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Gene Expression Signatures in Polyarticular Juvenile Idiopathic Arthritis Demonstrate Disease Heterogeneity and Offer a Molecular Classification of Disease Subsets

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Abstract

Objective—Microarray analysis was used to determine whether children with recent onset polyarticular juvenile idiopathic arthritis (JIA) exhibit biologically or clinically informative gene expression signatures in peripheral blood mononuclear cells (PBMC).

Methods—Peripheral blood samples were obtained from 59 healthy children and 61 children with polyarticular JIA prior to treatment with second-line medications, such as methotrexate or biological agents. RNA was extracted from Ficoll-isolated mononuclear cells, fluorescently labeled and hybridized to Affymetrix U133 Plus 2.0 GeneChips. Data were analyzed using ANOVA at a 5% false discovery rate threshold after Robust Multi-Array Average pre-processing and Distance Weighted Discrimination normalization.

Results—Initial analysis revealed 873 probe sets for genes that were differentially expressed between polyarticular JIA and controls. Hierarchical clustering of these probe sets distinguished three subgroups within polyarticular JIA. Prototypical subjects within each subgroup were identified and used to define subgroup-specific gene expression signatures. One of these signatures was associated with monocyte markers, another with transforming growth factor β -inducible genes, and a third with immediate-early genes. Correlation of gene expression signatures with clinical and biological features of JIA subgroups suggests relevance to aspects of disease activity and supports the division of polyarticular JIA into distinct subsets.

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Conclusions—PBMC gene expression signatures in recent onset polyarticular JIA reflect discrete disease processes and offer a molecular classification of disease.

Polyarticular juvenile idiopathic arthritis (JIA) is chronic arthritis in more than four joints for more than six weeks with onset before the 16th birthday in a child without other known causes of arthritis (1-3). Polyarticular JIA is divided into rheumatoid factor (RF) positive and negative sub-types, with the RF+ sub-type having positive tests for serum IgM RF on two occasions at least three months apart within the first six months of disease. Ravelli and Martini have recently proposed that RF- polyarticular JIA be divided into 3 subsets: one similar to adult rheumatoid arthritis, another with “dry synovitis” and a third similar to ANA + early-onset oligoarticular JIA (3). Given this heterogeneity, it is not surprising that children with polyarticular JIA have a wide variety of disease courses and outcomes, ranging from self-limited arthritis with no long-term disability to relentless and destructive arthritis with severe disabilities (4). Unfortunately, our present ability to predict course and outcome is limited, with treatment typically tailored to current disease activity, assessment of which is also imperfect.

Global gene expression profiling is a molecular technique that measures in parallel genome-wide expression of thousands of genes in a sample of cells. This technology holds promise for dramatically advancing knowledge of many diseases, including JIA. This approach has already provided important information regarding classification and pathogenesis of several JIA sub-types in studies that generally used small numbers of subjects with varying degrees of clinical diversity (5-9). In the present study, global gene expression profiling of peripheral blood mononuclear cells (PBMC) was used to characterize a relatively large population of children with recent onset polyarticular JIA (both RF- and RF+) who had not been treated with methotrexate, biologics or other disease modifying anti-rheumatic drugs (DMARDs). The goals of applying this technology to JIA are to advance understanding of disease pathogenesis, improve assessment of disease activity, predict response to medications and foresee long-term outcomes. The present work takes a step toward these goals by defining gene expression signatures that appear to be associated with distinct disease processes in subgroups of children with polyarticular JIA.

Patients and Methods

Subjects and clinical data collection

Sixty-one children with polyarticular JIA, classified by ILAR criteria (2), were recruited at five clinical sites: 24 from Cincinnati Children's Hospital Medical Center (CCHMC), 16 from Schneider Children's Hospital, 9 from Children's Hospital of Philadelphia, 6 from Toledo Children's Hospital and 6 from Children's Hospital of Wisconsin. Of these 61 patients, 46 were taking scheduled NSAIDs, 3 were taking prednisone, and none had ever been treated with methotrexate, other DMARDs or biologics. Informed consent was obtained and clinical data was collected, including the following disease activity measures: erythrocyte sedimentation rate (ESR), active joint count (tender and limited, and/or swollen), Childhood Health Assessment Questionnaire (CHAQ), physician global assessment of disease activity, and parent/patient global assessment of well-being. All JIA subjects were tested for RF, including a second test at least 3 months later for classification if the first test was positive. Most JIA subjects were tested for anti-CCP and ANA (Table 1). If frozen serum was available and RF or anti-CCP testing was not performed at the collecting center, these tests were performed at CCHMC. The specific joints involved with arthritis at the time of sampling were documented. Fifty-nine normal controls were recruited at CCHMC. Twenty-nine controls provided blood samples as part of a demographically representative Cincinnati population recruited and screened to serve as healthy normal controls. Thirty controls provided blood samples and limited clinical data when blood was

drawn at the CCHMC Test Referral Center for clinical purposes. These thirty subjects were screened by questionnaire and deemed to be free of inflammatory disease.

