by whole genome analyses and verified by Q-PCR in autoimmune diabetes and performed a hierarchical cluster analysis.

RESULTS—Of 24 genes abnormally expressed in childhood-onset type 1 diabetes, we revalidated abnormal expression in 16 of them in diabetic twins including distinct sets of downregulated ($P < 0.03$) and upregulated ($P < 0.02$) genes. Of these 16 genes, 13 were abnormally expressed in nondiabetic twins, implicating these genes in diabetes susceptibility ($P < 0.044$ for all). Cluster analysis of monocyte gene-expression in nondiabetic twins identified two distinct, mutually exclusive clusters, while diabetic twins had a network of positively correlated genes.

CONCLUSIONS—Patients with childhood-onset type 1 diabetes show abnormal monocyte gene–expression levels with an altered gene–expression network due to gene-environment interaction. Importantly, perturbed gene–expression clusters were also detected in nondiabetic twins, implicating monocyte abnormalities in susceptibility to diabetes. ***Diabetes*** **59:1751–1755, 2010**

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The destructive autoimmune process associated with type 1 diabetes involves both the innate and adaptive immune response represented by monocytes, dendritic cells, macrophages, and T-cells, which infiltrate the islets at disease onset (1). Patients with type 1 diabetes show functional abnormalities of monocytes and monocyte-derived cells (2–8), which are assumed to promote the immunogenic potential of the cells.

Recently, we reported that type 1 diabetic patients show abnormal monocyte gene–expression profiles involving 24 inflammatory-related genes (5). Two distinct sets of correlating genes were found. One cluster consisted of downregulation of 12 core inflammatory cytokine/compound genes strongly correlated to the expression of *PDE4B* (the *PDE4B*-associated cluster). A second cluster was most prevalent in childhood-onset type 1 diabetes and consisted of 10 upregulated genes strongly correlated to the expression of *FABP5* (the *FABP5*-associated cluster).

These aberrant expression profiles in monocytes could be either familial, through shared genetic and/or environmental factors, or disease-associated. To resolve this issue, we studied monocytes of monozygotic (MZ) twin pairs discordant for childhood-onset type 1 diabetes, i.e., one twin had type 1 diabetes and the other did not. Gene-expression abnormalities associated with type 1 diabetes susceptibility would be found in both type 1 diabetic twins and their nondiabetic twins (such similarities between MZ twins being due to shared genes, shared environment, or both), but not in normal healthy individuals (twin pair case–control design). Changes associated with type 1 diabetes progression would only be found in the type 1 diabetic twin (co-twin case–control design).

RESEARCH DESIGN AND METHODS

Twins and control subjects. MZ twin pairs were selected from the British Diabetic Twin Study (9). Of 451 twin pairs, we selected MZ pairs discordant for type 1 diabetes and eligible according to the following criteria: *1*) European origin, *2*) affected twins had type 1 diabetes (diagnosed according to American Diabetes Association guidelines), *3*) both twins of each pair were available for study, *4*) neither twin was receiving drugs other than human insulin in the index case, and *5*) the nondiabetic twin had a low disease risk, which is a risk less than 2% based on the lack of diabetes-associated antibodies (9,10). Of the selected 10 MZ pairs discordant for type 1 diabetes ($n = 10$, mean age 32 years, range 18–50 years, three males), the diabetic twins were treated with insulin from the time of diagnosis and were taking highly purified human insulin at least twice daily (means [± SD] duration of diabetes 22 years [11] in the diabetic twin). Monozygosity was established in twin pairs using both clinical data and at least 22 blood groups as described previously (9,10). Control MZ twin pairs ($n = 12$, mean age 37 years [11], range 17–53 years, four males) were recruited from the local population through advertising. Childhood-onset type 1 diabetes singletons ($n = 30$, mean age 24 years, range 5–50 years, 11 males) were identified from our previous study (5).

