

BRIEF REPORT

Two Broad Categories Overlapping With Rheumatoid Arthritis Observed in Synovial Biopsies from Patients With Juvenile Idiopathic Arthritis

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Objective. The objective is to characterize transcriptomic profiles and immune cell composition and distribution in juvenile idiopathic arthritis (JIA) synovial biopsies, assess for associations of these features with clinical parameters, and compare JIA and rheumatoid arthritis (RA) synovial features.

Methods. RNA sequencing (RNASeq) was performed on 24 samples, with pathway analysis and inference of relative abundance of immune cell subsets based on gene expression data. Two multiplex fluorescence immunohistochemistry (IHC) panels were performed on 28 samples (including 13 on which RNASeq was performed), staining for CD206[−] classical and CD206⁺ nonclassical macrophages, and CD8⁺ and CD4⁺ T and B lymphocytes. Data were compared to a published series of early RA synovial biopsies.

Results. Pathway analysis of the most variably expressed genes (n = 339) identified a B and plasma cell signature as the main driver of heterogeneity in JIA synovia, with strong overlap between JIA and RA synovitis. Multiplex IHC confirmed heterogeneity of immune cell infiltration. M1-like macrophage-rich synovial lining was associated with greater lining hypertrophy and higher (CD45⁺) pan-immune cell and CD8⁺ T cell infiltration.

Conclusion. Our study indicates significant similarities between JIA and RA synovitis. Similar to RA, JIA synovia may be broadly categorized into two groups: (1) those with an inflammatory/adaptive immune transcriptomic signature, M1-like macrophage and CD8⁺ T cell infiltration, and thicker, M1-like macrophage-rich synovial lining, and (2) those with an M2-like macrophage transcriptomic signature, greater M2/M1-like macrophage ratios, and thinner, M2-like macrophage-rich synovial lining. Synovial features were not significantly associated with clinical parameters, likely because of group size and heterogeneity.

INTRODUCTION

Juvenile idiopathic arthritis (JIA) refers to a heterogeneous group of chronic immune-mediated arthritis with onset before 16 years of age. The synovial membrane is the main seat of

aberrant inflammation in JIA, sometimes spreading to surrounding tendon sheaths and bone. Disease pathophysiology (especially in its early phase) has not been completely elucidated, but involvement of both distinct immune subsets and joint-resident cells in established arthritis is widely accepted.¹

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Nevertheless, it remains unclear whether these effectors contribute equally to disease activity in all individuals or if pathophysiology varies across patients with JIA and/or disease stages.

It is now widely considered that several JIA categories share similarities with rheumatoid arthritis (RA).² In RA, synovial tissue biopsies have improved our understanding of multiple aspects of the disease. We and others have shown that synovial tissue inflammation is highly variable across patients (in degree, cellular composition, and molecular profile), even in early disease-modifying antirheumatic drug (DMARD)–naïve RA.^{3–5} These synovial features correlate with multiple clinical parameters and possibly with response to therapies.^{3,6} The potential of synovial tissue in precision medicine is therefore being actively investigated in RA.⁶ In contrast, few studies have explored synovial tissue alterations in JIA (although minimally invasive biopsy techniques are available)⁷; most compare synovial pathology between different clinically defined JIA subtypes.^{8,9} Here, we characterized synovial transcriptomic profiles (by bulk RNA sequencing [RNA-Seq]) and immune cell composition (by multiplex fluorescence immunohistochemistry [IHC]) in two large, overlapping series of JIA samples. We assessed for the existence of pathophysiologically and clinically relevant patient subgroups using a data-driven approach.

