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Association of Dendritic Cell Signatures With Autoimmune Inflammation Revealed by Single-Cell Profiling

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Objective. To identify single-cell transcriptional signatures of dendritic cells (DCs) that are associated with autoimmunity, and determine whether those DC signatures are correlated with the clinical heterogeneity of autoimmune disease.

Methods. Blood-derived DCs were single-cell sorted from the peripheral blood of patients with rheumatoid arthritis, systemic lupus erythematosus, or type 1 diabetes as well as healthy individuals. DCs were analyzed using single-cell gene expression assays, performed immediately after isolation or after in vitro stimulation of the cells. In addition, protein expression was measured using fluorescence-activated cell sorting.

Results. CD1c+ conventional DCs and plasmacytoid DCs from healthy individuals exhibited diverse transcriptional signatures, while the DC transcriptional signatures in patients with autoimmune disease were altered. In particular, distinct DC clusters, characterized by up-regulation of *TAP1*, *IRF7*, and *IFNAR1*, were abundant in patients with systemic autoimmune disease, whereas DCs from patients with type 1 diabetes had decreased expression of the regulatory genes *PTPN6*, *TGFB*, and *TYROBP*. The frequency of CD1c+ conventional DCs that expressed a systemic autoimmune profile directly correlated with the extent of disease activity in patients with rheumatoid arthritis (Spearman's $r = 0.60$, $P = 0.03$).

Conclusion. DC transcriptional signatures are altered in patients with autoimmune disease and are associated with the level of disease activity, suggesting that immune cell transcriptional profiling could improve our ability to detect and understand the heterogeneity of these diseases, and could guide treatment choices in patients with a complex autoimmune disease.

INTRODUCTION

Autoimmunity occurs when the immune system mounts an unfavorable response toward a self antigen, which may lead to tissue damage and disease. The pathogenesis of autoimmune diseases is complex and the clinical manifestation of each disease varies between patients, which makes it difficult to predict the effectiveness of treatments or preventative strategies (1). For type 1 diabetes (T1D), a pancreas-specific autoimmune disease, immunotherapy has had limited success in reversing the disease or preventing progression to end-stage disease (2–4). In contrast, in many systemic autoimmune diseases, such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), treatment with immunomodulatory and immunosuppressive agents has optimally achieved clinical remission. However, even with continued therapy, remission will often not last, and therefore regular, lifelong reassessment of disease activity may be required. Moreover, the very same drug that yields measurable benefits in one patient with the disease may have no measurable effect in other patients with the same disease.

Dendritic cells (DCs) are important regulators of immunity that provide immunogenic and tolerogenic signals, which shape the adaptive immune response (5,6). In animal models, the absence of DCs or their abnormal function has been shown to induce autoimmunity, while alterations in the number of DCs, cytokine secretion, transcriptional signaling, and cell migration have been

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associated with development and progression of autoimmune diseases in humans (7–9). The DC compartment in mice and humans comprises several specialized subsets with different origins, localizations, morphologies, cytokine secretion patterns, and immunologic functions (5,6). Because these functionally distinct subsets have the potential to influence autoimmune responses in multiple ways, it has been proposed that diverse DC abnormalities could explain the broad spectrum of immunopathologic features and therapeutic responses in patients with autoimmune disease (7). Therefore, altered DC profiles may be associated with disease heterogeneity and could serve as a useful biomarker for monitoring disease pathogenesis or for predicting the response to treatment.

To explore this hypothesis, we used single-cell gene expression assays to analyze the diversity of blood-derived DCs, assess how the DC phenotype is altered in autoimmunity, and determine whether the alterations are correlated with the extent of disease activity. We profiled blood-derived CD1c+ conventional (or classical) DCs (cDCs) and plasmacytoid DCs (pDCs) from patients diagnosed as having either RA, SLE, or recent-onset T1D, using single-cell reverse transcription–polymerase chain reaction (RT-PCR) with a select panel of genes. The transcriptional profile of both of these DC subsets was altered in patients with systemic autoimmunity. Moreover, the frequency of CD1c+ cDCs that were characterized by a transcriptional signature associated with autoimmune disease varied among RA patients and was correlated with the extent of disease activity. Thus, our study shows a relationship between DC transcriptional profiles and autoimmune disease, and highlights the feasibility of profiling DCs or other immune cells to better understand the clinical heterogeneity of these diseases.

PATIENTS AND METHODS

Subjects. Samples of peripheral blood were collected from children with recent-onset T1D who were enrolled in the Diabetes Mellitus Incidence Cohort Registry (DiMelli) study (10), and from age-matched healthy children from the Prospective Evaluation of Risk Factors for the Development of Islet Autoimmunity and Type 1 Diabetes during Puberty (TEENDIAB) study (11). Protocols were approved by the Ethikkommission der Bayerischen Landesärztekammer (approval no. 08043) and the Ethikkommission der Fakultät für Medizin der Technischen Universität München (approval no. 2149/08). In addition, blood samples were collected from consenting patients with SLE or RA who fulfilled the respective American College of Rheumatology (ACR) (12) or ACR/European League Against Rheumatism (13) classification criteria, with approval provided by the ethics committee of TU Dresden (protocol no. EK 337122008). Routine clinical assessment parameters and C-reactive protein (CRP) values were obtained from the patients' charts.

Blood samples from healthy adults were provided by the Deutsches Rotes Kreuz Blutspendedienst Ost (Dresden, Germany) after the subjects had given their informed consent and approval was provided by the ethics committee of TU Dresden (protocol no. EK 240062016). All research was performed in accordance with the Declaration of Helsinki.

Cell stimulation with Toll-like receptor 7 (TLR-7). Frozen peripheral blood mononuclear cells (PBMCs) were thawed and seeded into a 48-well tissue culture plate at 2.5×10^6 cells/ml in RPMI medium containing 10% fetal bovine serum, 1% streptomycin, 1% penicillin, and 1% L-glutamine with or without 2.5 μg/ml R848, a TLR-7 agonist (InvivoGen). Thereafter, the cells were incubated at 37°C in an atmosphere of 5% CO_2 for 3 hours, and then harvested, washed, and prepared for single-cell sorting of pDCs.

Intracellular staining. PBMCs were isolated by density centrifugation of sodium-heparinized peripheral venous blood samples over Ficoll-Hypaque. PBMCs were incubated with a Live/Dead Fixable Blue Dead Cell Stain Kit (ThermoFisher) for 20 minutes, and then washed, fixed, permeabilized, and incubated with a cocktail of anti-human monoclonal antibodies (mAb) (allophycocyanin [APC]–conjugated LIN, BV650-conjugated CD123, and V450-conjugated GZM mAb [BioLegend], phycoerythrin [PE]–Cy7–conjugated HLA–DR and BV785-conjugated CD303 mAb [BD Biosciences], AF700-conjugated IFNAR1 mAb [R&D Systems], PerCP–eF710–conjugated IRF8 mAb [eBioscience], and PE-conjugated IRF7 and PE–Vio770–conjugated IRF7[pS477/pS479] mAb [Miltenyi]) using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience).

Cells were acquired and analyzed using a BD LSRII fluorescence-activated cell sorter, with the results analyzed using FACSDiva and FlowJo software (BD Biosciences). Aliquots of frozen PBMCs from a healthy donor were stained and analyzed concurrently with the study samples to control for interexperimental variation (see Statistical Analysis for more details). CST beads (BD Biosciences) were used to calibrate the instrument before each analysis.