Sample processing

PBMC were isolated by Ficoll gradient centrifugation and frozen at -80°C in TRIzol (Invitrogen, Carlsbad, CA) at the collecting centers with documentation of both time of phlebotomy and time of freezing. Time to Freezing (TTF) is defined as the interval between these two times and is expressed in minutes. Frozen samples were batched and shipped on dry ice to CCHMC and stored at -80°C until RNA was extracted and purified on RNeasy columns (QIAGEN, Germantown, MD). RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) according to CCHMC Affymetrix GeneChip Core protocols. NuGEN Ovation Version 1 (NuGEN Technologies, San Carlos, CA) was used with 20 ng starting RNA to produce fluorescently labeled cDNA that was assayed by 54,675 probe sets on Affymetrix U133 Plus 2.0 GeneChips (Affymetrix, Santa Clara, CA) scanned with an Agilent G2500A GeneArray Scanner (Agilent Technologies). A Universal Standard was prepared from a pooled mixture of RNA from 35 healthy adult volunteers (6) and included in each batch of 12 samples to provide technical replicates for assessing batch-to-batch variation.

Data analysis

Data were imported into GeneSpring GX 7.3.1 (Agilent Technologies) with Robust Multi-Array Average (10) pre-processing, referenced to the median of each gene's adjusted value across all samples, followed by Distance Weighted Discrimination (DWD) to adjust for batch-to-batch variation (11). Probe sets for differentially expressed genes were identified by ANOVA at a 5% false discovery rate (12). Tukey post-hoc testing identified probe sets for genes differentially expressed between JIA subjects and controls. The complete microarray dataset has been deposited in NCBI's Gene Expression Omnibus and is accessible through GEO Series accession number GSE13849.

Flow cytometry

Aliquots of PBMC from 143 JIA subjects and controls (45 polyarthritis, 21 oligoarthritis, 25 enthesitis-related arthritis, 6 psoriatic arthritis, 12 systemic arthritis and 34 controls) were frozen in 10% DMSO at the collecting centers, shipped to CCHMC on dry ice and stored in liquid nitrogen. These samples represented a subset of subjects involved in the larger study that compared PBMC gene expression profiles of various JIA sub-types with controls, and included 45 out of the 61 subjects with polyarticular JIA studied here (Barnes, et al., manuscript submitted). Sixteen polyarticular JIA subjects were not sampled for flow cytometry due to phlebotomy volume limits relative to age. After thawing and washing PBMC in FACS buffer (PBS with 0.2% BSA), cells were stained with the following monoclonal antibodies in appropriate combinations (BD Biosciences, San Jose, CA): CD3-PerCP, CD8a-FITC, CD4-APC, CD15-PE, CD16-FITC, CD33-APC, CD34-FITC and CD45-PerCP. Stained cells were analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences). PBMC sub-populations of interest were captured by standardized polygonal gates. Linear correlation analysis was performed between the average normalized expression of the 50 probe sets of Signatures I, II and III (described in Results) with proportions of PBMC sub-populations determined by flow cytometry.

Results

General approach to data analysis

A preceding study of gene expression differences between various sub-types of JIA and controls used ANOVA to identify 873 probe sets for genes differentially expressed between RF- polyarticular JIA (n=45) and normal controls (n=59) (Barnes, et al, manuscript submitted). This comparison is the starting point for the current work, which utilizes an iterative approach with several rounds of identifying probe sets for genes differentially expressed between comparison groups, each time refining the analysis based on hierarchical clustering of differentially expressed genes, ultimately defining prototypical gene expression signatures. The first iteration included RF+ polyarticular JIA subjects, the next involved subgroups of polyarticular JIA, and the final comparisons involved small cohorts of prototypical subjects within each subgroup. Prototypical gene expression signatures were then applied to all subjects to assess the association of each signature with clinical and biological features within the entire population.

Identification of polyarticular JIA subgroups clustered by PBMC gene expression patterns

For the first comparison, RF+ polyarticular JIA subjects were included as it was observed that many RF+ subjects co-clustered with a subset of RF- subjects rather than clustering separately, suggesting that RF status did not clearly distinguish a subset of polyarticular JIA assessed by PBMC gene expression profiling. Figure 1 shows an expression heat map of the starting 873 probe sets (Barnes, et al., manuscript submitted) in 61 polyarticular JIA subjects (both RF+ and RF-) and 59 normal controls. Subjects are ordered by hierarchical clustering using Pearson correlation with average linkage. Three clusters predominantly composed of JIA subjects are boxed in Figure 1. The JIA subjects within these three clusters comprise Groups A, B and C. An alternative approach to clustering was tested in which 1,523 probe sets identified by ANOVA as differentially expressed between all polyarticular JIA subjects (RF- and RF+, n=61) and controls were used (data not shown). This approach also demonstrated Groups A, B and C with few differences in group assignments compared to the original clustering. Most importantly, all prototypical subjects for each group remained co-clustered, and thus the prototype signatures that are ultimately derived from all probe sets are identical using either approach.

