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Biological Predictors of Extension of Oligoarticular Juvenile Idiopathic Arthritis from Synovial Fluid Cellular Composition and Gene Expression

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Abstract

Objective To identify biomarkers in the first synovial fluid aspirate of children with oligoarticular juvenile idiopathic arthritis (OA-JIA) that can be used to identify children whose disease is likely to extend to a more severe phenotype.

Methods Recent onset OA-JIA patients were identified and grouped according to persistence or extension at one year from diagnosis. Flow cytometry was used to delineate differences in mononuclear cell populations between first blood and synovial fluid in the same patient and between outcome groups. Lymphocyte proportions in the joint were modeled on chemotaxis of lymphocytes to CCL5 using transwell migration assays. Synovial fluid CCL5 was quantified by ELISA. RNA from synovial fluid mononuclear cells was compared between groups using Affymetrix Gene Chip HGU133plus2.0.

Results Compared to blood, synovial fluid cells had an expansion of CD8+ T cells, reduced B cells and an expansion CD16- NK cells. The lower CD4:CD8 ratio in synovial fluid was recapitulated *in vitro* by migration of blood T cells to CCL5. Synovial CCL5 levels were higher in patients who extended. The CD4:CD8 ratio in synovial fluid was significantly lower in patients who extended (0.90 vs. 0.57, difference=0.33, 95%CI=0.04 to 0.62). 344 genes were > 1.5-fold differentially expressed between outcomes ($P<0.05$) and included genes associated with inflammation and macrophage differentiation increased in patients who had extended at one year and genes associated with immune regulation increased in the patients who persisted.

Conclusions Synovial lymphocyte proportions, levels of CCL5 and gene expression yielded potential biomarkers with which to predict extension of OA-JIA.

Introduction

Inflammatory arthritis in children presents with a diverse range of phenotypes, of which the most common is arthritis that starts in 4 or less joints, known as oligoarticular juvenile idiopathic arthritis (OA-JIA) (1). Although sometimes thought of as a benign condition, OA-JIA may in fact lead to a wide spectrum of outcomes ranging from complete remission off medication, to a severe, extended form of JIA which spreads to involve many joints (≥5). Extended OA-JIA can be highly erosive and destructive and may be difficult to control with conventional DMARDs thus requiring long term treatment with biological therapies. When OA-JIA remains limited to 4 or less joints, so called persistent OA-JIA, it is typically relatively easily controlled with local intra-articular steroid and non steroidal medication (2;3).

A large study of remission rates in different subtypes of JIA found that after ≥ 4 year follow up, only 31% of children with extended OA-JIA achieved remission off medication, compared to 68% of those with persistent OA-JIA (4). In a long term retrospective outcome study in adults who had had JIA, those with extended OA-JIA had significantly worse HAQ scores than those whose disease had remained persistent (<4 joints) (5). Extension to 5 or more joints in this form of childhood arthritis has been reported to be as high as 50% at 5 years and carries a far higher risk of chronic disability (6). Several clinical factors have been proposed as predictors of extension, such as high ESR, upper limb involvement and involvement of more than one or symmetrical joints at presentation (6;7). At present, no simple clinical algorithm can predict extension but meeting this need would represent a major step forward in the care of children with arthritis.

We have previously shown that once extension to many joints has occurred, these two subtypes of OA-JIA have significantly different immunological characteristics in their

synovial infiltrate. Thus we demonstrated that the pro-inflammatory T cell subset that produces IL-17, IL-21 and IL-22 (Th17) is enriched in the joints of children with JIA compared to blood and that this cell type is found in significantly higher numbers in the joints of children with extended OA-JIA than those with persistent OA-JIA (8). In contrast we and others have shown that immuno-regulatory T cells (Treg), which express high levels of CD25 (IL2Ralpha) and the transcription factor Foxp3, are present at significantly higher numbers in the joints of children with persistent OA-JIA than those with extended disease (9;10). Interestingly we also found that these two specific subpopulations show a directly reciprocal relationship with each other within the joint (8).