MATERIALS AND METHODS

Patients and samples. This study was approved by the ethics committee of the Université catholique de Louvain (P1200_30; 2017/09FEV/081). Patients with active JIA were recruited at Cliniques Universitaires Saint-Luc (Brussels, Belgium) and Hospital Santa Maria at Centro Hospitalar Universitário Lisboa Norte (Lisbon, Portugal). Synovial tissue from a clinically affected joint was obtained using needle arthroscopy or ultrasound-guided biopsy before intra-articular steroid infiltration. Demographic and clinical characteristics of patients with JIA are shown in Supplementary Table 1. Demographic and clinical characteristics of a previously published series of patients with early stage RA³ are summarized in Supplementary Table 2.

Transcriptomic profiling. Total RNA was extracted from synovial biopsies using the NucleoSpin RNA II extraction kit (Macherey-Nagel) including DNase treatment. RNA quality control was performed on a 2100 Bioanalyzer (Agilent). RNASeq was performed on all ($n = 24$) samples that yielded sufficient quantity (350 ng) and quality (RNA Integrity Number ≥ 5) of RNA (Supplementary Methods). RNASeq data were deposited in the GEO (National Center for Biotechnology Information) and are available via accession number GSE253553.

Multiplex fluorescence IHC. Two multiplex fluorescence IHC panels were performed on 5- μ m serial sections each of

formalin-fixed, paraffin-embedded (FFPE) synovial biopsies ($n = 28$, including $n = 13$ on which RNASeq had also been performed; Supplementary Methods). Multispectral image acquisition was performed using an Axioscan.z1 (Zeiss, 20 $\times$ objective).

Quantitative and semiquantitative evaluation of multiplex fluorescence IHC.

Images were quantified using QuPath software (Supplementary Methods). Semiquantitative evaluation (by a single-blinded scorer) of images was performed using Zen lite software (Zeiss). Biopsies were classified based on semiquantitative evaluation of the following: (1) macrophage composition of the synovial with CD45⁺CD68⁺CD206[−] (ie, “M1-like” macrophage predominant lining [M1-L]) or CD45⁺CD68⁺CD206⁺ (ie, “M2-like” macrophage predominant lining [M2-L]); (2) synovial lining thickness (visualized using CD68 staining) on a scale of 1 to 4 from low to high; and (3) pattern of distribution of pan-T lymphocytes (CD45⁺CD3⁺), CD8⁺ T lymphocytes (CD45⁺CD3⁺CD8⁺), CD4⁺ T lymphocytes (CD45⁺CD3⁺CD8[−]), and B lymphocytes (CD45⁺CD20⁺), as well as the number of “B-T lymphoid aggregates” (defined as dense clusters of pan-T and B cells with a diameter $\geq 100 \mu$ m).

Statistical analyses. Raw RNASeq read counts were normalized using DESeq2 Bioconductor Package and further analyzed on Qlucore Omics Explorer (Supplementary Methods). Pathway enrichment analyses were performed on Metascape, and CIBERSORTx was used to extrapolate relative abundance of immune cell subsets from gene expression data. All other statistical analyses were performed on GraphPad Prism version 9.

RESULTS

Patient and sample characteristics. A total of 39 patients with JIA were included in this study (Supplementary Table 1). RNASeq was performed on synovial biopsies from 24 patients, and multiplex IHC was performed on biopsies from 28 patients, with an overlap of 13 patients with both transcriptomic and histological analyses. Based on International League of Associations for Rheumatology (ILAR) classification, 76.3% had oligoarticular JIA, 13.1% had psoriatic JIA, and 10.5% had polyarticular JIA. Biopsies were all harvested from the knee, at diagnosis (36.1%) or at a subsequent episode of arthritis during follow-up (63.9%). No biopsy-related complications were reported. At the time of biopsy, 28.9% of patients had ongoing treatment with DMARDs (methotrexate in 11 patients, sulfasalazine in 1 patient, and TNF blockade in 2 patients). Early relapse, defined here as the onset of an active episode of arthritis (articular swelling and pain) within three months of steroid infiltration at the time of biopsy, was observed in 44.4% of patients.