The next round of comparisons identified gene expression differences between each JIA subgroup (Groups A, B and C) and controls. Probe sets for genes identified as differentially expressed by t test using a 5% false discovery rate (multiple testing correction) comprised 2,111 for Group A (1,025 up/1,086 down), 6,713 for Group B (2,900 up/3,813 down), and 11,485 for Group C (5,319 up/6,166 down). Notably, comparison of controls with the 24 polyarticular JIA subjects that did not cluster into any subgroup did not identify any probe sets for differentially expressed genes, even when using a 20% false discovery rate. Comparisons among the groups indicated some overlap, particularly between Groups B and C (582 probe sets between A and C, 756 probe sets between A and B, and 2,555 probe sets between B and C).

Characteristics of polyarticular JIA subgroups

Table 1 compares JIA subjects in Groups A, B and C, as well as JIA subjects that did not cluster into any subgroup ("Other Polys"). Notable characteristics include a greater proportion of RF positivity and a trend toward a greater proportion of anti-CCP+ subjects in Group A, a younger age and greater proportion of ANA+ subjects in "Other Polys", and a trend toward higher ESR, CHAQ scores and physician global scores in Groups A and B. Generally, there was proportionate representation of subjects from each clinical center in Groups A and C. Distribution of the 9 subjects in Group A from 5 centers was 1/2/3/1/2.

Distribution of the 11 subjects in Group C was 5/2/2/0. Conversely, Group B had a disproportionate distribution (3/11/1/1), with 11 of 17 subjects coming from one center.

Identification of prototypical subjects for each polyarticular JIA subgroup

For the final round of comparisons, prototypical subjects were identified within each subgroup that maximized the gene expression signature for that subgroup and minimized overlap with other subgroups. Subjects were included if the geometric mean of the 50 most up-regulated probe sets for that subgroup was at least 2-fold greater than controls. Subjects were excluded if they did not remain co-clustered using several alternative hierarchical clustering methods (Pearson, Standard, and Distance correlations), or if the geometric mean of the 50 most up-regulated probe sets for more than one subgroup was more than 2-fold increased over controls. Using this method, we identified 4 prototypical subjects for Group A, and 5 prototypical subjects each for Groups B and C (bullets on the right side of Figure 1).

Determination of probe sets that define subgroup-specific gene expression signatures

Probe sets for genes differentially expressed between prototypical subjects for each subgroup and controls were identified using t tests with a 5% false discovery rate. For this comparison, 10 controls were excluded that had more than 2-fold increase in geometric mean of the 50 most up-regulated probe sets for any JIA subgroup (A, B or C), yielding a cohort of 49 signature-free controls. This comparison produced lists of 4,098 probe sets for Group A, 7,290 for Group B and 16,462 for Group C (Supplementary Tables 1, 2 and 3). Gene expression signatures for each subgroup (Signatures I, II and III for Groups A, B and C, respectively) were defined as the 50 most up-regulated probe sets from these lists, excluding redundant probe sets for the same genes (using the probe set with the lowest P value), and excluding probe sets that were up-regulated more than 1.5-fold in any of the other prototypical groups. Down-regulated probe sets were not included, as these probe sets had similar patterns to up-regulated probe sets in each subgroup, and inclusion of down-regulated probe sets would not have significantly changed the discriminatory power of the signatures, though they may provide important clues to understanding the processes represented by each signature and are included in the Supplementary Tables. The top 25 probe sets for Signatures I, II and III are presented in Table 2, and all 50 probe sets for each signature are indicated in the Supplementary Tables.

Quantification of subgroup-specific gene expression signatures

Signatures I, II and III were quantified for all subjects by calculating the average of the geometric mean of fold increase over controls for the 50 probe sets that comprise each signature. A value of 1 equates to the average signature observed in the 49 signature-free controls. The magnitudes of each signature in every subject are graphed in Figure 2, using the same order of subjects in Figure 1 for comparison. As expected, subjects in Groups A and C predominantly express Signatures I and III, respectively. Conversely, subjects in Group B expressed not only Signature II, but also many subjects in Group B expressed significant amounts of either Signature I or III, suggesting overlap between Groups B and A and between Groups B and C. Overall, the numbers of JIA subjects that exhibited at least a 1.5-fold increase in a particular signature were 16 (26.2%) for Signature I, 17 (27.9%) for Signature II, and 20 (32.8%) for Signature III. Quantification of signatures enabled correlation with clinical characteristics and abundance of PBMC subsets determined by flow cytometry.