Single-cell sorting of DC populations. PBMCs were isolated by density centrifugation of sodium-heparinized peripheral venous blood samples over Ficoll-Hypaque. Nonspecific binding was blocked by incubating cells with a human Fc blocking reagent (Miltenyi) before adding a cocktail of anti-human mAb (APC-conjugated CD3, APC-conjugated CD19, PE–Cy7–conjugated CD14, PE–Cy7–conjugated CD56, and APC–Cy7–conjugated HLA–DR mAb [BD Biosciences], AF700-conjugated CD11c mAb [eBioscience], and BV650-conjugated CD123, BV605-conjugated CD15, BV421-conjugated CD141, and AF488-conjugated CD1c mAb [Biolegend]). Ten minutes before acquisition, 7-aminoactinomycin D (BD Biosciences) was added to the samples to enable discrimination of dead cells. Cells were acquired and sorted using

a BD FACSAria III, with results analyzed using FACSDiva software (BD Biosciences). Doublets and clumps were excluded using the side scatter–height versus side scatter–width plot. Two DC subsets, pDCs (CD3–CD19–CD56–CD14–CD16–HLA–DR+CD11c–CD123+) and CD1c+ cDCs (CD3–CD19–CD56–CD14–CD16–HLA–DR+CD11c+CD141–CD1c+), were single-cell sorted into 96-well PCR plates containing 5 μl of EB elution buffer (Qiagen). The cells were then frozen at –80°C for RT-PCR analysis.

Gene expression analysis. Gene expression analysis of single-cell–sorted DCs was performed as previously described, with some modifications (14). Total complementary DNA was preamplified for 20 cycles (1 cycle at 95°C for 1 minute, 20 cycles at 95°C for 15 seconds, 60°C for 1 minute, and 72°C for 1.5 minutes, and 1 cycle at 72°C for 10 minutes) with TATAA GrandMaster Mix (TATAA Biocenter) and 29 primer pairs (see Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40793/abstract) at a final concentration of 25 n*M* in a total reaction volume of 35 μl. Raw data were preprocessed as previously described (14) to regress out plate effects on each individual gene while controlling for group effects. Thus, all gene expression values are shown as regressed C_t values, where a value of 0 indicates no gene expression.

Statistical analysis. Gene and protein expression levels are reported as the mean with 95% confidence intervals (95% CIs) or mean ± SEM. Various statistical tests were used (as described in the figure legends). To adjust for interexperimental variability, the fluorescence intensity values recorded during index sorting are displayed as the z-scores of data from each independent experiment. Single-cell gene expression correlation analyses were performed using corrplot, with a significance threshold of 0.001 (15). To identify biologically meaningful transcriptional profiles, t-distributed stochastic neighbor embedding (t-SNE) dimensions were calculated with Rtsne (16,17), and unsupervised Ward hierarchical clustering was performed with hclust.

The significance of differential gene expression was determined using the Hurdle model (18), with correction for false-discovery rate and with a significance threshold of 0.001. Protein expression, as measured by the mean fluorescence intensity (MFI), was normalized for each fluorescence channel by dividing the MFI by the value for an internal control and then multiplying by the mean MFI value for all samples. All statistical analyses were performed using GraphPad Prism version 5 or RStudio software.

RESULTS

Single-cell expression analysis of DC populations. To investigate the transcriptional profile of DCs, CD1c+ cDCs and pDCs were freshly isolated from PBMCs by single-cell sorting (Figure 1A) and then individually analyzed for gene expression using single-cell RT-PCR analysis, as previously described (14). We selected a panel of 29 genes involved in various aspects of DC function, including pathogen recognition, antigen uptake and processing, type I interferon (IFN) signaling and response, negative regulation, and cytokine/chemokine signaling.

The analysis of 327 CD1c+ cDCs and 325 pDCs isolated from 9 healthy adults (1 male, 8 female; median age 47 years [interquartile range (IQR) 30 to 59 years]) showed subset-specific expression of several genes, including *CD1C* in CD1c+ cDCs and *GZMB* in pDCs (Figure 1B). A common pDC marker at the protein level, *NRP1*, was included in the gene panel, but limited and variable expression of *NRP1* was observed in single cells, suggesting that the detection of this gene may be compromised in this single-cell assay. Furthermore, t-SNE analysis based on our gene panel showed that the gene signatures of the 2 DC subsets were distinct (Figure 1C).

The *CD1C* transcript was exclusively, but not universally, detected in CD1c+ cDCs (Figure 1B) and its abundance was correlated with CD1c protein expression, which was recorded as the fluorescence intensity (i.e., the MFI) by index sorting (for results, see Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40793/abstract). CD1c protein expression was significantly lower in CD1c+ cDCs with undetectable *CD1C* transcript expression than in CD1c+ cDCs with *CD1C* transcript expression (MFI 4,486 [95% CI 3,629, 5,344] versus MFI 8,258 [95% CI 7,310, 9,206]; $P < 0.0001$) (Supplementary Figure 1A [http://onlinelibrary.wiley.com/doi/10.1002/art.40793/abstract]). In cells with detectable *CD1C* transcripts, the gene expression was positively correlated with CD1c protein expression (Spearman's $r = 0.26$, $P < 0.0001$) (Supplementary Figure 1B [http://onlinelibrary.wiley.com/doi/10.1002/art.40793/abstract]), indicating that the quantitative difference in transcript expression measured using this analytic method is likely to be a reflection of true biologic variation.

Heatmap analysis based on the restricted gene panel revealed genetic heterogeneity in both DC subsets (Figure 1B). For example, we observed a subpopulation of pDCs that expressed *CD86* but not *GZMB*. These *CD86*-expressing DCs within the pDC flow cytometry gate also displayed reduced CD123 protein expression and increased CD141 protein expression (Supplementary Figure 1C [http://onlinelibrary.wiley.com/doi/10.1002/art.40793/abstract]). This subset of cells may correspond to the newly identified pre-DC subset, which is functionally distinct from pDCs (19,20).

We examined the coexpression of genes at the single-cell level using Spearman's correlation analysis (Figure 1D). The regulatory genes *PTPN6*, *TYROBP*, *STAT3*, *IRF8*, and *TGFB* were significantly coexpressed in both DC subsets. Expression of *TGFB* and *TYROBP* in pDCs was also significantly correlated with the expression of other genes, including the type I IFN–related