As well as these cellular correlates, there are significant genetic differences between these two subtypes of JIA. Within the HLA region, which codes for multiple genes critical to immune function, the haplotype HLA-DRB1*0801-DQA1*0401-DQB1*0402 is associated with both persistent and extended oligarticular JIA although the effect size is greater within the extended subgroup, while the HLA-*1301-DQA1*01-DQB1*06 haplotype confers an increased risk of persistent but not extended OA-JIA (11). Similarly an IL-10 promoter polymorphism which is associated with low IL-10 production (ATA haplotype) has been found to be significantly associated with extended OA-JIA (12).

Given these cellular and genetic associations which are relatively specific for these two subtypes of JIA, we reasoned that there may be differences in molecular or cellular features within the inflamed joint, early in OA-JIA, which would be predictive of extension from mild to more severe disease. If so these might form the basis of a diagnostic test, which combined with clinical features might be used to generate an algorithm allowing selection of those children who need more frequent follow up and early intervention with DMARDs. Previous studies using gene expression profiling in JIA have focused upon polyarticular disease (13) or have not distinguished between persistent

and extended OA-JIA (14). To our knowledge no previous study has specifically chosen to look for predictors of extension, before it occurs, by analysis of the transcriptome. Such an approach to the development of predictive biomarkers has been highly successful in other fields, notably oncology (15;16).

Here we tested the hypothesis that synovial fluid (SF) cellular composition, gene expression and/or cytokine/chemokine profile could reveal significant differences which are predictive of extension before it occurs. We used SF samples taken at first therapeutic aspiration and before use of DMARDs and we chose early extension (within one year from diagnosis) as the endpoint. We have opted to use the term ‘extended-to-be’ for those children who were studied at a time when disease was still limited to 4 or less joints but whose oligoarthritis had extended by 1 year of follow up.

Patients and Methods

Patients and samples

Samples from a total of 38 children (26 females/12 males) who met ILAR criteria for OA-JIA (1) and 6 healthy controls were included in this study. Patients attended either Great Ormond Street Hospital, London, or the Royal Victoria Infirmary, Newcastle, the latter as part of the Childhood Arthritis Prospective Study (CAPS) (17). The study had approval from the local and multicentre ethical review committees (LREC and MREC). Full informed consent was obtained from the parents of each child.

The 38 JIA patients in this study had a mean age of 8.8 years (range 1.3 – 13 years) and median disease duration of 7 months (range 2 – 16 months). All 38 children were undergoing their first knee aspiration of synovial fluid in order to receive intra-articular injection of triamcinolone hexacetonide and none of the children had yet received methotrexate, steroids or any other DMARD (Table 1). Synovial fluid (SF) samples were obtained at the time of clinically indicated arthrocentesis and, where available, samples of peripheral blood (PB) were collected at the same time. Samples were processed within 1 hour of removal from the patient. PB mononuclear cells (PBMC) were isolated by density centrifugation using Lymphoprep (Axis-Shield, Oslo, Norway). For preparation of synovial fluid mononuclear cells (SFMC), samples were first treated with 10 U/mL hyaluronidase (Sigma, UK) for 30 minutes at 37°C, before isolation as described for PBMC. For some patients, samples of plasma or synovial fluid were also obtained for the analysis of cytokines and chemokines. These were spun to remove cells and frozen at -80C within 1 hour of removal from patient.

Analysis by flow cytometry

Standard five-colour flow cytometry was performed for surface markers using directly labelled monoclonal antibodies against the following human proteins: CD3-PECy7 (UCHT1, Southern Biotech, Birmingham, AL), CD3-APC (S4.1, Caltag, Burlingame, CA), CD4-PerCP (L200, BD Biosciences, San Diego, CA), CD4-APC (S3.5, Southern Biotech), CD8-FITC (G42-8, BD Biosciences), CD8-PE (DK25, Dako, Glostrup, Denmark), CD8-PECy7 (RPA-T8, BD Biosciences), CD8-APC (RTF8, Southern Biotech), CD13-APC (WM15, BioLegend, San Diego, CA), CD14-PerCP (MΦP9, BD Biosciences), CD16-PerCPCy5.5 (3G8, BD Biosciences), CD19-FITC (HIB19, BD Biosciences), CD25-PE (ACT-1, Dako), CD25-PECy5 (M-A251, BD Biosciences), CD56-PE (B159, BD Biosciences), CD195-PE (2D7/CCR5, BD Biosciences) and γδTCR-FITC (11F2, BD Biosciences).