Correlation of Signature I with rheumatoid factor and monocytes

Group A contained the highest proportion of RF+ subjects (5 out of 9) (Table 1), which suggests that Signature I correlates with RF positivity. To test this, the average magnitude of each signature was compared between RF+ and RF- JIA subjects, and indeed RF+ subjects had statistically greater Signature I than RF- subjects (Figure 3A). Signature II did not differ between RF+ and RF- subjects, and Signature III trended toward being greater in RF- subjects, which is consistent with the absence of any RF+ subjects in Group C (Table 1). RF + polyarticular JIA is more likely than RF- JIA to involve joint damage (13), raising the possibility that Signature I is an indicator of active joint injury, which is also consistent with Group A trending to have higher ESR and CHAQ scores than the other subgroups (Table 1). Along these lines, the genes that comprise Signature I suggest that this signature comes from monocytes, and the presence of Signature I may indicate that these cells are either mediating or responding to joint damage. In particular, there is increased expression of monocyte markers FCGR1A (CD64) (4.09 fold, Table 2) and CD14 (2.33 fold, Supplementary Table 1) in prototypical Group A subjects (14). While flow cytometry analysis did not include CD64 or CD14 markers, other markers of monocyte lineage subsets correlated with Signature I better than with the other signatures, demonstrating an association of Signature I with increased abundance of monocyte populations (Figure 3B) (15).

Correlation of Signature II with prolonged sample processing

The biological basis for Signature II was first suggested by the observation that subjects from one clinical center were over-represented in this subgroup, which led to the discovery that samples in Group B tended to have longer processing times, as measured by Time to Freezing (TTF). The average TTF for Group B was significantly greater than that for other polyarticular JIA subjects or controls (Figure 3C). However, prolonged TTF was not sufficient to produce Signature II, as a number of controls and JIA samples did not exhibit this signature despite having relatively prolonged TTF, as shown in Figure 3D, which suggests differential susceptibility of samples to the length of processing. Interestingly, this graph also suggests that if all samples were processed promptly, with TTF around 60 minutes, Signature II would not have been observed at all (Figure 3D). Signature II did not correlate with any PBMC subpopulations analyzed by flow cytometry (all correlation coefficients <0.3), indicating that this signature is not associated with abundance of any particular PBMC subset. Rather, the association with longer TTF suggests that Signature II is due to activation of PBMC after phlebotomy.

Correlation of Signature III with reduced CD8⁺ T cells and increased plasmacytoid dendritic cells

As noted above, each JIA subgroup had both up-regulated and down-regulated genes, and up-regulated genes were sufficient to define the gene expression signatures. Nevertheless, down-regulated genes are likely to provide valuable information regarding disease processes, and along these lines CD8b was noted to be among the most highly down-regulated genes in Group C (0.44-fold compared to controls, Supplementary Table 3). CD8a was also decreased, though not as strongly as CD8b (0.76-fold compared to controls, Supplementary Table 3). Since abundance of CD8⁺ T cells had been measured by flow cytometry, it was of interest to assess correlation of CD8b expression with the proportion of CD8⁺ T cells in Group C. Indeed, both CD8b expression and CD8⁺ T cell abundance were significantly lower in Group C compared to either all other polyarticular JIA subjects or controls (Figures 4A and B). Additionally, CD8b expression and abundance of CD8⁺ T cells trended to be lower in subjects that exhibited strong expression of Signature III (Figures 4C and D). The correlation coefficient for Signature III with abundance of CD8⁺ T cells was -0.14, supporting an inverse relationship. Conversely, Signature III exhibited its strongest positive correlation with Lin⁻BDCA4⁺ plasmacytoid dendritic cells (16), with a correlation

coefficient of 0.30. These results suggest that reduced CD8⁺ T cells and increased plasmacytoid dendritic cells are important features of the disease process represented by Signature III, which may involve transforming growth factor β (TGF β) action, as discussed below.

Discussion

Global gene expression analyses of PBMC from a large cohort of polyarticular JIA patients that had not been treated with DMARDs or biologics revealed striking subgroups within an otherwise uniform collection of subjects with recent onset polyarthritis. These gene expression profiles demonstrate biologically significant heterogeneity within the JIA population, with distinct subgroups of patients expressing distinct gene expression signatures. These signatures appear to be manifestations of biological processes that are occurring in some but not all patients, and in the long run they may prove to be valuable tools for classifying and managing individuals with polyarticular JIA.