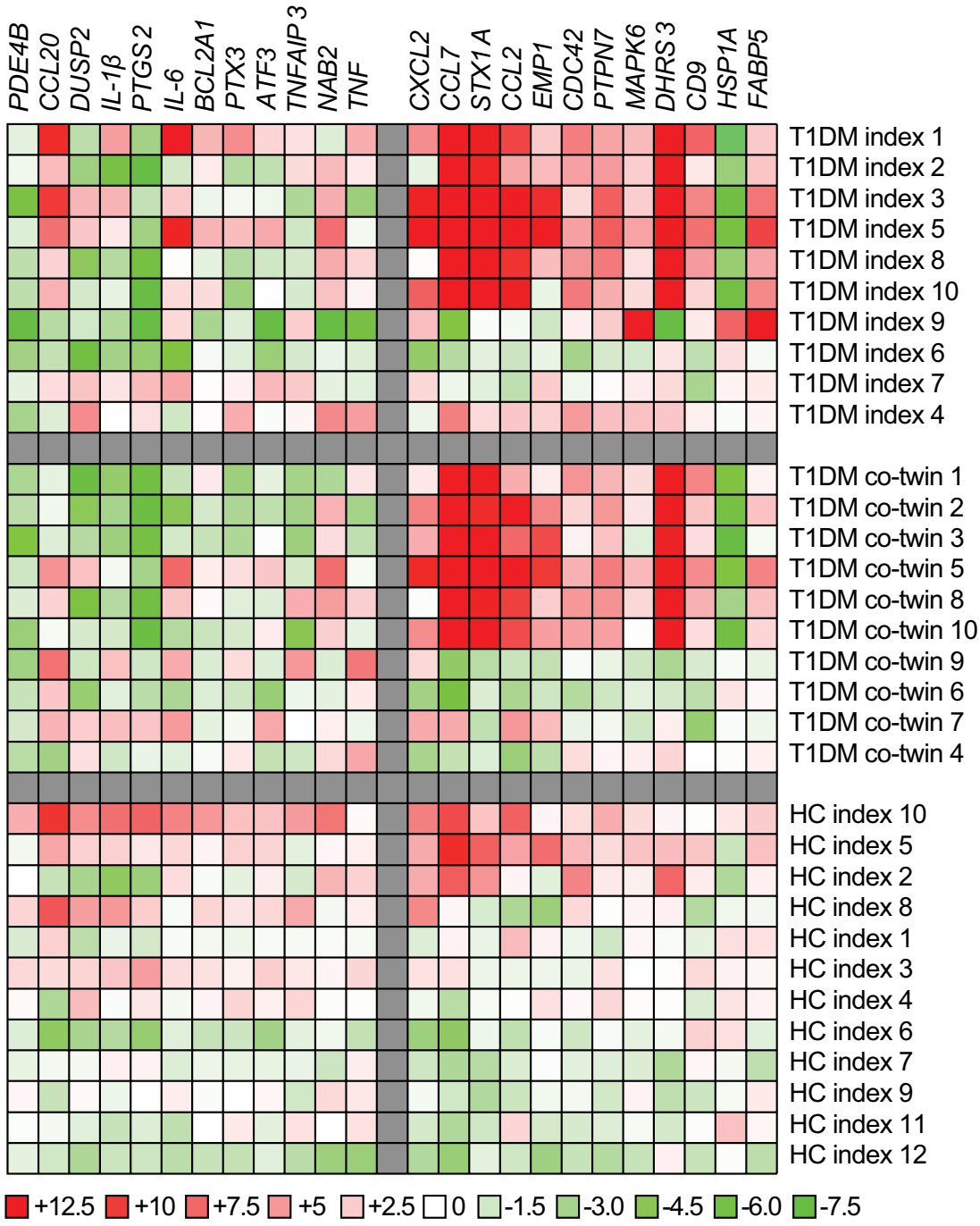

FIG. 1. This figure shows monocyte gene–expression levels from diabetic index twins, nondiabetic co-twins, and the index twin of a healthy control (HC) twin pair. Fold changes (analyzed as in Table 1) are presented similarly with increased (red) or decreased (green) expression levels (the color intensities code for the strength of the expression levels). A gray line separates two distinct gene–expression profiles (*PDE4B*- and *FABP5*-associated clusters). For the majority, diabetic and nondiabetic twins show similar increased or decreased gene–expression levels compared with HC twins. (A high-quality digital representation of this figure is available in the online issue.)