The following intracellular proteins were detected by flow cytometry following fixation and permeablization of cells: Foxp3-APC (PCH101, eBioscience, San Diego, CA), Ki67-FITC (B56, BD Biosciences) and IL-17-Alexa Fluor 647 (64CAP17, eBioscience). For detection of Foxp3, buffers from the Foxp3 Staining Set (eBioscience) were used. For Ki67 and IL-17, cells were fixed with 4% paraformaldehyde (BDH, VWR, UK) in PBS (Sigma) and permeablized with 0.1% saponin in PBS containing 1% fetal calf serum (Invitrogen, Renfrew, UK) and 0.1% sodium azide (Sigma). For analysis of IL-17 production by T cells, the SFMC or PBMC were cultured for 3 hours in the presence of 50 ng/mL of phorbol myristate acetate (PMA) (Sigma), 500 ng/mL of ionomycin (Sigma) and 5 μg/mL of Brefeldin A (Sigma) before analysis by intracellular staining and flow cytometry as described above.

Levels of apoptosis in cell populations were estimated by detecting active caspases using the Vybrant FAM Poly Caspase Assay Kit (Molecular Probes, Eugene, OR). VAD (valine-alanine-aspartic acid) binds to the groove in most caspases (including caspase-1

and caspases 3 through 9) that is exposed by cleavage to their active forms (18). The FLICA reagent combines VAD with FMK to create a covalent link with the target caspase and FAM for fluorescence detection. Cells were resuspended in RPMI 1460 (Invitrogen) at 1 x 10^6/mL, incubated at 37ºC 5% CO_2 for 1 hour with FLICA reagent, washed with the kit buffer and stained for CD3, CD4 and CD8 as above. PBMC that had been stimulated with anti CD3/anti CD28 beads (Miltenyi Biotech, Bergisch Gladbach, Germany) for 4 days followed by 20 hour treatment with Brefeldin A (Sigma) was used as a positive control for caspase activity.

Flow cytometric data were collected on an LSRII (BD Biosciences) or Cyan ADP (Beckman Coulter, Fullerton, CA); 100,000 to 200,000 events were collected for each condition and cells gated by scatter properties. Data were analysed using FlowJo (TreeStar Inc, Ashland, OR).

ELISA

Detection of CCL5 in plasma and synovial fluid by ELISA (R&D Systems, Abingdon, UK) was performed according to manufacturer's instructions.

Chemotaxis Assay

PBMC were allowed to adhere to plastic for 3 hours at 37ºC 5% CO_2 in RPMI/5% human AB serum in order to deplete monocytes and enrich for lymphocytes. Non-adherent cells at a starting concentration of 5 x 10^6/mL were exposed to 20 – 500 ng/mL CCL5 (R&D Systems) in RPMI/0.5% BSA, or RPMI/0.5% BSA alone in the lower chamber of a 5μm pore polycarbonate filter Transwell chamber (Corning Life Sciences, Schiphol-Rijk, The Netherlands). After 90 minutes, migrated cells were recovered and stained with anti-human CD3, CD8 and CD4 as above. Immediately prior to acquisition of data by flow

cytometry, 20 000 FACS counting beads (Perfect Count, Cytognos, Salamanca, Spain) were added per sample. The number of cells acquired was standardised relative to bead numbers.

Gene expression profiling

SFMC was thawed and total RNA was prepared from 2 - 5 x 10^6 cells using RNeasy spin columns (Qiagen, Hilden, Germany). Quality and purity of RNA was assessed using the Bioanalyser 2100 (Agilent Technologies, Palo Alto, CA). cDNA and subsequent cRNA synthesis was then performed as previously described (19). Purified cRNA transcripts were fragmented and hybridized to human U133 plus 2.0 GeneChips according to Affymetrix standard protocols (http://www.affymetrix.com). Signal values were calculated using MAS 5.0, scaled to 100 and normalized to the median prior to analysis with GeneSpring GX 10 software (Agilent Technologies). Probesets were excluded if the signal strength did not significantly exceed background values and if expression did not reach a threshold value for reliable detection [based on the relaxed Affymetrix MAS 5.0 probability of detection; $P<0.1$ (20) in 5 out of 21 samples. A list of probesets where the difference between mean expression levels of the two patient groups was greater than 1.5 fold was statistically analyzed by the Student's *t* test with Welch's correction and a *P* value cut-off of 0.05 was applied. Functional categories for corresponding genes were assigned using gene ontology terms from the Gene Ontology Consortium (www.geneontology.com). Hierarchical clustering was performed using average linkage. Complete gene expression data for each patient are available on the GEO database (www.ncbi.nlm.nih.gov).