Three distinct gene expression signatures were identified with variable expression among polyarticular JIA subjects. Signature I was strongly expressed in many RF+ JIA subjects, but also in a number of RF- JIA subjects (Figures 2 & 3A). In fact, Signature I may prove useful for identifying a subset of RF- JIA patients that have a disease phenotype similar to RF+ patients, as described by Ravelli and Martini (3). Signature I contains many genes specifically expressed in monocytes, and thus this signature may be a manifestation of increased abundance and/or activation of peripheral blood monocytes. Furthermore, a number of genes in Signature I have been identified as being regulated in monocytes in other rheumatic diseases. For example, FCGR1A (CD64), the third ranked gene in Signature I, was shown by Wijngaarden, et al. to be dramatically down-regulated in monocytes in rheumatoid arthritis after methotrexate treatment (17). Likewise, MS4A4A, the top ranked gene in Signature I, and FCGR1A were the top two ranked cell surface marker genes in isolated monocytes that Abe, et al. found to be significantly down-regulated in Kawasaki disease after treatment with intravenous immunoglobulin (18). Additionally, MS4A4A, CLU, DYSF and IL8RB were found by Ogilvie, et al. to be up-regulated in PBMC of patients with active systemic JIA (7). A very interesting comparison with 25 genes up-regulated in rheumatoid arthritis finds 16 of these also up-regulated in Group A prototypes, including CD14, AQP9 and several S100 proteins, while in contrast 14 of these up-regulated RA genes were down-regulated in Group C prototypes, emphasizing the contrast between Groups A and C (Supplementary Tables 1 and 3) (19). Thus, Signature I may be indicative of monocyte activity in a number of autoimmune inflammatory diseases, and it may be useful for assessing disease activity and monitoring response to treatments in these diseases.

Rigorous protocols were followed for isolating and freezing PBMC as quickly as possible to minimize effects of processing on gene expression. Nevertheless, some of the observed gene expression differences that contribute to Signature II were associated with prolonged processing times. Still, this signature does not appear to be solely a function of processing, but also depends on immunological differences between subjects, noting that this signature was observed almost exclusively in JIA subjects and not controls, and many of those JIA subjects also expressed Signatures I and/or III. Signature II contains many immediate-early genes, including several FOS-related genes, JUN and EGR1 (20), consistent with very recent cellular responses. The association of Signatures I and III with antigen presenting cells (monocytes and plasmacytoid dendritic cells, respectively), suggests that Signature II may develop after phlebotomy in samples that have pathologically primed antigen presenting cells that are able to readily stimulate other cells in the “test tube” environment, leading to rapid induction of immediate-early genes. Furthermore, Signature II's pathological relevance is supported by the presence of many genes that have been previously

associated with autoimmune arthritis. For example, NR4A2 (also called NURR1), the first-ranked gene in Signature II, is markedly up-regulated in rheumatoid arthritis synovium (21). Likewise, OSM (Oncostatin M), the third-ranked gene in Signature II, is detectable in JIA synovial fluid and it can induce joint inflammation in a murine adenoviral gene transfer model (22). Thus, Signature II may provide valuable information regarding pathological processes in samples with prolonged processing that may not otherwise be observed if processing were prompt.

Signature III was associated with absence of rheumatoid factor (Figure 3), and appears to be distinct from Signature I. In fact, few subjects expressed both Signatures I and III together (Figure 2), suggesting that these signatures are manifestations of independent biological processes. Like Group A, the average age of Group C was greater than that of the entire polyarticular JIA population, supporting the concept that Signature III identifies a distinct subset of JIA patients. Additionally, Group C had a trend toward lower ESR, lower CHAQ scores and lower physician global assessment of disease activity (Table 1), all of which are consistent Signature III identifying a less inflammatory subset of polyarticular JIA, possibly similar to the “dry synovitis” subset described by Ravelli and Martini (3). Signature III was associated with low numbers of CD8⁺ T cells (Figure 4), increased abundance of BDCA-4⁺ plasmacytoid dendritic cells, and it contains many genes that are inducible by TGFβ and potentially involved in mediating or regulating TGFβ action. These include the first ranked gene, DAPK1, which is a pro-apoptotic protein that can be induced by TGFβ via SMAD activation (23). Other Signature III genes that are TGFβ-inducible include SMAD3, BCL2, MAPK1 and FOXO3A (24-27). These observations suggest that TGFβ may be responsible for reducing levels of CD8⁺ T cells via a pro-apoptotic influence, and increasing plasmacytoid dendritic cells via an activating effect. Notably, increased levels of TGFβ have been detected in synovial fluid in juvenile arthritis (28), where it has been conjectured to serve an immunosuppressive role in countering synovial inflammation (29). Interestingly, while one might conjecture that PBMC exposure to TGFβ occurs in inflamed synovium, another intriguing possibility is via regulatory T cells that express TGFβ (30).