Heterogeneity in JIA synovial tissue transcriptome driven by a B and plasma cell signature versus an “M2-like” macrophage-associated signature. We first performed a principal component analysis (PCA) on the expression of $n = 58,735$ genes in JIA synovial tissue samples, which showed a continuous distribution along PC1 and 2, rather than discrete subgroups (Figure 1A). To identify genes with the most variable expression across samples, we filtered on variance in expression across the dataset; a heat map showing the expression pattern of these genes ($n = 339$, with $\sigma/\sigma_{\max} \geq 0.4$) across samples is shown in Figure 1B (listed in Supplementary Table 3A). Pathway enrichment analysis of these genes showed highly significant enrichment of Ig production and other B and plasma cell-related pathways in the largest cluster of genes (gene cluster 1 [GC1]) (Figure 1C, Supplementary Figure 1A–C, and Supplementary

Table 3B). Because Ig genes account for a large proportion of GC1 genes, potentially overwhelming other pathways, we performed the same analysis after removing all Ig genes. The remaining genes were strongly enriched in the RA and proinflammatory and profibrotic mediator pathways and included several key genes involved in inflammatory synovitis (eg, *IL6*, *CXCL10*, *MMP1*, and *MMP3*) (Supplementary Figure 1C). A second cluster of genes (gene cluster 2 [GC2]) instead displayed enrichment in lipolysis and peroxisome proliferator-activated receptor (PPAR) signaling pathways, which have been associated with M2-like (in vitro) or nonconventional/tissue-resident macrophages (in vivo)^{10,11} (Figure 1B, Supplementary Figure 1B, and Supplementary Table 3C). Finally, a cluster of genes located on the Y chromosome distinguished male from female patients (in black; Figure 1B).