Healthy control singletons (n = 51, mean age 39 years, range 21–67 years, 23 males) were recruited from enrolling laboratory staff, medical staff, and medical students. The inclusion criteria for the healthy controls were no family history of diabetes or other autoimmune disease; no illness at the time of testing or for at least two weeks prior to the blood withdrawal including acute infections and allergic reactions; taking no drugs; and on a normal diet. All the subjects gave informed consent. The ethics committees of Bart's and The London National Health Service Trust and Royal Hospital Trusts, Heinrich-Heine University Düsseldorf, and the Erasmus MC Medical Centre Rotterdam approved the study.

Blood collection and monocyte isolation. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by the standard Ficoll procedure (11). Purified PBMCs were then frozen in 10% DMSO and stored in liquid nitrogen. This enabled us to store the samples in order to batch samples for assays. CD14+ monocytes were isolated by positive selection as described from frozen PBMCs using a magnetic cell sorting system (Miltenyi Biotec, U.K.); monocyte purity was >95% (determined by morphological screening after trypan blue staining and fluorescence-activated cell sorter).

Quantitative RT–PCR. In this present study, RNA was isolated from monocytes using RNeasy columns (Qiagen, Hilden, Germany), and both this method and quantitative RT-PCR has been described in detail elsewhere (11). Genes under study are the sets of genes previously described as aberrantly expressed in type 1 and type 2 diabetes. All Taqman probes and consensus primers were preformulated and custom designed by Applied Biosystems (Supplemental Table 1). PCR amplification of the housekeeping gene *ABL* was performed for each sample to allow normalization between the

TABLE 1
Q-PCR analysis of monocytes of MZ twins with and without type 1 diabetes and healthy control (HC) MZ twin pairs. The quantitative values are expressed as fold changes; in essence, the quantitative value obtained from Q-PCR is a CT. The fold change values between different groups were determined from normalized CT values as previously described (5). Data were standardized to 51 healthy control singletons (used as the calibrator). Significance was tested by ANCOVA so that values >1 (*) reflect higher expression and <1 (†) lower expression in twins than in these control singletons

	Index diabetic twin			Nondiabetic co-twin		
	fold change	*P* value vs. HC		fold change	*P* value vs. HC	*P* value vs. co-twin
PDE4B	0.29†	0.001	*PDE4B*	0.32†	0.001	0.741
CCL20	0.45	0.482	*CCL20*	0.27	0.129	0.425
DUSP2	0.74	0.067	*DUSP2*	0.44†	0.018	0.142
IL-1B	0.16†	0.001	*IL-1B*	0.12†	0.001	0.650
PTGS2	0.23†	0.014	*PTGS2*	0.25†	0.014	0.899
IL-6	1.99	0.448	*IL-6*	0.86	0.999	0.216
BCL2A1	0.42†	0.021	*BCL2A1*	0.29†	0.001	0.076
PTX3	0.66	0.129	*PTX3*	0.46†	0.027	0.360
ATF3	0.86	0.224	*ATF3*	1.00	0.697	0.654
TNFAIP3	0.54†	0.007	*TNFAIP3*	0.35†	0.001	0.266
NAB2	1.26	0.499	*NAB2*	1.75	0.601	0.569
TNF	0.35†	0.026	*TNF*	0.48†	0.039	0.561
CXCL2	0.41	0.035	*CXCL2*	0.29†	0.016	0.364
CCL7	75.58*	0.009	*CCL7*	43.71*	0.033	0.425
STX1A	13.83*	0.002	*STX1A*	9.00	0.081	0.019*
CCL2	14.83*	0.017	*CCL2*	8.94	0.074	0.380
EMP1	5.43*	0.001	*EMP1*	4.56*	0.007	0.544
CDC42	2.19	0.329	*CDC42*	1.60	0.754	0.015*
PTPN7	3.36*	0.004	*PTPN7*	2.22*	0.043	0.023*
MAPK6	1.75	0.106	*MAPK6*	0.77	0.218	0.082
DHRS3	6.11*	0.017	*DHRS3*	7.06	0.203	0.741
CD9	3.94*	0.005	*CD9*	2.81*	0.014	0.040*
HSP1A	0.28†	0.001	*HSP1A*	0.18†	0.001	0.108
FABP5	7.84*	0.001	*FABP5*	2.89*	0.002	0.064

samples. For details of the primers and probes used, see supplementary Table 1 in the online appendix available at http://diabetes.diabetesjournals.org/cgi/content/full/db09-1433/DC1. The quantitative value obtained from Q-PCR is a cycle threshold (CT). The fold change values between different groups were determined from the normalized CT values (CT gene − CT housekeeping gene).