Many polyarticular JIA subjects did not express Signatures I, II or III (24 out of 61, or 39%). These subjects were statistically younger and had a higher rate of ANA positivity than the rest of the polyarticular JIA subjects. It is uncertain as to whether these patients represent a unified group, but one can speculate that they do not have the same disease phenotype as subjects that express either Signature I and/or III, and given their age and ANA status it is likely that many of these subjects comprise a polyarticular JIA subset that closely resembles early-onset ANA positive oligoarticular JIA, described by Ravelli, et al. (3,31). It is also noted that a few normal controls clustered into Groups A, B or C. While control subjects were deemed free of inflammatory disease by screening questionnaire, there was no assurance that every control subject was completely healthy and this may account for co-clustering of some controls with JIA subjects.

In summary, PBMC gene expression signatures have been demonstrated within a large population of children with recent onset polyarticular JIA that correlate with differences in disease characteristics. These signatures support the sub-classification of polyarticular JIA offered by Ravelli and Martini (3), with Signature I identifying both RF+ and RF- patients with disease similar to adult rheumatoid arthritis, Signature III identifying a less inflammatory subset, and patients with ANA+ early-onset disease expressing neither signature. Thus, these signatures offer a molecular classification of polyarticular JIA, and may prove to be valuable tools for assessing disease activity, predicting response to medications and forecasting long-term outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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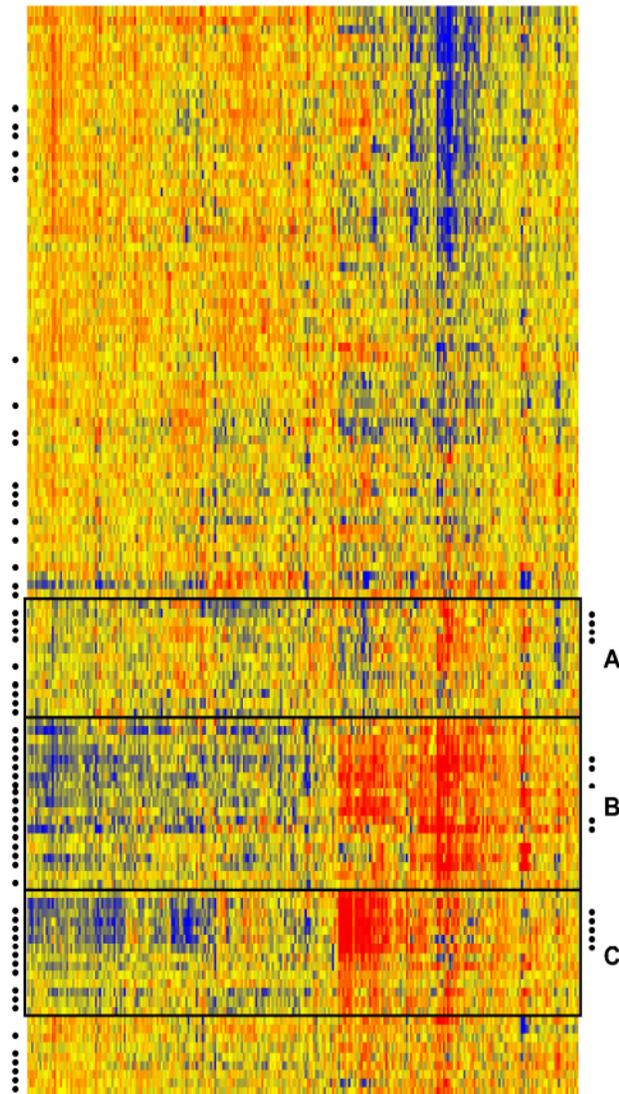


Figure 1.

Expression heat map for 873 probe sets identified by ANOVA as differentially expressed between RF- polyarticular JIA subjects (n=45) and controls (n=59). Relative intensity of red or blue indicates greater or lower expression compared to the mean of all samples for that probe set (yellow). Sixty-one subjects with either RF+ or RF- polyarticular JIA, and 59 normal controls are represented. Polyarticular JIA subjects are indicated by bullets on the left. Subjects are ordered by gene expression clustering using Pearson correlation with average linkage. Three clusters with predominantly JIA subjects are boxed, and the JIA subjects from these clusters comprise Groups A, B and C. Bullets on the right side of the figure indicate the JIA subjects from each group that served as prototypes for defining Signatures I, II and III.