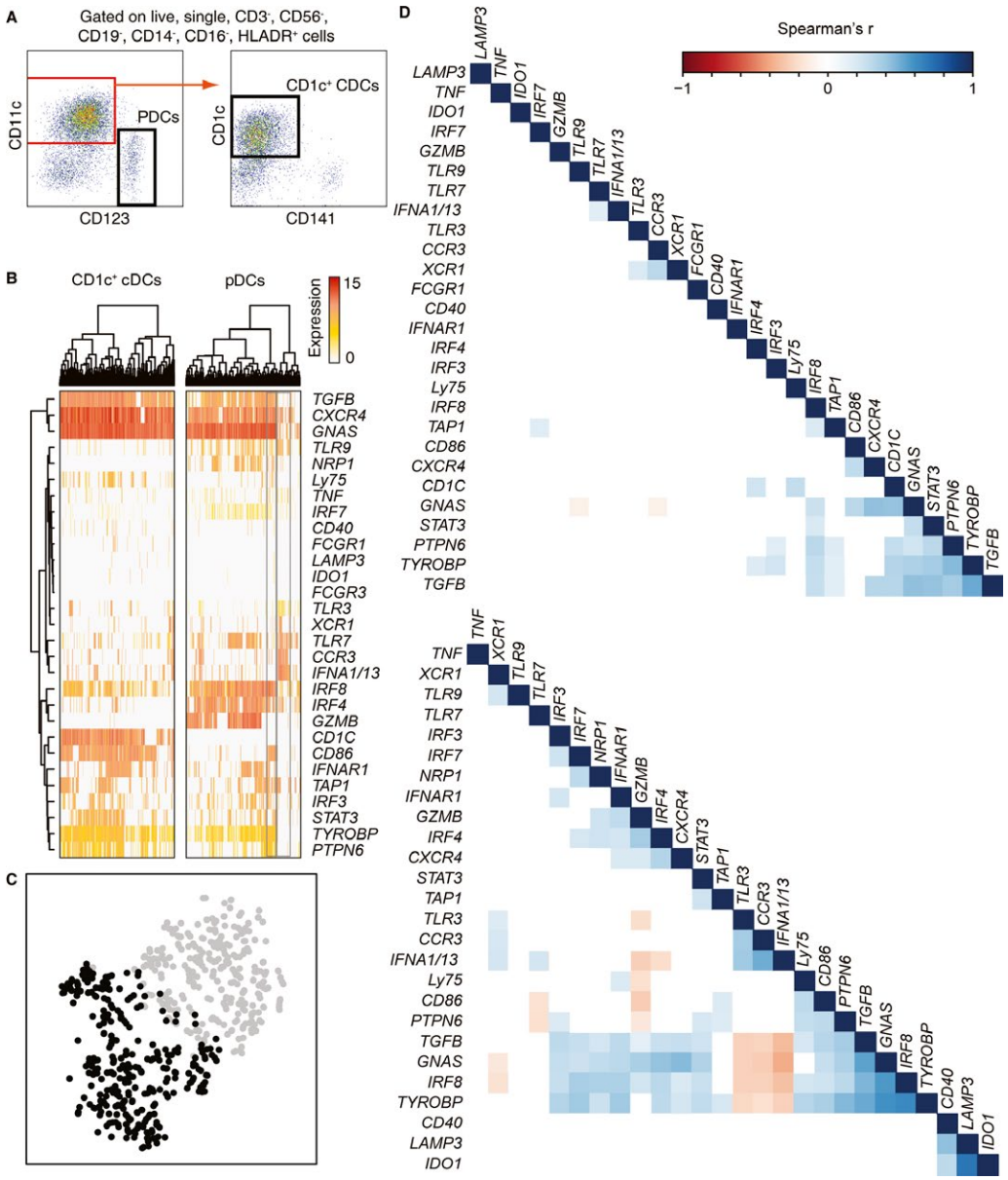

Figure 1. Transcriptional profiles of dendritic cell (DC) subsets isolated from the peripheral blood of healthy adults. **A**, Subsets of DCs were prepared by single-cell sorting of fresh peripheral blood mononuclear cells (PBMCs). After removing doublets, dead cells, and cells expressing CD3, CD56, CD19, CD14, and CD16, plasmacytoid DCs (pDCs) were identified as $CD11c^{low}CD123+$ cells, and CD1c+ conventional DCs (cDCs) were identified as $CD11c^{high}CD141-CD1c+$ cells. **B**, Heatmaps of single-cell gene expression data in CD1c+ cDCs and pDCs from the PBMCs of healthy adults are shown. Unsupervised Ward hierarchical clustering was applied to cells and genes. Clusters of gene coexpression are denoted by adjoining lines on the top and left. **C**, Analysis by t-distributed stochastic neighbor embedding shows gene expression data in CD1c+ cDCs (gray-shaded circles) and pDCs (black circles). Each circle represents a single cell. **D**, Correlation matrices of genes expressed by CD1c+ cDCs (top) and pDCs (bottom) from the PBMCs of 9 healthy adults are shown. Different colors represent the significance of the correlations ($P < 0.001$) by Spearman's correlation analysis.

genes *IFNAR1*, *IRF7*, and *IRF3*. The pDCs also significantly coexpressed *TLR3*, *CCR3*, and *IFNA1/13*, and this correlation appeared to arise from a distinct subpopulation of cells observed on the heatmap (Figure 1B).

These findings confirm that there is marked diversity within the CD1c+ cDC and pDC populations in peripheral blood. This diversity can be defined based on a relatively small number of genes, and the quantitative differences in transcript expression are likely to be biologically relevant.

DC transcription phenotypes in systemic and organ-specific autoimmunity. To investigate the changes in DC transcriptional profiles in patients with autoimmune disease, we sorted fresh peripheral blood–derived pDCs and CD1c+ cDCs

from patients with systemic or organ-specific autoimmunity as well as age-matched healthy individuals. Our previous data set obtained from healthy adults was reanalyzed in combination with pDCs and cDCs from patients with RA (550 pDCs and 524 cDCs from 13 patients [2 male, 11 female]; median age 59 years [IQR 45 to 62 years]), patients with SLE (286 pDCs and 282 cDCs from 7 patients [2 male, 5 female]; median age 40 years [IQR 32 to 50.5 years]), patients with recent-onset T1D (248 pDCs and 245 cDCs from 7 patients [3 male, 4 female]; median age 13.7 years [IQR 11.2 to 15.8 years]), and a second group of healthy individuals comprising healthy children who were age-matched to the T1D cohort (262 pDCs and 265 cDCs from 7 healthy children [4 male, 3 female]; median age 9.8 years [IQR 2.8 to 12.4 years]).

We identified 6 clusters of CD1c+ cDCs and 9 clusters of pDCs, based on unsupervised Ward hierarchical clustering of t-SNE dimensions generated from the single-cell gene expression data (Figures 2A–D and 3A–D). These clusters had distinct gene signatures. Within the CD1c+ cDC population, cluster 1 was defined by a lack of *CD1C* transcript and, along with cluster 2, exhibited reduced expression of *IRF3* relative to the other clusters. Clusters 2, 4, 5, and 6 were defined by increased expression of *CD86* transcript as compared to clusters 1 and 3. Clusters 4 and