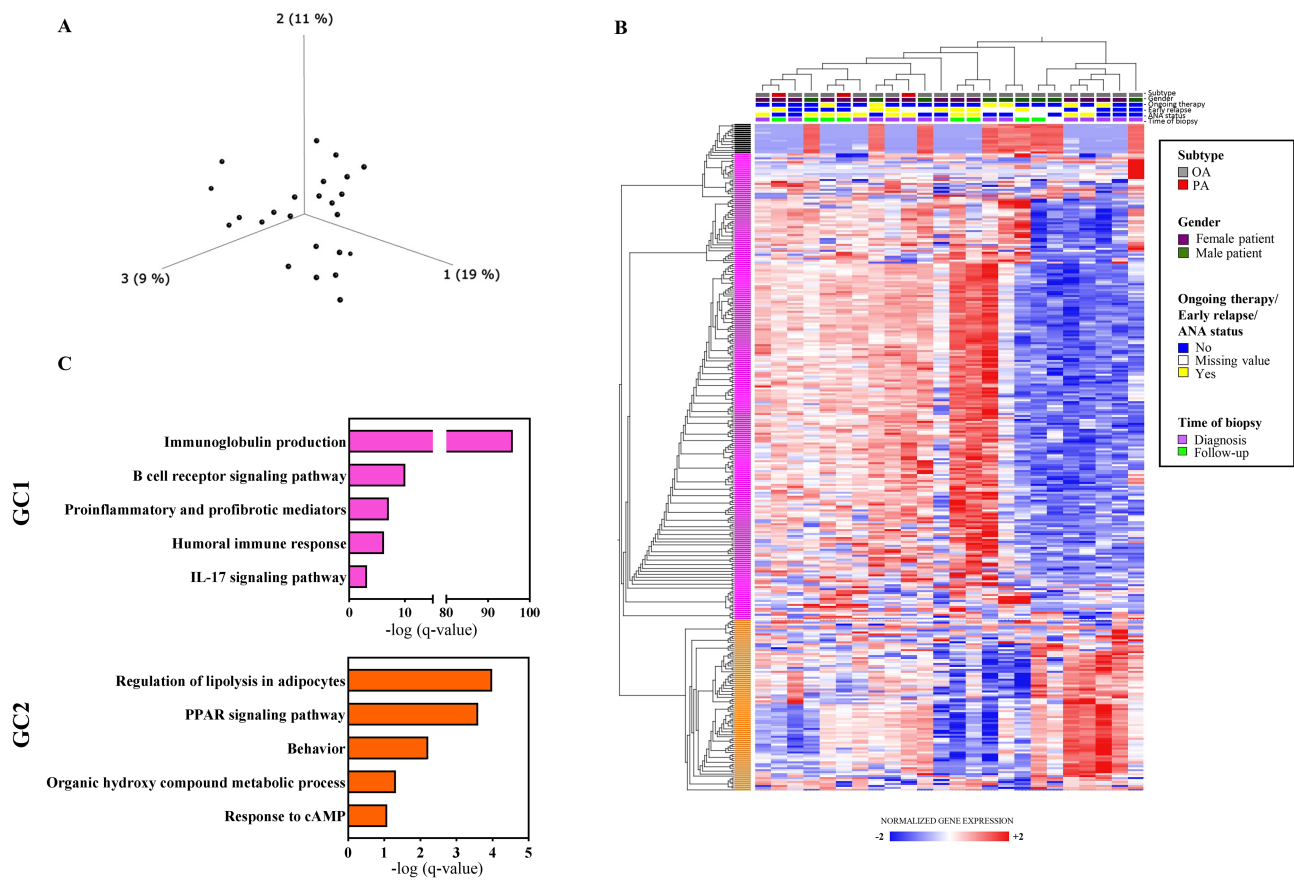


Figure 1. Heterogeneous transcriptomic profiles in JIA synovium. (A) PCA on expression levels of $n = 58,735$ genes in $n = 24$ JIA synovial biopsies. The proportion of variation explained by each PC is shown on the axes. (B) Heat map of scaled, normalized expression of the 339 most variable genes across samples. Genes and samples are classified using unsupervised clustering (average linking rule). JIA subtype (OA, gray; PA, red), sex (female patients, maroon; male patients, dark green), ongoing therapy (DMARD) at biopsy (yes, yellow; no, blue), early relapse (yes, yellow; no, blue; missing value, white), ANA (yes, yellow; no, blue; missing value, white), and time of biopsy (at diagnosis, purple; during follow-up, light green; missing value, white) are indicated above the heat map. Genes fall into two main groups (GC) using unsupervised clustering (average linking rule): GC1 (pink) and GC2 (orange). Genes in black are located on the Y chromosome and expressed exclusively in male patients. (C) Pathway enrichment analysis using Metascape (top five based on q value) on GC1 and GC2 genes, colored as in panel B. ANA, anti-nuclear antibody; GC, gene cluster; JIA, juvenile idiopathic arthritis; OA, oligoarticular; PA, polyarticular; DMARD, disease-modifying antirheumatic drug; PC, principal component; PCA, principal component analysis.

JIA synovial transcriptomic profile highly similar to that in RA. The pathways enriched in the two main gene clusters identified in JIA (GC1 and GC2) are highly reminiscent of those identified using the same strategy on 74 early, DMARD-naïve RA synovia.³ Indeed, 92.9% of the most heterogeneously expressed genes in JIA were also among the most variable genes identified in early RA (Supplementary Figure 2A). PCA (on the whole transcriptome or on the 620 most variable genes across all 98 JIA and RA samples) did not distinguish between the two diseases (Supplementary Figure 2B–C). Accordingly, supervised comparison of JIA versus RA synovial gene expression profiles yielded few differentially expressed genes (Supplementary Figure 2D), without robust pathway enrichment (data not shown). We used CIBERSORTx to compare inferred immune cell frequencies between JIA and RA synovia and found similar relative abundance of immune cells subsets (Supplementary Figure 2E). As in RA,³ synovial M2-like macrophage signature is negatively correlated with M0- and M1-like macrophage signatures as well as multiple adaptive immune cell subtypes (most strongly, plasma cells, CD4⁺ T memory cells, and CD8⁺ T cells) (Supplementary Figure 3).