Statistical analysis. Data were assembled using Microsoft Excel software and are expressed as means (± SD). Data were analyzed using SPSS/the Prism statistical package (SPSS 15.0 for Windows/GraphPad Prism, version 3). Data were tested for normal distribution using the Kolmogorov-Smirnov test. Hierarchical gene analysis and heat maps were determined using a Pearson correlation matrix. Depending on the distribution pattern and the total number of subjects, parametric (normal distribution and ≥50 subjects) or nonparametric tests (skewed distribution or <50 subjects) were used. Levels of significance were set at $P < 0.05$ (two-tailed).

RESULTS

Q-PCR analysis. A preliminary observation and validation study identified 24 genes abnormally expressed in monocytes from type 1 diabetic patients (5). These 24 genes were examined in this revalidation study of MZ twins and the gene expression levels of 16 of these 24 monocyte genes were abnormally expressed in twins with childhood-onset type 1 diabetes when compared with 12 normal MZ twin pairs and 51 normal singletons (Fig. 1) (Table 1). These 16 abnormally expressed genes were *PDE4B*, *IL-1B*, *PTGS2*, *BCL2A1*, *TNFAIP3*, *TNF*, *CXCL2*, *HSP1A* (all downregulated) ($P < 0.04$ for all) and *CCL2*, *CCL7*, *STX1A*, *EMP1*, *PTPN7*, *CD9*, *FABP5*, *DHRS3* (all upregulated) ($P < 0.02$ for all) (Fig. 1) (Table 1).

To determine whether the changes were familial, i.e., due to gene-environment interaction independent of diabetes and hyperglycemia, we examined the nondiabetic MZ twins of these patients, selected to be at low disease risk. Of 16 genes that were altered in the validation study in the index twins, 13 were also altered in the nondiabetic twins compared with control subjects: *PDE4B*, *IL-1B*, *PTGS2*, *BCL2A1*, *TNFAIP3*, *TNF*, *CXCL2*, *HSP1A* (all downregulate) ($P < 0.04$ for all) and *CCL7*, *EMP1*, *PTPN7*, *CD9*, *FABP5* (all upregulated) ($P < 0.044$ for all) (Fig. 1) (Fig. 1) (Table 1); 2 additional genes were abnormally expressed in nondiabetic, but not diabetic, twins, i.e., *DUSP2* and *PTX3* (both downregulated, $P < 0.05$ for each) (Table 1). Only four genes (*STX1A*, *CDC42*, *PTPN7*, and *CD9*) were abnormally expressed in diabetic twins compared with both nondiabetic twins and control subjects ($P < 0.041$ for all), consistent with a diabetic effect. These four genes are also abnormally expressed in monocytes of type 2 diabetic patients, consistent with a metabolic effect (5).

Cluster analysis. Diabetic twins had a similar gene-expression profile and gene order in cluster analysis to that found previously in childhood-onset type 1 diabetes singletons (Fig. 2*A* and *C*); strongly positively correlated genes clustered in both diabetic groups with the dominant cluster extending from *CCL2* to *NAB2* (Fig. 2*A* and *C*). Nondiabetic twins showed similar abnormalities of gene expression (13 of 16) (data not shown) and gene order to their diabetic twins and a similar positively correlating dominant gene cluster (Fig. 2*B*); by contrast, the gene order differed from control twins (data not shown), and the latter had no comparable dominant cluster (Fig. 2*D*). Both diabetic twins and singletons had two genes (*PTGS2* and *HSPA1A*) strongly negatively correlated with the dominant cluster (Fig. 2*A* and *C*); in the nondiabetic twins, these negatively correlated genes extended into a cluster from *PDE4B* to *CCL20* (Fig. 2*B*). The two gene-expression clusters evident in the nondiabetic twins broadly corresponded to the *PDE4B*-associated and *FABP5*-associated gene clusters with the latter previously reported by us as the major gene cluster in childhood-onset