Statistics

All statistics including logistic regression were performed in Statistical Package for the Social Sciences version 15 (SPSS Inc., Chicago, IL). A *P* value of less than 0.05 was considered significant.

Results

In this study of the first joint aspirate obtained from children with oligoarticular JIA, we defined the composition of the mononuclear cell populations in the SF compared with the PB. We then looked for looked for demonstrable differences in SFMC composition in children whose arthritis would remain mild (persistent oligoarticular JIA) compared to those who would go on to extend to a more severe phenotype (extended-to-be oligoarticular JIA). At the time of sampling, there were no significant differences between clinical parameters in these two groups (Table 1).

The composition of mononuclear cells in synovial exudate differs markedly from PBMC in children with OA-JIA

Flow cytometry was used to compare the proportions of mononuclear cell subsets in blood versus synovial fluid aspirated from patients with OA-JIA. The most prominent differences were observed in the paucity of B cells (1.8 ± 1.3% S.D. in synovial fluid vs. 14.5 ± 4.8% in blood) (Figure 1A) and the dominance of CD8 T cells (Figure 1B) in synovial fluid so that there was a reversal of the typical CD4:CD8 T cell ratio observed in blood of children (21). Within the natural killer cell subset, identified as expressing CD56, we observed a preponderance of CD16- cells (Figure 1C). The majority of blood-borne natural killer cells express the Fc receptor. The downregulation of CD16 on NK cells in an inflammatory site has been described in RA (22).

Reversal of the CD4:CD8 ratio in synovial T cells early in disease correlates with likelihood of progression to more severe disease phenotype

We compared synovial fluid T cell, B cell, natural killer cell, monocyte/macrophage and dendritic cell subset proportions between the patient group whose disease had remained persistent (<5 joints) (n=21) and those whose disease had extended to 5 or more joints at one year (n=11), (demographics shown in Table 1). A statistically significant difference was observed in the proportion of the total SFMC that were CD4+ T cells (Figure 2A). While both groups had the same proportion of synovial fluid T cells overall (Figure 2A), the CD4:CD8 ratio was lower in the group that had extended at one year (difference=0.33, 95% CI=0.038 – 0.62, P=0.009)(Figure 2B). Since this ratio is a simple laboratory measure that can be routinely performed, we considered its merit as a predictive test for extension in OA-JIA. A receiver-operator curve was used to establish a ratio of 0.67 as optimal for maximizing the sensitivity and specificity of the test (Figure 2D). In our data set, a patient with a CD4:CD8 ratio of less than 0.67 was 2.5 (95% CI=1.16 – 5.4) times more likely to extend. The predictive power of joint cell proportions was further increased using a logistic regression model that used B cell percentages in addition to CD4 T cell subsets. B cell proportions were higher, but not significantly, in the group that had extended at one year (Figure 2A) but in concert with the CD4+ T cell proportions, the regression model correctly assigned 27 out of 32 of the patients (84%) (Figure 2C). Other cell subsets, including the CD8+ T cell proportion, were able to improve the accuracy of prediction but did not contribute significantly to the model (P<0.05). The addition of joint count at time of sample or ESR, did not significantly improve the model. The results of the logistic regression and the equation used to calculate the probability of extending are shown as supplementary data (Supplementary Table 1).

We have previously shown that once extension occurs, there are significant differences in proportions of both Treg and Th17 cells in the joint (8;9). Therefore we examined the Treg (Foxp3+CD4+) and IL-17+CD4+ T cells subsets in these two patient groups. Neither Treg nor Th17 measured at presentation predicted extension although the reciprocal relationship between the numbers of these two cells types was maintained (Supplementary figure 1).