Transcriptionally defined groups (samples with upregulation of GC1 genes vs those with up-regulation of GC2 genes) did not show significant differences in terms of clinical parameters such as age at diagnosis or at biopsy, disease duration at the time of biopsy, ILAR classification, antibody positivity, treatments undergone before synovial biopsy, and early relapse (data not shown).

Heterogeneity of histological features including synovial lining composition and immune cell infiltration in JIA synovitis. We performed two multiplex fluorescence IHC panels on a series of JIA synovia (n = 28, including n = 13 with RNASeq data) to assess for infiltration (percentage of marker-positive cells/total cells) and spatial distribution (in the synovial lining and sublining) of the following immune cell types: “M1-like” (CD45⁺CD68⁺CD206[−]) and “M2-like” macrophages (CD45⁺CD68⁺CD206⁺), pan-T lymphocytes (CD45⁺CD3⁺), CD8⁺ T lymphocytes (CD45⁺CD3⁺CD8⁺), CD4⁺ T lymphocytes (CD45⁺CD3⁺CD8[−]), and B lymphocytes (CD45⁺CD20⁺). These data corroborated interpatient heterogeneity in immune cell infiltration (suggested by transcriptomic profiling) of JIA synovia

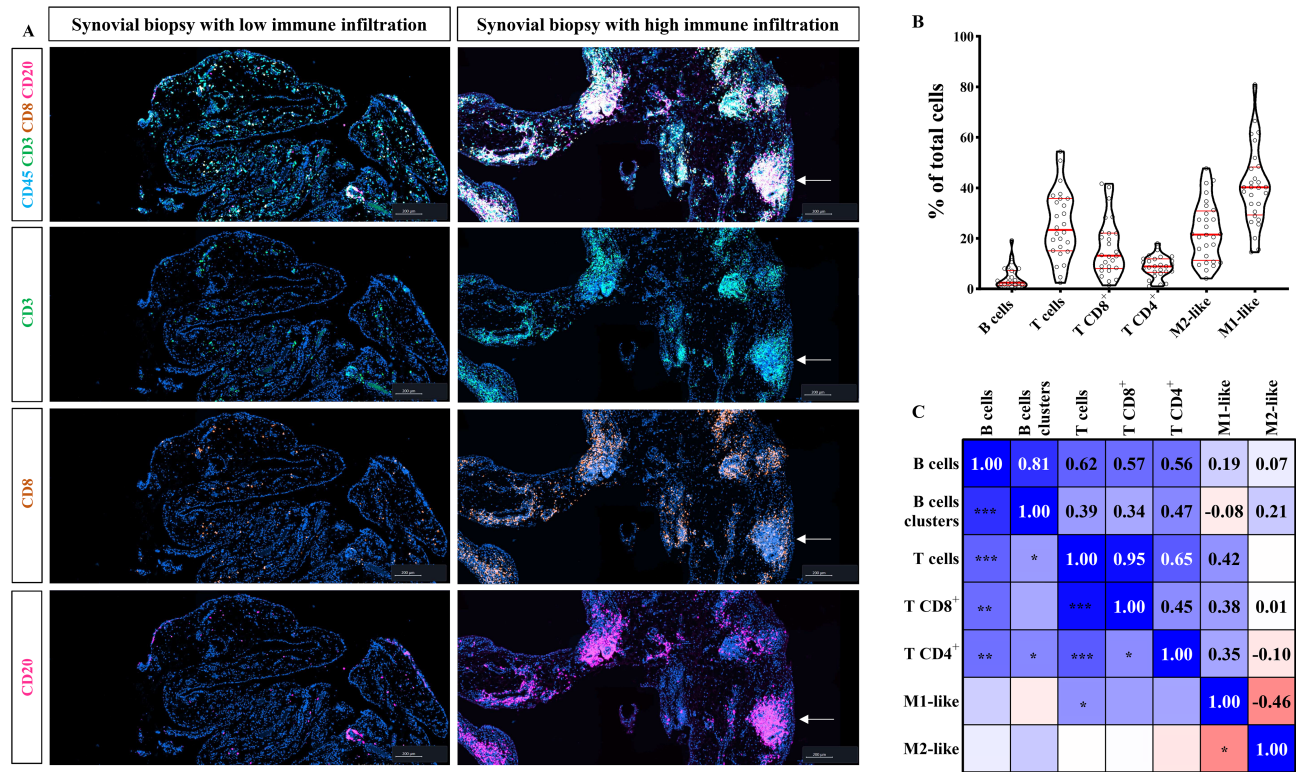


Figure 2. Immune cell infiltration of JIA synovial biopsies. (A) Representative images from two synovial biopsies stained with a four-plex fluorescence IHC panel (CD45, CD3, CD8, and CD20, in cyan, green, orange, and pink, respectively; nuclei in dark blue). Left panel, biopsy with low inflammation; right panel, biopsy with high inflammation. Arrow shows an example of the “B/T lymphoid aggregates” noted in this biopsy. (B) Truncated violin plot of immune cell subsets (marker-positive cells as a percentage of total cells) in JIA synovial biopsies (n = 28) stained for two multiplex fluorescence IHC panels (CD3, CD8, CD20, CD45 and CD45, CD68, CD206) on FFPE serial sections: pan-T lymphocytes (CD45⁺CD3⁺), CD8⁺ T lymphocytes (CD45⁺CD3⁺CD8⁺), CD4⁺ T lymphocytes (CD45⁺CD3⁺CD8[−]), B lymphocytes (CD45⁺CD20⁺), M1-like macrophages (CD45⁺CD68⁺CD206[−]), and M2-like macrophages (CD45⁺CD68⁺CD206⁺). Red lines, median