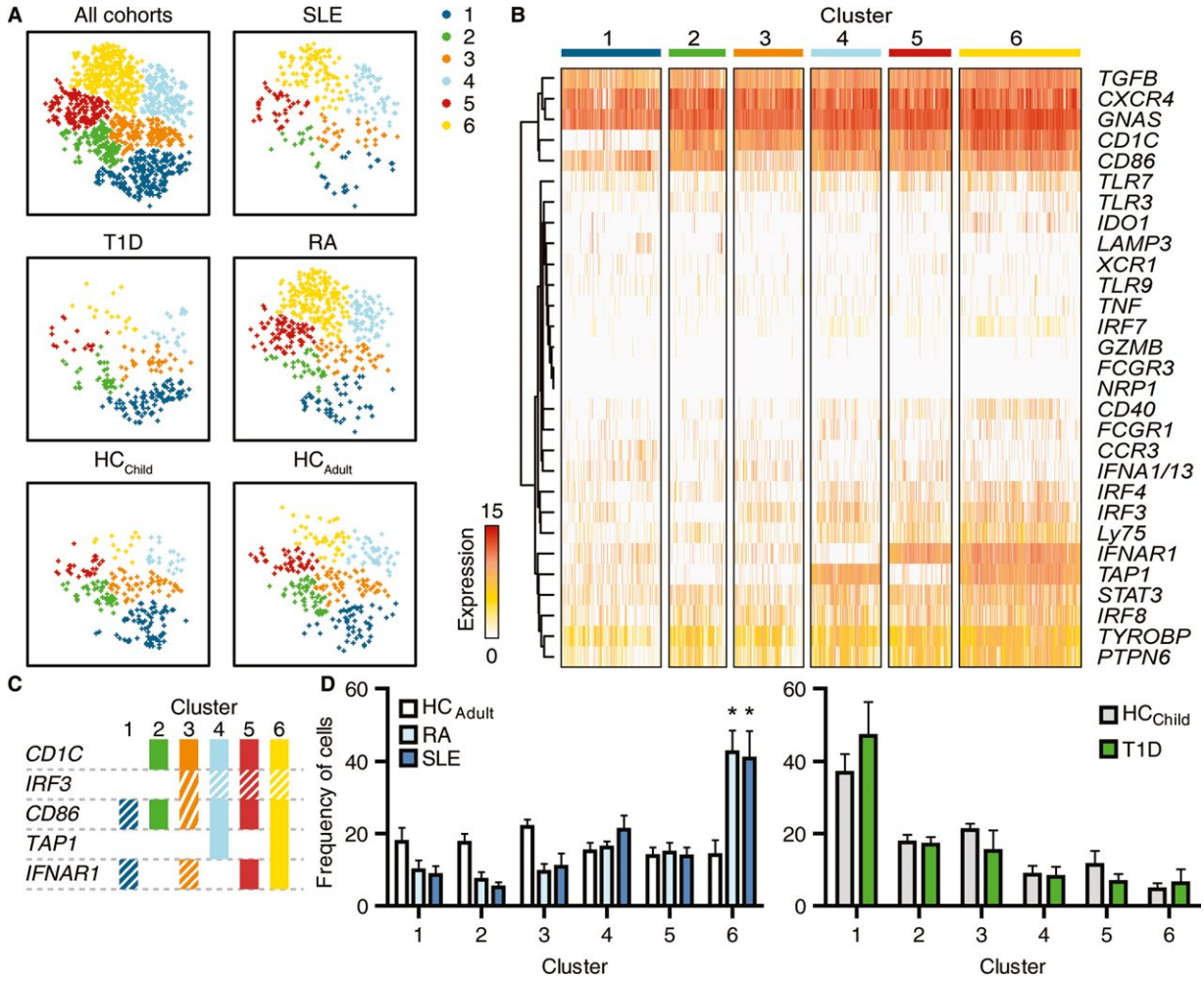

Figure 2. Distinct gene signatures of cDCs from patients with systemic autoimmune diseases. CD1c+ cDCs were single-cell sorted from freshly isolated PBMCs from a control cohort of healthy adults (HC_{Adult}), patients with rheumatoid arthritis (RA), patients with systemic lupus erythematosus (SLE), or patients with recent-onset type 1 diabetes (T1D) and a second healthy control cohort of children (HC_{Child}) age-matched to the T1D cohort. Sorted cells were subjected to single-cell reverse transcription–polymerase chain reaction analysis. **A**, Analysis by t-distributed stochastic neighbor embedding reveals CD1c+ cDC gene expression clusters (6 clusters, determined according to unsupervised Ward clustering analysis) in all cohorts and in each individual cohort. **B**, Heatmaps of single-cell gene expression data are sorted into the clusters defined in **A**. **C**, Transcriptional profiles of the clusters defined in **A** are shown. Colored bars represent the percentage of cells expressing the indicated gene (expression defined as a corrected C_t >0). Solid bars = >75% of cells expressing the indicated gene in that cluster; hatched bars = 25–75% of cells expressing the indicated gene in that cluster. **D**, Frequency of CD1c+ cDCs in each cluster from PBMCs from each cohort is shown. Results are the mean ± SEM of 7–13 individuals per cohort. * = $P < 0.05$ versus HC_{Adult}, by two-way analysis of variance. See Figure 1 for other definitions.