Synovial CD4 and CD8 T cells are matched in cell turnover rates

Having established the predominance of CD8 T cells in the joint and the implications for predicting disease course, the cellular dynamics that were potentially driving CD8 T cell accumulation were investigated. We hypothesized that CD8 T cells might be expanding through proliferation or undergoing apoptosis to a lesser extent compared to CD4 T cells. Ki67 is an antigen that is expressed during S and M phases of the cell cycle and can thus report the fraction of a population undergoing cell division (23). No difference in proportion of Ki67+ cells was observed between CD4 and CD8 T cells from the same SFMC samples (Figure 3A). Similarly, we estimated apoptotic activity by detecting activated caspases using VAD-FAM reagent. We observed no significant difference in VAD+ cells between CD4 and CD8 SF T cells (Figure 3B). Therefore, we concluded that the observed dominance of CD8 T cells over CD4 T cells in the joint could not be explained by differences in cell death or proliferation.

Migration to synovial chemokines may contribute to altered CD4:CD8 ratio within the joint

We considered mechanisms that might enrich for CD8 T cells through chemoattraction from blood to the inflamed joint. The CC-type chemokine receptor 5 (CCR5/CD195) is

expressed on a higher proportion of CD8 T cells compared to CD4 T cells in the blood of healthy adults and children (24), in the patients in this study (Figure 3C) and in our previous study (25). We and others have described a role for CCL5 (RANTES), a ligand of CCR5, in JIA and indeed, high levels in blood and synovial fluid have been reported to anticipate flares (26;27). CCL5 was significantly higher in the synovial fluid of extended-to-be patients compared to patients who remained persistent (Figure 3D). Synovial fluid CCL5 levels showed weak correlation with synovial fluid CD8+ T cell proportions (Figure 3E) suggesting that CCL5 could play a role in the enrichment of CD8 T cells in the joint. To test the effect of CCL5 on T cell migration *in vitro*, non-adherent PBMC from healthy adults was exposed to increasing concentrations of CCL5 in a transwell assay. CCL5 was able to enrich for CD8+ T cells compared to CD4+ T cells, thus decreasing the CD4:CD8 ratio in a dose-dependent fashion (Figure 3F).

Differential synovial mononuclear cell gene expression in children who will go on to extend to more severe disease

Microarray generated gene expression levels were obtained for 13 persistent and 8 extended-to-be SFMC samples. A total of 362 probesets covering 344 individual genes were at least 1.5-fold differentially expressed at a significance level of $P<0.05$. The annotated probesets are listed in Supplementary Table 2. The expression of 155 genes was increased and the expression of 189 genes was decreased in patients that extended compared to patients that persisted at one year. Probesets with greater than two-fold expression were clustered by average linkage along with patients (Figure 4).

Some of the differentially expressed genes associated with immune function and inflammation are listed in Table 2 and labelled on Figure 4. In general, genes encoding proteins with involvement in inflammation tended to be more highly expressed in the

extended-to-be group and genes associated with immune regulation were more highly expressed in the persistent group. One interesting exception in the persistent group included RORC, a transcription factor involved in the differentiation of pro-inflammatory IL-17-secreting cells (28).

A subset of genes that were more highly expressed in the extended-to-be group included markers of macrophage differentiation to both M1 (CXCL9, SLC31A2, ATF3) and M2 (CCL18, fibronectin) effector types (29). Several transcripts of genes for components of the complement pathway, also likely derived from macrophages, were overrepresented in the extended-to-be group. The proportion of monocytes/macrophages as identified by coexpression of CD13 and CD14 in the sample SFMC did not significantly differ between the persistent and extended-to-be sample sets that were subjected to microarray analysis or in the complete patient groups (Figure 2A). The cartilage glycoprotein-39 (chitinase 3-like 1), found in serum and synovium of both rheumatoid and osteoarthritis patients, tends to correlate with disease severity (30) so it was interesting to find more of its transcript in the extended-to-be patients in this study.