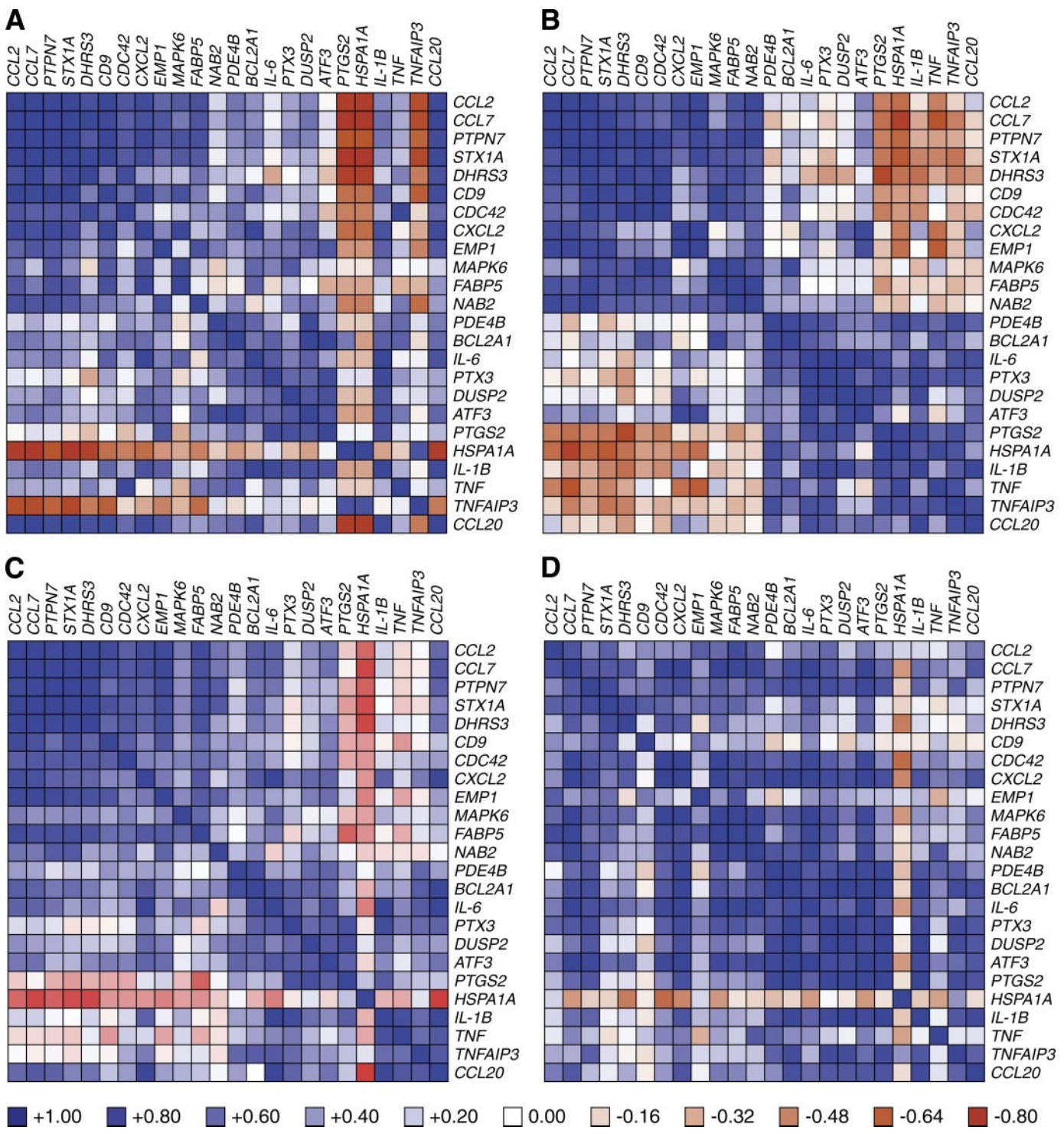

FIG. 2. This figure shows a heat map of correlations between gene-expression levels for 24 genes initially observed to be abnormally expressed in type 1 diabetes in: index diabetic twins (*A*), their nondiabetic co-twins (*B*), diabetic singletons (*C*), and healthy control twins (*D*). Correlations in gene-expression level are presented as positive (blue) or negative (red) expression levels (the color intensities code for the strength of the correlations). The hierarchy of gene expression (found in cluster analysis) in all figures is ordered according to that in the index diabetic twins; two distinct clusters are noted in nondiabetic co-twins. (A high-quality digital representation of this figure is available in the online issue.)