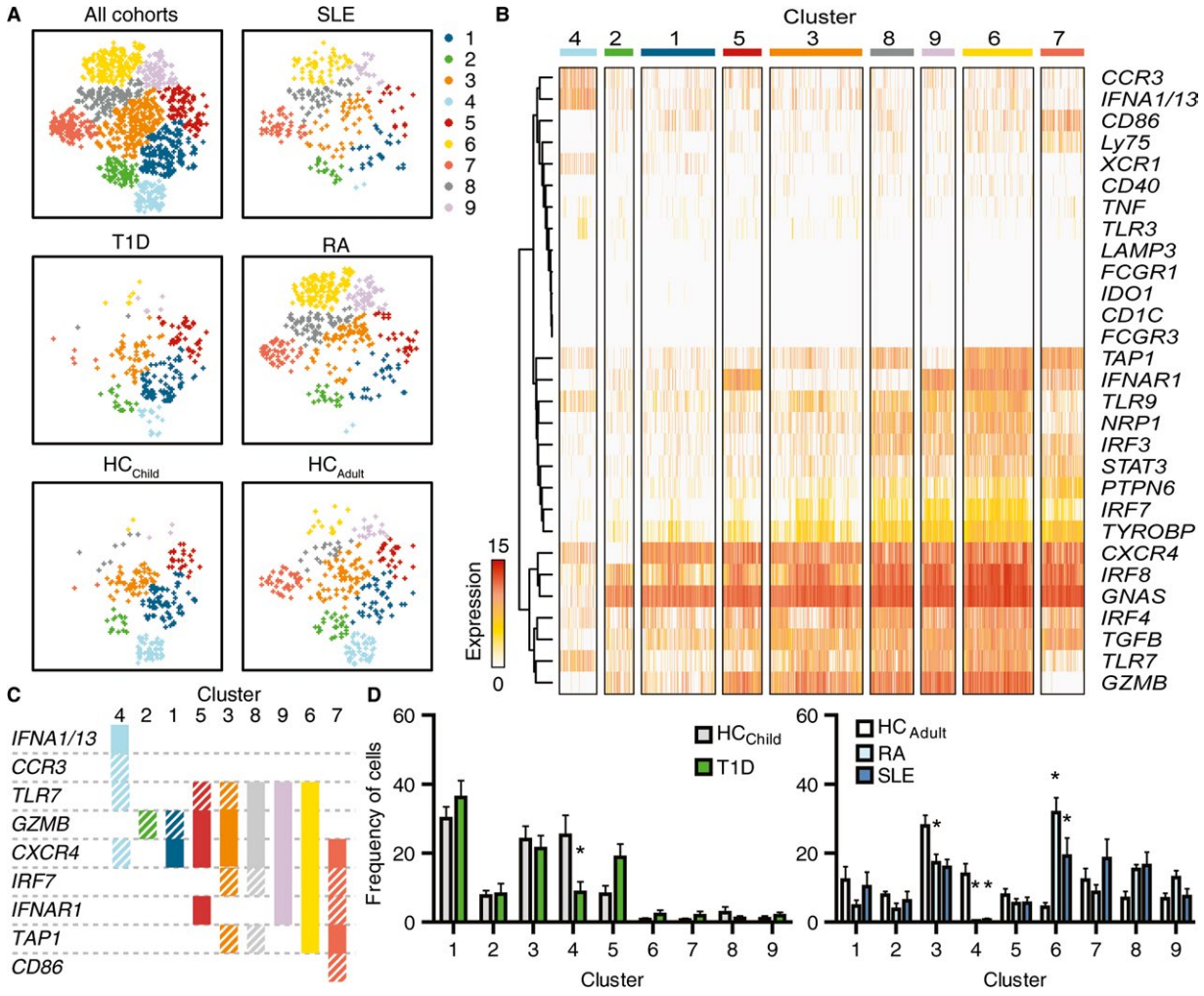

Figure 3. Distinct gene signatures of plasmacytoid dendritic cells (pDCs) from patients with systemic autoimmune disease. The pDCs were single-cell sorted from freshly isolated peripheral blood mononuclear cells (PBMCs) from a control cohort of healthy adults (HC_{Adult}), patients with rheumatoid arthritis (RA), patients with systemic lupus erythematosus (SLE), or patients with recent-onset type 1 diabetes (T1D) and a second healthy control cohort of children (HC_{Child}) age-matched to the T1D cohort. **A**, Analysis by t-distributed stochastic neighbor embedding reveals pDC gene expression clusters (9 clusters, determined according to unsupervised Ward clustering analysis) in all cohorts and in each individual cohort. **B**, Heatmaps of single-cell gene expression data are sorted into the clusters defined in **A**. **C**, Transcriptional profiles of the clusters defined in **A** are shown. Colored bars represent the percentage of cells expressing the indicated gene (expression defined as a corrected C_t >0). Solid bars = >75% of cells expressing the indicated gene in that cluster; hatched bars = 25–75% of cells expressing the indicated gene in that cluster. **D**, Frequency of pDCs in each cluster from PBMCs is shown for each cohort. Results are the mean ± SEM of 7–13 individuals per cohort. * = $P < 0.05$ versus HC_{Adult}, by two-way analysis of variance.

6 were further defined by overexpression of *TAP1*, while clusters 5 and 6 were defined by overexpression of *IFNAR1*.

Within the pDC population, clusters 1, 2, and 4 were characterized by lower transcript expression of most of the genes analyzed, relative to that in clusters 3, 5, 6, 7, 8, and 9. The expression profiles of pDCs were similar between clusters 1 and 2, although cells in cluster 2 lacked *CXCR4* expression. Cluster 4 had a distinct profile characterized by expression of *CCR3* and *IFNA1/13*. Compared with clusters 3 and 5, clusters 6, 7, 8, and 9 were defined by increased expression of *IRF3*, *PTPN6*, and *STAT3*. Other distinguishing genes for these clusters included *IRF7* (absent from cluster 5), *IFNAR1* (absent from clusters 3 and 8), and *TAP1* (up-regulated in clusters 6 and 7), while pDCs in cluster 7 were characterized by low expression of *TLR7* and *GZMB* as well as increased expression of *CD86*.

When we separated the t-SNE data (shown in Figures 2A and 3A) into the separate cohorts, we found that the profiles of CD1c+ cDCs and pDCs were altered in patients with RA and patients with SLE relative to those in healthy adults. In particular, we found a higher proportion of CD1c+ cDCs and pDCs in cluster 6 in samples from patients with RA (CD1c+ cDCs, mean 42.5% [95% CI 29.5%, 55.4%], $P < 0.0001$; pDCs, mean 31.8% [95% CI 22.4%, 41.1%], $P < 0.0001$) and patients with SLE (CD1c+ cDCs, mean 40.7% [95% CI 22.3%, 59.1%], $P < 0.0001$; pDCs, mean 19.2%

[95% CI 6.3%, 32.0%], $P < 0.01$) relative to that in healthy adults (CD1c+ cDCs, mean 14.1% [95% CI 5.7%, 23.5%]; pDCs, mean 4.4% [95% CI 1.4%, 7.3%]) (Figures 2D and 3D). For the pDCs, this was accompanied by a relative decrease in cells within clusters 3 and 4 in patients with RA (pDCs in cluster 3, mean 17.1% [95% CI 11.8%, 22.6%], $P < 0.05$; pDCs in cluster 4, mean 0.4% [95% CI –0.2%, 1.0%], $P < 0.01$) and a relative decrease in cells within cluster 4 in patients with SLE (mean 0.7% [95% CI –0.4%, 1.8%], $P < 0.05$) relative to that in healthy adults (pDCs in cluster 3, mean 27.9% [95% CI 20.8%, 35.0%]; pDCs in cluster 4, mean 13.9% [95% CI 7.0%, 20.8%]).

In contrast, the t-SNE profiles of both cell types were similar between patients with T1D and the corresponding healthy children (Figures 2A and 3A). A significant difference in the frequency of DC clusters between patients with T1D and healthy children was seen only for pDC cluster 4, which was decreased in patients with T1D compared with healthy children (mean 25.2% [95% CI 11.0%, 39.3%] versus 8.6% [95% CI 1.0%, 16.2%], $P < 0.001$) (Figure 3D).

DC gene transcription in autoimmunity. In addition to analyzing the gene signatures, we compared expression levels of individual genes between the disease cohorts and healthy subjects by applying the Hurdle model, which accounts for the bimodal expression of single-cell populations (Table 1 and Supplementary Figures 2A and B, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40793/abstract). The up-regulated genes in CD1c+ cDCs from patients with RA were *IFNAR1*, *CD1C*, and *IRF3*, which is consistent with the transcription profile observed for CD1c+ cDCs in cluster 6 (Figures 2B and C). *CXCR4* was down-regulated in CD1c+ cDCs from

Table 1. Differential gene expression in dendritic cells from patients versus age-matched healthy controls*

	CD1c+ cDCs			pDCs		
	RA	SLE	T1D	RA	SLE	T1D
CD1C	<0.0001†	0.132	0.7068	–	–	–
IRF8	<0.0001†	<0.0001†	<0.0001‡	<0.0001†	<0.0001†	0.2383
IFNAR1	<0.0001†	0.0497	0.0001†	0.0001†	0.1021	0.0024
IRF3	<0.0001†	0.0655	0.1584	0.0037	0.3604	0.6945
IRF7	0.0254	0.1255	0.6804	<0.0001†	<0.0001	0.4884
IRF4	0.0014	0.166	0.3764	<0.0001†	<0.0001‡	0.0015
GZMB	0.5951	0.3435	0.343	0.0006†	0.1829	0.6353
TLR7	0.0093	0.0073	0.0001†	0.0002†	0.6607	0.8875
TAP1	0.0014	0.0039	0.0142	0.0412	0.0008†	0.6353
CD86	0.0025	0.1206	<0.0001†	0.6789	0.1259	0.001†
LAMP3	0.1816	0.1055	<0.0001†	0.207	0.6494	0.6335
CCR3	0.0732	0.4197	<0.0001†	0.0004‡	0.5867	0.1565
IFNA1/13	0.0189	0.8275	0.0005†	0.0002‡	0.1591	0.6353
TNF	0.137	0.0206	0.3357	<0.0001‡	<0.0001‡	0.6353
TLR3	0.006	0.1055	0.4132	0.0007‡	0.0057	0.0002†
XCR1	0.0165	0.132	0.7921	0.0005‡	0.0128	0.1565
CXCR4	<0.0001‡	0.0001‡	0.0019	0.0048	0.6607	0.4884
TYROBP	0.0114	0.0039	<0.0001‡	0.0679	0.1591	<0.0001‡
PTPN6	0.0024	0.0206	<0.0001‡	0.8917	0.5867	0.4884
TGFB	0.3466	0.01	<0.0001‡	0.9009	0.2105	0.6353
CD40	0.0027	0.0039	0.013	0.9009	0.9518	0.9201
FCGR1	0.0016	0.0014	0.0688	–	0.6607	0.6353
IDO1	0.0025	0.0028	0.1135	0.2997	0.6607	–
Ly75	0.1061	0.4245	0.3349	0.3066	0.1114	0.6353
NRP1	–	–	–	0.1331	0.0738	0.6353
STAT3	0.0025	0.8054	0.1135	0.2153	0.498	0.641