There was good agreement between gene expression and protein measurements for the CD8 antigen. It was more highly expressed in the extended-to-be group that had a higher proportion of CD8 T cells. Similarly, higher levels of interferon-γ (IFNγ) messenger RNA corresponded to a trend towards more IFNγ in the SF of the extended-to-be patients as detected by Luminex assay (data not shown).

Discussion

This study has sought predictors of future worsening of disease in children with an initially benign arthritis by screening for molecules in affected joints. Our results have revealed candidates with previously described roles in inflammatory or regulatory processes and novel factors whose role in JIA are not yet clearly defined.

This report documents the dominance of CD8 T cells in the joint and the corresponding reversal of the CD4:CD8 ratio in blood, typically 1.5 – 3 in healthy individuals (31), to less than 1 in the majority of synovial fluid samples analysed, all from children with OA-JIA. In this study, the extent to which this reversal occurs in the joint is a predictor of unfavourable outcome. Measuring CD4:CD8 ratio is a simple procedure and could be easily adopted as a prognostic test if validated in a larger study group. Logistic regression on all mononuclear cell types revealed that B cells, even their reduced proportions compared to blood, can also function prognostically, independent of the CD4:CD8 ratio.

The predominance of CD8 T cells in synovial tissue of JIA patients, and in the oligoarticular subtype in particular, has been previously described (32). In adult RA, CD4+ T cells are retained in the synovium while CD8+ T cells accumulate in the fluid (33). Unlike type I diabetes, where CD8 T cells participate in the destruction of pancreatic islet cells through specific lysis (34), no clear role for the cytotoxic lymphocyte has yet been demonstrated in the joint. Furthermore, synovial T cells have been reported to be anergic *ex vivo* (35). Since the level of cell turnover in synovial T cells did not differ between subsets, we favour a model in which the distribution of CD4 and CD8 T cells in the joint is a reflection of cytokine and chemokine milieu attracting migration into the joint. Our work supports a role for CCL5 (RANTES), shown here to bias migration of CD8 T cells over CD4 T cells and to be a predictor of extension in OA-JIA. Therefore, while we cannot exclude a distinct role for CD8 T cells, a low CD4:CD8 ratio may simply

be an indirect measure of the effects of chemokines affecting migration to the joint. Once in the joint, CD8+ T cells and not CD4+ T cells produce CCL5 (26). This may therefore provide a positive feedback loop enhancing the selective migration of CD8+ T cells. Further support for a role for CCL5 derives from the finding of TPSAB1, the gene encoding beta-tryptase, to be more highly expressed in patients that persisted. Beta-tryptase is a serine protease whose pro-arthritic potential (demonstrated in mice) (36) is thought to be due to its similarity to other matrix-destroying proteinases (37). However, Pang et al have recently described a role for beta-tryptase in the abrogation of CCL5 by specific cleavage (38). Thus this protein could play a role in limiting chemokine activity by cleavage within the joint.

The profile of differentially expressed genes between patients who persisted and those whose disease became worse gives a snapshot of early disease in which the shift toward pro- versus anti-inflammatory mechanisms has already been made. A significant subset of the genes expressed in the extended-to-be group overlapped with genes expressed in *in vitro* activated macrophages (29). Differentiation of macrophages, particularly the M1 type, is associated with greatly increased transcriptional and metabolic activity (29). Since the proportions of monocyte/macrophage phenotyped cells did not significantly differ in the samples subjected to microarray analysis, it is possible that the level of activation or differentiation in these cells was higher in the patients whose disease would later extend. These macrophage-associated genes together with complement and cartilage glycoprotein 39 form a group of genes that anticipate extension and link OA-JIA to more severe forms of JIA such as polyarticular and systemic onset (39-41).

Genes that were more highly expressed in persistent OA-JIA revealed factors with roles in immune regulation not previously examined in JIA. Adiponectin and the vasoactive intestinal peptide receptor both have anti-inflammatory signalling properties that correlate

with less severe rheumatoid arthritis and the amelioration of collagen-induced arthritis in mice (42-46). Our data implicate new pathways to disease control that may act independently or in concert with previously characterized activity of regulatory T cells. It was interesting that neither regulatory T cell proportions nor the Foxp3 protein levels in these cells predicted outcome in this study, however the association of SMAD3 with a more favourable outcome is consistent with its role as a transducer of the TGFβ signal that leads to Foxp3 expression and regulatory function (47). Similarly, proportions of Th17 cells were not predictive in this patient group and RORC, the transcription factor associated with Th17 development, was more highly expressed in the patients who persisted. Like TGFβ, the transcription factor RUNX1 has been linked to both Foxp3 and RORC transcriptional activity (48;49). RUNX1 and SMAD3, which were associated with extension and persistence, respectively, could therefore be functioning to modulate the transcriptome to tip the balance in favour of heightened inflammation or a more quiescent environment in the joint.