type 1 diabetes (5). No dominant cluster in diabetic and nondiabetic twins or diabetic singletons was found in control twins. Importantly, the disease discordant twin pairs showed significant correlations for 12 of 16 abnormally expressed genes (r [range] 0.68–0.95; $P < 0.05$ for all 12) (data not shown), which was consistent with a strong shared gene-environment interaction.

DISCUSSION

We previously reported that monocyte gene–expression profiles are abnormal in type 1 diabetes and vary according to age at diabetes onset, suggesting heterogeneity in disease pathogenesis (5). We revalidated abnormal monocyte gene–expression levels for 16 genes in twins with

childhood-onset type 1 diabetes. Cluster analysis found that the gene order was similar in both twins and singletons with childhood-onset type 1 diabetes, but was distinct from that found in control twins and singletons. Diabetic twins and singletons showed a dominant cluster of 12 abnormally expressed monocytes genes, including *FABP5*. Our failure to revalidate all the abnormally expressed monocyte genes initially reported could be due to the relatively small numbers of highly selected twin pairs, including selection of only childhood-onset type 1 diabetic patients, which will have limited the study power.

The present observations in type 1 diabetes indicate that much of the altered monocyte gene–expression profile is due to shared gene-environment interaction since similar changes were detected in their genetically identical twins who were not diabetic and selected to be at low disease risk. Such similarities between MZ twins are not necessarily due to shared genes as shared environmental effects or shared gene/environment interaction would give the same result. Changes in these nondiabetic twins likely reflect monocyte changes associated with disease susceptibility without necessarily predicting type 1 diabetes. Of 16 monocyte genes abnormally expressed in type 1 diabetic twins, 13 were abnormal in their nondiabetic twins and gene-expression levels for the majority were strongly correlated between diabetic and nondiabetic twins. Moreover, in contrast to control subjects, the gene order and dominant gene cluster on cluster analysis were broadly similar in co-twins. It follows from our observations that most abnormalities in monocyte gene expression in childhood-onset type 1 diabetes are associated with familial predisposition to the disease, consistent with a previous study of monocyte responses in disease-discordant twins (7). Resolution of the degree of genetic contribution to altered monocyte gene expression must await a classic twin study; but this would require substantially more MZ twins discordant for type 1 diabetes—as well as matched dizygotic twins—than we studied.

Cluster analysis showed a striking disparity between nondiabetic twins and other groups in that they had two clear gene–expression clusters and one cluster negatively correlated with the other. The dominant monocyte gene–expression cluster, with an upregulated *FABP5*, included genes involved in adhesion, chemotactic, and metabolic factors, while the less prominent negatively correlated and downregulated *PDE4B*-associated cluster involved proinflammatory cytokines and chemokines (5). These two gene clusters were perturbed in the diabetic twins into a network of predominantly positively correlated genes with a weaker downregulation of the *PDE4B*-associated gene cluster, potentially representing a progression marker to clinically overt type 1 diabetes. Longitudinal studies of at-risk individuals could clarify if such changes reflect disease progression.

Our aberrant gene–expression data implicate altered monocyte function in the pathogenesis of childhood-onset type 1 diabetes through gene-environment interaction. The character of the genes and previous studies (6,7,12) suggest that these functional changes include altered monocyte integrin expression, adhesion to endothelial cells and fibronectin, lipid raft and immune synapse formation, cell motility and assembly, and induction of proinflammatory cells. In-depth functional studies using transfection or small interfering RNA regulating the expression of selected key genes in human monocytes will need to be performed to define their exact function in diabetes pathogenesis. A substantially expanded classic twin study, also using dizygotic twins, would be required to determine to what extent genetic factors alone determine the altered gene expression. Nevertheless, identification of key monocyte genes involved in the perturbed gene–expression networks predisposing to type 1 diabetes suggests possible therapeutic targets to prevent progression to clinical disease.