* Values are *P* values from the Hurdle model for the significance of differential gene expression between patients with rheumatoid arthritis (RA), patients with systemic lupus erythematous (SLE), and patients with type 1 diabetes (T1D) compared with age-matched healthy controls. cDCs = conventional dendritic cells; pDCs = plasmacytoid dendritic cells.
† Up-regulated genes.
‡ Down-regulated genes.

patients with RA and/or patients with SLE. Although we found no robust gene signature in patients with T1D, the transcripts *IFNAR1*, *CCR3*, *CD86*, *IFNA1/13*, *LAMP3*, and *TLR7* were up-regulated and *IRF8*, *PTPN6*, *TGFB*, and *TYROBP* were down-regulated in CD1c+ cDCs from patients with T1D relative to the expression levels in healthy children (Table 1 and Supplementary Figure 2A [http://onlinelibrary.wiley.com/doi/10.1002/art.40793/abstract]).

In pDCs, transcript levels of *IRF7* and *IRF8, TAP1, GZMB*, *IFNAR1*, and *TLR7* were up-regulated ($P < 0.0001$) in patients with SLE or patients with RA (Table 1 and Supplementary Figure 2B [http://onlinelibrary.wiley.com/doi/10.1002/art.40793/abstract]). Again, the up-regulated genes corresponded to those that were highly expressed in cluster 6 (Figures 3B and C). *IRF4* and *TNF* were down-regulated ($P < 0.0001$) in patients with RA and patients with SLE relative to healthy adults. Additionally, pDCs from patients with RA exhibited lower expression levels of *CCR3*, *IFNA1/13*, *XCR1*, and *TLR3* (Table 1 and Supplementary Figure 2B [http://onlinelibrary.wiley.com/doi/10.1002/art.40793/abstract]), all of which are genes that are characteristic of cluster 4. Similar to the findings in CD1c+ cDCs, *CD86* was up-regulated ($P = 0.001$) and *TYROBP* was down-regulated ($P < 0.0001$) in pDCs from patients with T1D relative to healthy children.

We subsequently analyzed the protein expression levels of IFN regulatory factor 7 (IRF-7; total protein or phosphorylated IRF-7 [pS477/pS479]), IRF-8, IFN-alpha-1/13 receptor 1 (IFNAR-1), and granzyme B by flow cytometry in pDCs from a second cohort of healthy adults (n = 9 [3 male, 6 female]; median age 49 years [IQR 30 to 59 years]), patients with RA (n = 10 [1 male, 9 female]; median age 60.5 years [IQR 57.3 to 67 years]), and patients with SLE (n = 9 [2 male, 7 female]; median age 43 years [IQR 36 to 48 years]) (see results in Supplementary Figures 3A–C, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40793/abstract). Consistent with the gene expression data, protein expression levels of IRF-7 and IRF-8 were increased in pDCs from patients with RA (for IRF-7, MFI 347.4 [95% CI 326.9, 367.9], $P < 0.01$; for IRF-8, MFI 12,909 [95% CI 10,936, 14,882], $P < 0.05$) and pDCs from patients with SLE (for IRF-7, MFI 354.9 [95% CI 311.7, 398.2], $P < 0.01$; for IRF-8, MFI 13,002 [95% CI 10,710, 15,293], $P < 0.05$) relative to that in healthy adults (for IRF-7, MFI 288.9 [95% CI 262.3, 315.5]; for IRF-8, MFI 9,422 [95% CI 8,078, 10,768]).

Gene expression of *IFNAR1* and *GZMB* showed a trend toward up-regulated transcription in patients with SLE, and both were up-regulated at the protein level in pDCs from patients with SLE (for IFNAR-1, MFI 1,547 [95% CI 1,232, 1,861], $P < 0.05$; for granzyme B, MFI 793.2 [95% CI 623.3, 963.1], $P < 0.05$) relative to that in healthy adults (for IFNAR-1, MFI 1,124 [95% CI 835.7, 1,412]; for granzyme B, MFI 567.5 [95% CI 488.3, 646.7]). In patients with RA, both *IFNAR1* and *GZMB* were up-regulated at the transcript level, but not at the protein level (for IFNAR-1, MFI 1,428 [95% CI 1,234, 1,621]; for granzyme B, MFI 662.2 [95% CI 552.1, 772.3]).

Taken together, these results indicate that the changes in gene expression for individual genes are reflected in the gene signatures characteristic of DCs from patients with RA and patients with SLE, and these gene signatures partially translated into protein signatures. DCs from patients with T1D exhibited altered expression of individual genes, but this did not yield an observable gene signature based on the gene panel studied herein.

Gene expression response of pDCs to stimulation with TLR-7 in patients with organ-specific autoimmunity. The robust differences in DC gene signatures in healthy adults compared with that in patients with RA or patients with SLE, in whom a systemic inflammatory environment is present, were not unexpected. However, only subtle changes were observed in patients with T1D. Therefore, we investigated how DC stimulation with the TLR-7 agonist R848 could affect pDC transcripts, and whether stimulation elicited more robust differences in gene signatures in patients with T1D. We chose R848 because we expected it to affect the expression of several genes in our panel based on the results of a previous study (21), and we used pDCs, which have high TLR-7 expression (22).

We cultured previously frozen PBMCs from 4 patients with recent-onset T1D (2 male, 2 female; median age 14.5 years [IQR 13.2 to 15.7 years]) and 4 age-matched healthy individuals (2 male, 2 female; median age 14.5 years [IQR 13.3 to 15.6 years]) in the presence or absence of the TLR-7 agonist R848 for 3 hours, and then sorted pDCs (CD3–CD19–CD14–CD56–CD16–HLA–DR+CD11c^{low}CD123+CD303+) for single-cell gene expression analysis. Stimulation with R848 in pDCs from healthy children significantly altered the expression of 10 genes in our restricted panel (Figure 4A). As expected from the results of a prior study (21), *TNF*, *LAMP3*, and *CD40* were up-regulated and *CXCR4* was down-regulated by R848 stimulation.

Analysis with t-SNE and unsupervised Ward hierarchical clustering of R848-stimulated pDCs from healthy individuals and patients with T1D identified 5 pDC clusters, including cluster 1, that reflected the transcriptional changes induced by R848 stimulation (Figures 4B and C). The frequency of pDCs in each cluster was not significantly different between patients with T1D and healthy children (Figure 4D). It can also be noted that the expression profiles of the R848-stimulated pDCs differed from those of pDCs from patients with SLE, patients with RA, and patients with T1D (shown in Figure 3), indicating that the transcriptional profiles of patients are unlikely to reflect the response to a single stimulus or pathway.