A possible limitation of using synovial cells for this analysis is that not all cases will have synovial fluid aspirated or available. However previous studies comparing gene expression profiling on peripheral blood cells in JIA have been unable to distinguish between persistent–OA-JIA and those who have already extended (at that time called polyarticular JRA)(14). For this reason and our previous work on synovial fluid differences between persistent and extended OA-JIA we chose to focus on synovial cells. Other studies looking for clinical predictors of extension have suggested that pattern and number of joints at presentation may be useful for prediction. All the subjects in this study had knee arthritis and therefore they may not represent a fully representative cross section of all types of oligoarticular JIA. In addition the study size may have been too small to detect such predictive value of these clinical factors. In our analysis in this group, adding

in such clinical indices did not improve our ability to predict those who would extend. In addition we have focused on outcome at only 1 year since this study aimed to uncover predictors of early extension: it is of course possible that the children in the persistent OA-JIA group in this study will later extend to have arthritis in 5 or more joints. However, we suggest that predictors of early extension may well be useful clinical /prognostic tools in the management of oligoarticular JIA.

In conclusion, we propose new ways to identify children at risk of developing a more severe form of JIA after an initially mild diagnosis by looking at transcripts and proteins in synovial fluid. These now deserve further investigation in prospective studies. While it is unlikely that any single measurement will individually discriminate between these two outcomes, it is possible that a 'biomarker set' of strong predictors could provide a valuable prognostic tool in this group of childhood arthritis.

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Table 1. Clinical Characteristics of patients with known outcome at one year

Outcome	Persistent (n=21)	Extended-to-be (n=11)
Female, n (%)	14 (66%)	8 (73%)
Mean age at time of sample, ± SD	6.8 ± 3.7 years	5.1 ± 3.5 years
Mean disease duration at time of sample ± SD	7.4 ± 3.7 months	8.1 ± 3.9 months
Erythrocyte sedimentation rate at time of sample, ± SD	21 ± 17	29 ± 23
MTX or prednisolone prior to or at time of sample	0	0
Mean number of joints involved at time of sample , ± SD	1.7 ± 1.0	2.0 ± 1.2
Mean cumulative number of joints involved by 1 year ± SD *	2.3 ± 1.0	5.6 ± 0.9
Mean number of actual joints involved at one year, ± SD	0.8 ± 1.3	3.8 ± 2.4

*As defined for persistent or extended status, (1)

Table 2. Genes differentially expressed in SFMC of extended-to-be vs. persistent OA-JIA patients

Functional category, Gene name	Symbol	Fold Change*
Cell surface proteins		
Interferon inducible 27	IFI27	4.2
Macrophage receptor with collagenous structure	MARCO	3.1
Interleukin 31 receptor alpha	IL31RA	2.7
T-cell immunoglobulin and mucin domain containing 4	TIMD4	2.1
Interferon alpha inducible protein	IFI6	1.8
Solute carrier family 31 member 2	SCL31A2	1.7
CD8 beta	CD8B1	1.6
CXCR6	CXCR6	-1.6
Vasoactive intestinal peptide receptor 1	VIPR1	-1.7
Leukocyte-associated Ig-like receptor 2	LAIR2	-2.0
CXCR2 (Interleukin 8 receptor beta)	IL8RB	-2.3
CCR3	CCR3	-2.7
Cytokines and chemokines		
CCL18	CCL18	3.0
CXCL9	CXCL9	3.0
Interferon gamma	IFNG	1.7
Small inducible cytokine subfamily E1	SCYE1	1.5