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No potential conflicts of interest relevant to this article were reported.

Author contributions to the study design include: H.B., H.A.D., and R.D.L. provided the samples; H.B., R.C.D., L.v.d.H.N., H.d.W., and R.C.P. researched the data; H.B., H.A.D., and R.D.L. wrote the initial manuscript; R.C.D. and N.C.S. reviewed/edited the manuscript; and all the authors contributed to the discussion.

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REFERENCES

1. Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. Lancet 2001;358:221–229
2. Devaraj S, Glaser N, Griffen S, Wang-Polagruto J, Miguelino E, Jialal I. Increased monocytic activity and biomarkers of inflammation in patients with type 1 diabetes. Diabetes 2006;55:774–779
3. Litherland SA, She JX, Schatz D, Fuller K, Hutson AD, Peng RH, Li Y, Grebe KM, Whittaker DS, Bahjat K, Hopkins D, Fang Q, Spies PD, North K, Wasserfall C, Cook R, Dennis MA, Crockett S, Sleasman J, Kocher J, Muir A, Silverstein J, Atkinson M, Clare-Salzler MJ. Aberrant monocyte prostaglandin synthase 2 (PGS2) expression in type 1 diabetes before and after disease onset. Pediatr Diabetes 2003;4:10–18
4. Skarsvik S, Tiittanen M, Lindström A, Casas R, Ludvigsson J, Vaarala O. Poor *in vitro* maturation and pro-inflammatory cytokine response of dendritic cells in children at genetic risk of type 1 diabetes. Scand J Immunol 2004;60:647–652
5. Padmos RC, Schloot NC, Beyan H, Ruwhof C, Staal FJ, de Ridder D, Aanstoot HJ, Lam-Tse WK, de Wit H, de Herder C, Drexhage RC, Menart B, Leslie RD, Drexhage HA, LADA Consortium. Distinct monocyte gene-expression profiles in autoimmune diabetes. Diabetes 2008;57:2768–2773
6. Bouma G, Lam-Tse WK, Wierenga-Wolf AF, Drexhage HA, Versnel MA. Increased serum levels of MRP-8/14 in type 1 diabetes induce an increased expression of CD11b and an enhanced adhesion of circulating monocytes to fibronectin. Diabetes 2004;53:1979–1986
7. Beyan H, Goodier MR, Nawroly NS, Hawa MI, Bustin SA, Ogunkolade WB, Londei M, Yousaf N, Leslie RD. Altered monocyte cyclooxygenase response to lipopolysaccharide in type 1 diabetes. Diabetes 2006;55:3439–3445
8. Zacher T, Knerr I, Rascher W, Kalden JR, Wassmuth R. Characterization of monocyte-derived dendritic cells in recent-onset diabetes mellitus type 1. Clin Immunol 2002;105:17–24
9. Hawa M, Rowe R, Lan MS, Notkins AL, Pozzilli P, Christie MR, Leslie RD. Value of antibodies to islet protein tyrosine phosphatase-like molecule in predicting type 1 diabetes. Diabetes 1997;46:1270–1275
10. Redondo MJ, Yu L, Hawa M, Mackenzie T, Pyke DA, Eisenbarth GS, Leslie RD. Heterogeneity of type I diabetes: analysis of monozygotic twins in Great Britain and the United States. Diabetologia 2001;44:354–362
11. Padmos RC, Hillegers MH, Knijff EM, Vonk R, Bouvy A, Staal FJ, de Ridder D, Kupka RW, Nolen WA, Drexhage HA. A discriminating messenger RNA signature for bipolar disorder formed by an aberrant expression of inflammatory genes in monocytes. Arch Gen Psychiatry 2008;65:395–407
12. Devaraj S, Dasu MR, Rockwood J, Winter W, Griffen SC, Jialal I. Increased toll-like receptor (TLR) 2 and TLR4 expression in monocytes from patients with type 1 diabetes: further evidence of a proinflammatory state. J Clin Endocrinol Metab 2008 93:578–583