Correlation of DC gene signatures with disease activity level in RA. The frequency of pDCs or CD1c+ cDCs in cluster 6 was increased in patients with RA and patients with SLE. However, there was substantial variability between individual

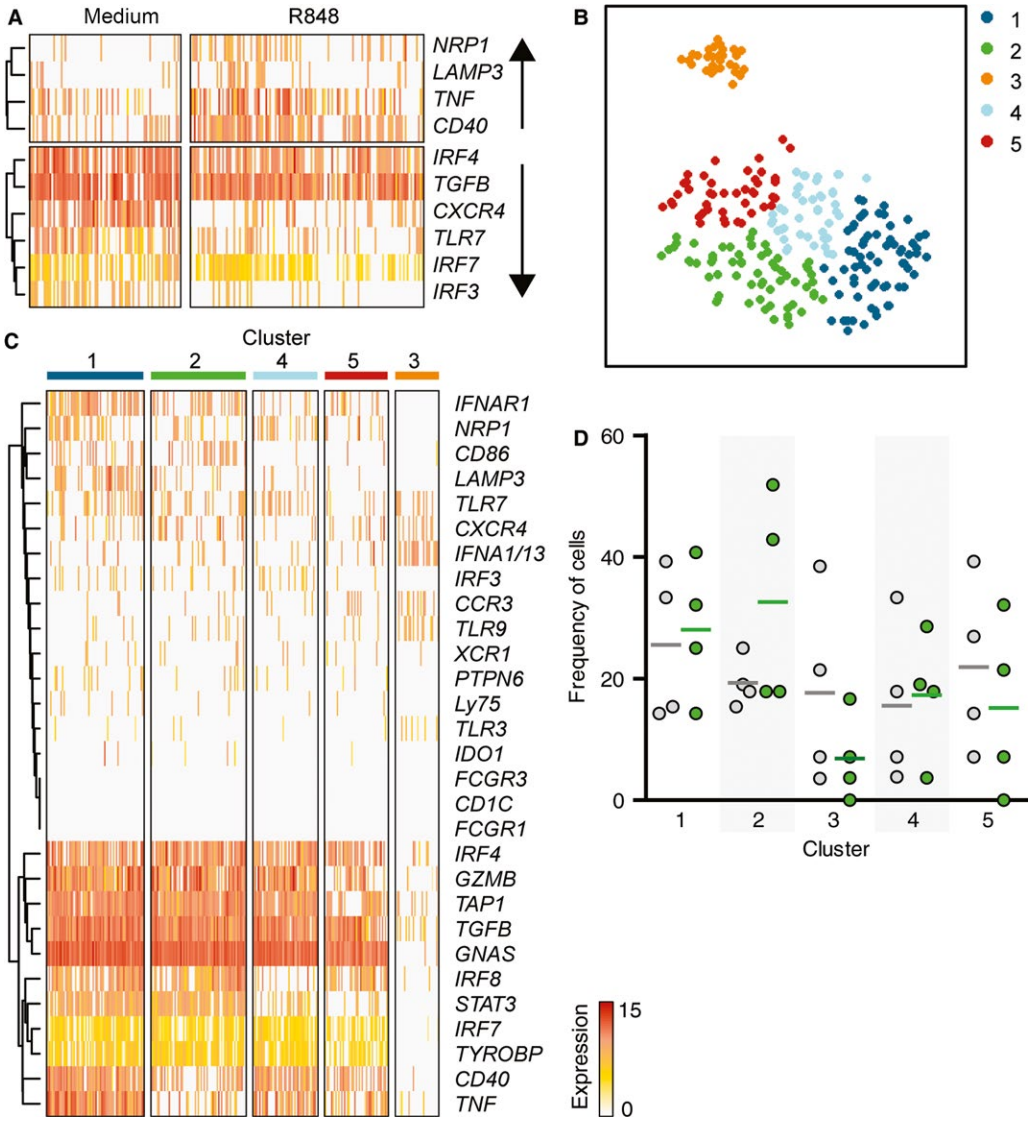

Figure 4. Single-cell analysis of R848-stimulated pDCs from patients with recent-onset type 1 diabetes (T1D) and healthy children. PBMCs from patients with recent-onset T1D and age-matched healthy children were cultured for 3 hours with a Toll-like receptor 7 agonist, R848. Single-cell–sorted pDCs were subjected to single-cell gene expression analysis. **A**, Heatmaps of gene expression in pDCs from healthy children are shown, separated according to the presence of stimulation with R848 or absence of stimulation (medium alone), with ordering according to unsupervised Ward hierarchical clustering analysis. Only genes that were significantly up-regulated (top) or down-regulated (bottom) after R848 stimulation are shown, based on the Hurdle model with correction for the false-discovery rate and a significance threshold of 0.001. **B**, Analysis by t-distributed stochastic neighbor embedding reveals gene expression clusters (5 clusters, determined according to unsupervised Ward hierarchical clustering analysis). **C**, Heatmaps of single-cell gene expression data are sorted into the clusters defined in **A**. **D**, Frequencies of cells in each cluster are shown for the healthy children (gray-shaded circles) and patients with T1D (green circles), where each circle represents an individual and the horizontal line represents the mean. There were no significant differences in the gene expression data, as determined by one-way analysis of variance. See Figure 3 for other definitions.

patients (Figure 5A). Therefore, we investigated whether this variability might be related to disease activity. Since disease activity in SLE is more difficult to measure, due to the multiorgan pattern of the disease, we focused on the larger cohort of patients with RA and used the Clinical Disease Activity Index (CDAI) as a measure of disease activity (23).

The frequency of CD1c+ cDCs in cluster 6 was positively correlated with the CDAI score in patients with RA (Spearman's r = 0.60, P = 0.03) (Figure 5B). Furthermore, in the group of patients with at least moderate disease activity (CDAI >10, n = 5) and a CRP concentration of >1 mg/liter, the frequency of CD1c+ cDCs in cluster 6 was higher (mean 65.6% [95% CI 57.5%, 73.7%]) than in the 8 patients with less severe inflammation (mean 28.0% [95% CI 18.1%, 38.0%], P < 0.0001) (Figure 5C). In contrast, the frequency of pDCs in cluster 6 was not correlated with the CDAI (Spearman's r = 0.22, P = 0.47) (Figure 5B), and segregation of