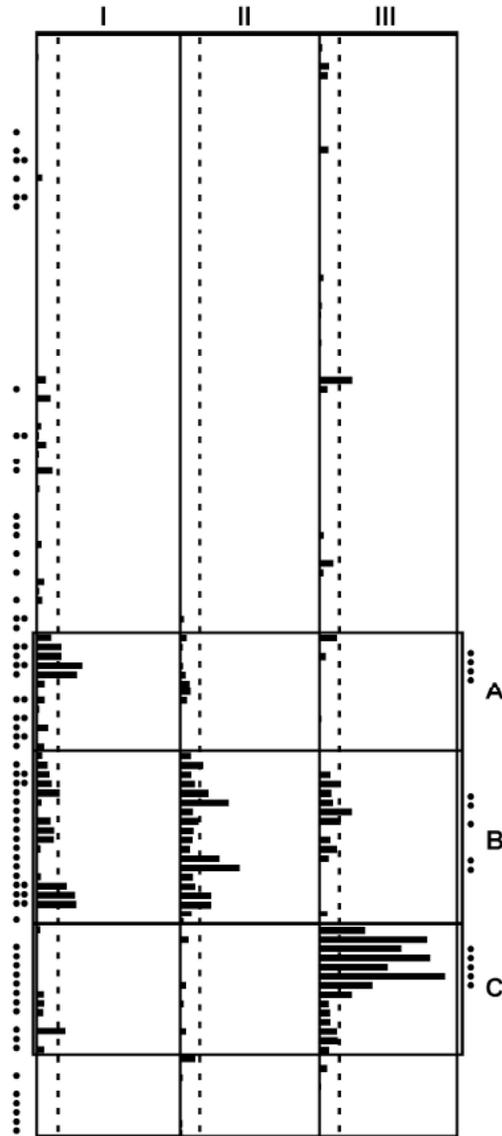


Figure 2. Comparison of Signatures I, II and III among subjects. The magnitude of each signature in individual subjects was calculated as the average fold increased expression of the 50 probe sets that define each signature over the mean of those 50 probe sets in the cohort of 49 signature-free controls. The dashed line indicates 2-fold increase over the mean of controls, and the baseline is the mean of controls (value of 1). Subjects are identical to and in the same order as Figure 1. Single bullets on the left indicate subjects with RF- polyarticular JIA, and double bullets indicate RF+ polyarticular JIA subjects. Groups A, B and C are boxed, and prototypical JIA subjects are indicated by bullets on the right, as in Figure 1.

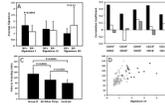


Figure 3.

Correlation of gene expression signatures with RF status, monocyte lineage markers and time to freezing. **A.** Average magnitudes of each gene expression signature (I, II and III) are compared between RF+ (n=14) and RF- (n=47) polyarticular JIA subjects. The only statistically significant difference by t-test was for Signature I. Error bars represent standard deviations. **B.** Correlation coefficients are shown for each gene expression signature (black=Signature I, clear=Signature II and grey=Signature III) in relation to monocyte lineage subsets determined by flow cytometry. **C.** Average TTF is compared between Group B (n=16), polyarticular JIA subjects not in Group B (All Other Polys, n=44) and controls (n=59). Statistically significant differences are indicated. Error bars represent standard deviations. **D.** TTF for each subject is plotted against magnitude of Signature II for that subject. Solid circles indicate subjects in Group B, and open circles indicate all other subjects including controls.

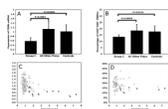


Figure 4.

Correlation of Signature III with CD8b expression and abundance of CD8⁺ T cells. **A.** Relative expression of CD8b mRNA is compared between Group C (n=11), JIA subjects not in Group C (All Other Polys, n=50) and controls (n=59). Statistically significant differences are indicated and error bars represent standard deviations. **B.** Percentage of CD3⁺CD8⁺ PBMC determined by flow cytometry is compared between Group C (n=8), All Other Polys (n=36) and controls (n=28), as in panel A. Only 72 out of 120 subjects had samples available for flow cytometry. **C.** Relative expression of CD8b mRNA is plotted against magnitude of Signature III for each subject. Solid circles indicate subjects in Group C, and open circles indicate all other subjects including controls. **D.** Percentage of CD3⁺CD8⁺ PBMC is plotted against magnitude of Signature III for each subject, using the same symbols as panel C.

Table 1

Characteristics of polyarticular JIA subgroups*

	Group A (n=9)	Group B (n=17)	Group C (n=11)	Other Polys (n=24)
Age (mean years \pm SD) [†]	11.3 \pm 3.6	9.8 \pm 4.4	11.3 \pm 4.6	7.0 \pm 4.5
Female	7 (78)	16 (94)	8 (73)	21 (88)
RF positive (two + tests, >3 mo apart) [‡]	5 (56)	5 (29)	0	4 (17)
Anti-CCP positive [§]	5 (56)	5 (29)	2 (20)	6 (30)
ANA positive [¶]	3 (33)	7 (41)	4 (36)	15 (65)
ESR (median mm/h (IQR)) [#]	26 (10-30)	22 (18-49)	15 (5-28)	17 (11-28)
Active joint count (median (IQR))	9 (9-14)	12 (8-21)	9 (8-11)	8 (5-11)
CHAQ (median, 0 to 3 (IQR)) ^{**}	1.063 (0.375-1.875)	0.813 (0.375-1.375)	0.500 (0.125-1.000)	0.375 (0.000-1.250)
Physician global (median, 0 to 10 (IQR))	6 (5-7)	7 (5-9)	4 (4-7)	5 (4-7)
Parent global (0 to 100 \pm SD, poor to well) ^{††}	65 \pm 33	62 \pm 26	57 \pm 26	67 \pm 23
Small joint involvement	8 (89)	16 (94)	10 (91)	21 (88)