Complement		
C1q gamma	C1QC	4.0
C1q beta	C1QB	3.5
C1q alpha	C1QG	3.1
C2	C2	3.2
Nuclear receptors and proteins		
Nuclear receptor subfamily 4, group A2	NR4A2	2.3
Activating transcription factor 3	ATF3	1.9
Runt-related transcription factor 1	RUNX1	1.7
RAR-related orphan receptor C	RORC	-3.4
Cytoplasmic proteins		
Fatty acid binding protein 3	FABP3	2.3
Endoplasmic reticulum aminopeptidase 2	ERAP2	2.2
Mothers against DPP (Drosophila) homologue 3	SMAD3	-1.7
Extracellular Mediators		
Chitinase 3-like 1 (cartilage glycoprotein 39)	CHI3L1	4.2
Fibronectin 1	FN1	2.8
Adiponectin	ADIPOQ	-2.5
Tryptase alpha/beta 1	TPSAB1	-6.0

* Fold change is the ratio of the means of the two groups. Positive values denote higher expression in extended-to-be OA-JIA and negative values indicate higher expression in persistent OA-JIA.

Figure Legends

Figure 1. Differences in mononuclear cell subsets in synovial fluid compared with blood from children with OA-JIA. Flow cytometry was used to enumerate proportions of **A,** T cells vs. B cells from the live cell gate, **B,** CD4+ and CD8+ T cell subsets from the CD3+ live cell gate (γδTCR-bearing cells were also enumerated with a specific antibody not shown), and **C,** natural killer (NK) cell subsets from the CD56+ live cell gate. Representative plots of blood (left panels) and synovial fluid (middle panels) are followed by graphs (right panels) of the mean and standard deviation of 14 paired patient samples.

Figure 2. Differences in synovial fluid mononuclear cell subsets in persistent OA-JIA (21 patients) compared to extended-to-be OA-JIA (11 patients) and their utility as predictors of outcome. **A.** Mean and standard error of T cell subsets, B cells, monocytes/macrophages (CD14+) and total myeloid cells (CD13+) derived from flow cytometry and reported as a percentage of total live cells. Persistent (pale grey) are compared with extended-to-be (dark grey) OA-JIA. **B.** Flow cytometry derived CD4:CD8 T cell ratios for persistent and extended-to-be OA-JIA. Lines indicate medians. **C.** A logistic regression model that used proportions of CD4+ and CD19+ lymphocytes correctly predicted 27 out of 32 (84%) outcomes. Probability of extending is plotted for each patient. Lines indicate medians. **D.** Receiver-operator characteristic curves comparing CD4:CD8 ratio (area=0.79) with the CD4+ T cell-B cell model derived from logistic regression (area=0.90). ***** $P<0.05$, ****** $P<0.01$, ******* $P<0.001$ by Mann-Whitney test.

Figure 3. Measures of proliferation, apoptosis and migration on T cell subsets in synovial fluid. OA-JIA patient synovial fluid (n=12) (A and B), or blood (n=7) (C) CD4+ and

CD8+ T cells were compared for **A,** intracellular expression of Ki67, **B,** binding of VAD tripeptide to active caspases, and **C,** surface expression of CCR5 by flow cytometry gated on live, CD3+ cells. **D,** CCL5 (RANTES) levels were measured in synovial fluid by ELISA (n=10,5). Lines indicate medians. **E,** Relationship between synovial CCL5 and CD8 T cell accumulation in the joint (n=14) r^2= 0.13 *P*=0.21. **F,** CCL5 lowers CD4:CD8 ratio in a transwell assay using non-adherent healthy adult PBMC. Mean and standard deviation of three individuals is shown. ****** *P*<0.01 by Mann-Whitney test.

Figure 4. Hierarchical clustering of genes differentially expressed between persistent OA-JIA (n=13) and extended-to-be OA-JIA (n=8) SFMC. Heat map contains genes that are 2-fold differentially expressed at a significance of $P < 0.05$ by the Student's *t*-test with Welch's correction. Each row represents a probeset and each column represents a patient. The normalized expression level for each gene and each patient is indicated by colour with red, yellow and blue reflecting expression levels greater than, equal to or less than the mean of the all patients. The boxes below the cluster indicate patients who persisted (light grey) versus those who had extended at one year (black).