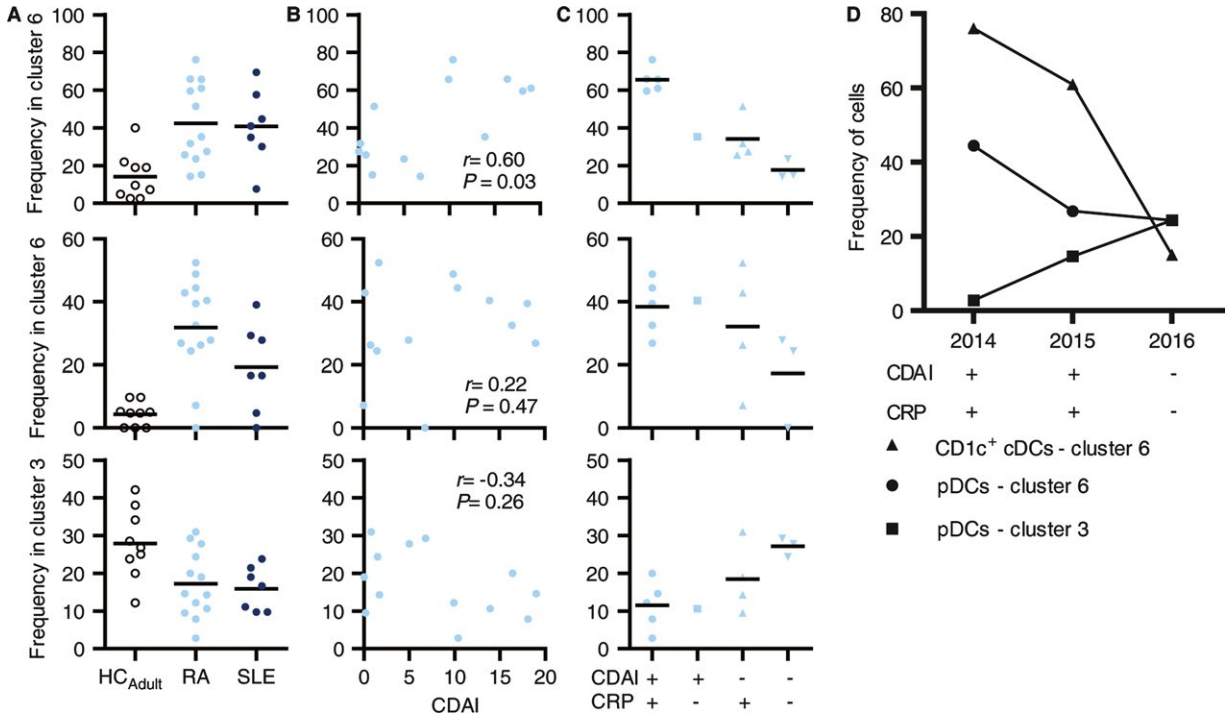

Figure 5. Correlation between cluster frequency and disease status. **A**, CD1c+ conventional dentritic cells (cDCs) and plasmacytoid DCs (pDCs) in cluster 6 (top and middle, respectively) and pDCs in cluster 3 (bottom) were examined for the frequency of enrichment in peripheral blood mononuclear cells (PBMCs) from healthy adults (HC_{Adult}), patients with rheumatoid arthritis (RA), and patients with systemic lupus erythematosus (SLE). Circles represent individual subjects, and the horizontal line represents the mean. **B**, Correlations between the frequency of CD1c+ cDCs and pDCs in cluster 6 and pDCs in cluster 3 and the Clinical Disease Activity Index (CDAI) in patients with RA were assessed by Spearman's correlation analysis. Circles represent individual subjects. *P* values are 2-tailed. **C**, Frequencies of CD1c+ cDCs and pDCs in cluster 6 and pDCs in cluster 3 were examined in patients with RA stratified according to CDAI score (+ = CDAI >10) and C-reactive protein (CRP) concentration in the blood (+ = >1 mg/liter). Symbols represent individual subjects, and the horizontal line represents the mean. **D**, Longitudinal changes in the frequencies of CD1c+ cDCs in cluster 6 and pDCs in clusters 3 and 6 in a single RA patient over 3 years are shown, including the patient's CDAI score (+ = CDAI >10) and CRP concentration in the blood (+ = >1 mg/liter) at each time point.

patients based on the CDAI and CRP level did not reveal a relationship between inflammation markers and cluster frequency (Figure 5C). However, the highest frequency of pDCs in cluster 3, which is characteristic of pDCs from healthy individuals (Figure 5A), was found in patients with RA who had low CDAI scores and a CRP concentration of <1 mg/liter (Figure 5C).

A relationship between increased disease activity and high frequency of cD1c+ cDCs in cluster 6 or a low frequency of pDCs in cluster 3 was observed in an individual patient over 3 consecutive years (Figure 5D). These findings suggest that the DC transcriptional profile is correlated with the severity of inflammation and disease.

DISCUSSION

In this study using single-cell gene expression analysis, we identified transcriptome heterogeneity of blood-derived DC subsets, and found transcriptional profiles of DC subsets that are associated with autoimmunity. We also found that the transcriptional profiles of the DC subsets are associated with disease activity in patients with RA.

We could identify distinct subpopulations of DCs using a restricted gene set, which was selected on the basis of DC markers and DC function. We observed transcriptionally distinct subpopulations within the pDC and CD1c+ cDC subsets. This included a population within the pDC subset with gene and protein expression patterns that corresponded to a T cell–activating DC population that has been newly identified by single-cell RNA sequencing (19,20). We also observed a unique pDC subpopulation characterized by increased expression of *IFNA1/13* and *CCR3*, which has not been previously described. Further studies are required to identify protein markers that may enable us to isolate these cells for functional characterization.

Our novel findings include identification of a DC gene expression signature with the potential to become a measure of disease activity in systemic autoimmune diseases such as RA and SLE, and a subtle alteration of DC gene expression in patients with T1D, an organ-specific autoimmune disease. We chose SLE and RA as systemic autoimmune disease models because these diseases, when active, often show systemic inflammation and immune complex–mediated IFN signatures (24–26). Consistent with this, patients with RA and those with SLE had a significantly greater

proportion of DCs with a transcriptional profile characterized by the concomitant up-regulation of multiple genes (cluster 6), such as *CD86* in CD1c+ cDCs, *TLR7* and *IRF7* in pDCs, and *TAP1* and *IFNAR1* in both subsets. Overexpression of these genes suggest that DCs with this cluster profile have an increased capacity to activate T cells, produce type I IFNs, and process antigens; these observations have previously been reported in RA and/or SLE (27–31). The profiles observed in patients could not be attributed to responses to a single stimulus, such as a TLR-7 agonist.

In contrast to that seen in patients with RA or SLE, the cluster profile of DCs isolated from patients with recent-onset T1D was not distinct from that of DCs from healthy children. Instead, we found changes in the expression of a small number of genes in CD1c+ cDCs and pDCs. As in patients with RA or SLE, *CD86* expression was increased in both DC subsets from patients with T1D. However, unlike in RA and SLE, we found decreased expression of *PTPN6* and *TGFB* in CD1c+ cDCs and decreased expression of *TYROBP* in both DC subsets from patients with T1D. These genes encode proteins with important roles in the negative regulation of the immune response, and their down-regulation or abnormal function can promote inflammatory or autoimmune responses (32–37). We speculate that the subtle changes in DCs from patients with T1D may occur downstream of genetic susceptibility, rather than being a reflection of generalized inflammation, and could therefore represent therapeutic targets.

Transcriptional profiles that can be used to monitor disease development or predict response to treatment would be valuable for developing personalized therapies for autoimmune diseases. Multiple studies have attempted to identify signatures that can clinically stratify patients for this purpose (38–41). A recent study, for example, showed that the IFN-stimulated genes associated with SLE were markedly different between populations of European ancestry and those of East Asian ancestry (42). Most of these studies used microarray or RNA sequencing to analyze bulk mixed-cell populations, but this approach can mask clinically relevant biologic complexity and heterogeneity at the single-cell level. In our study, we found that disease activity in patients with RA was correlated with the frequency of DCs expressing particular transcriptional signatures. Patients with more severe inflammation had higher frequencies of CD1c+ cDCs expressing an "autoimmune" profile and lower frequencies of pDCs expressing a "healthy" transcriptional profile. These are promising findings that require validation in prospective studies. It will also be important to determine whether the changes in DC transcriptional profile are secondary to changes in the inflammatory environment, and therefore might provide an indirect measure of the degree of inflammation, or whether they reflect functional abnormalities that may affect the choice and outcome of treatment.

Single-cell gene expression analyses can identify heterogeneity and distinct cell populations within phenotypically similar cells. As these technologies improve and downstream analyses become more robust and standardized, it is becoming more feasible to screen patients based on their immune cell transcriptional profiles. As we have shown in the present study, this approach may yield new disease markers and therapeutic targets in patients with autoimmune disease.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Bonifacio had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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