* Except where indicated, values are the number of subjects (%).

[†] P=0.018 by one-way ANOVA.[‡] P=0.008 by Fisher Exact test comparing Group A vs. Group C.[§] One subject in Group C and 4 subjects in Other Polys were not tested for anti-CCP.[¶] P=0.026 by Fisher Exact test comparing combined Groups A, B and C vs. Other Polys. One subject in Other Polys was not tested for ANA.[#] IQR=inter-quartile range. Two subjects in Group A, one subject in Group C, and 6 subjects in Other Polys were not tested for ESR.^{**} One subject each in Group A, Group B, and Other Polys were not assessed by CHAQ.^{††} One subject each in Groups A and B, and two subjects in Other Polys were not assessed by parent global.

Table 2

Top 25 out of 50 probe sets that define gene expression Signatures I, II and III

Signature I			Signature II			Signature III		
Probe Set	Gene Symbol	Fold ↑*	Probe Set	Gene Symbol	Fold ↑	Probe Set	Gene Symbol	Fold ↑
155728_a_at	MS4A4A	4.63	216248_s_at	NR4A2	16.38	239162_at	DAPK1	8.05
205568_at	AQP9	4.27	38037_at	HBEGF	10.36	237001_at	NIBP	7.71
216951_at	FCGR1A	4.09	205239_at	AREG	7.93	240544_at	ZFAND3	7.35
214021_x_at	ITGB5	4.08	230170_at	OSM	7.93	1557811_a_at		7.23
241981_at	FAM20A	3.89	208078_s_at	SNF1LK	6.59	239448_at	SMAD3	7.20
210873_x_at	APOBEC3A	3.79	201694_s_at	EGR1	6.35	1556865_at	PACSIN2	6.87
208791_at	CLU	3.75	242904_x_at		5.55	244860_at		6.78
206655_s_at	GP1BB	3.62	242397_at	OLR1	5.53	241154_x_at	MTSS1	6.74
218660_at	DYSF	3.55	202768_at	FOSB	5.47	232522_at	TCF7L2	6.73
210119_at	KCNJ15	3.45	1557285_at	LOC653193	4.92	1559723_s_at	C9orf3	6.58
233749_at	MSN	3.32	241824_at	FOSL2	4.41	206548_at	FLJ23556	6.39
206026_s_at	TNFAIP6	3.28	214696_at	MGC14376	4.38	1561166_a_at	FOXPI	6.17
212651_at	RHOBTB1	3.27	204470_at	CXCL1	4.37	235701_at	R3HDM2	6.16
211163_s_at	TNFRSF10C	3.27	1556874_a_at	RKHD2	4.32	1561167_at	ETV6	6.16
206343_s_at	NRG1	3.21	209189_at	FOS	4.25	238544_at	IGFIR	6.10
207808_s_at	PROS1	3.13	1568665_at	RNF103	4.19	215597_x_at	MYST4	5.99
214469_at	HIST1H2AE	3.09	204014_at	DUSP4	4.18	227062_at	TncRNA	5.97
1555659_a_at	TREML1	3.08	226578_s_at	DUSP1	4.17	240254_at	TNIK	5.85
206493_at	ITGA2B	3.08	237082_at	DDEF1	3.99	232882_at	FOXO1A	5.80
237563_s_at	LOC440731	3.02	1559203_s_at	KRAS	3.70	1557555_at	MAD1L1	5.71
231711_at		3.02	204794_at	DUSP2	3.65	238812_at	ZA20D3	5.56
216243_s_at	IL1RN	2.97	243213_at	STAT3	3.61	1556493_a_at	JMJD2C	5.53
229967_at	CMTM2	2.96	207630_s_at	CREM	3.51	244682_at	CAMSAP1	5.51
226303_at	PGM5	2.95	202861_at	PER1	3.38	242801_at	WVOX	5.49
214073_at	CTTN	2.94	201465_s_at	JUN	3.28	1569578_at	ANKRD11	5.48

* Fold ↑ represents the ratio of the geometric mean of prototype samples for each group to the geometric mean of 49 signature-free controls.