and interquartile range. (C) Correlation matrix (Spearman’s coefficient and asterisks showing P value if significant) between fractions of different immune cell subsets in synovial tissue. *P < 0.05, **P < 0.01, ***P < 0.001. FFPE, formalin-fixed, paraffin-embedded; IHC, immunohistochemistry; JIA, juvenile idiopathic arthritis.

(Figure 2A–B). As suggested by RNASeq data-based inference (Supplementary Figure 3), abundance of pan-T lymphocytes (as well as T cell subtypes) and B lymphocytes were positively correlated. M1-like macrophage abundance correlated positively with T cell abundance and negatively with M2-like macrophage abundance (Figure 2C).

In addition to the abundance of different immune cell types, multiplex fluorescence IHC allowed us to assess for spatial distribution patterns of immune cells, which also varied across samples (Figure 2A). We observed “B/T lymphoid aggregates” (dense clusters of pan-T and B cells with a diameter $\geq 100 \mu\text{m}$) in 57.7% of samples; these were abundant (>4 clusters) in 34.6% of samples (Figure 2A). There was a significant positive correlation between the abundance of B cells and the number of B/T lymphoid aggregates (Figure 2C). T cells (CD8^+ and CD4^+

subpopulations) either co-localized with B lymphocytes in aggregates and/or were scattered in the synovial sublining (Figure 2A). Intriguingly, in a small subset of biopsies ($n = 10$), abundant CD8^+ T lymphocytes were distributed in a layer just below the synovial lining (Supplementary Figure 4).

As previously observed in early RA synovia,³ we found striking differences in macrophage composition of the synovial lining in JIA. Samples were therefore classified as M1-L ($n = 14$) or M2-L ($n = 10$) (Figure 3A); four samples showed mixed composition of the lining and could not be assigned to either category. Samples in the M1-L group displayed the following: (1) higher M1 cell infiltration ($\text{CD68}^+\text{CD206}^-$ cells as a percentage of total cells; data not shown) and, accordingly, higher M1:M2 ratios over the entire tissue; (2) higher immune cell infiltration (CD45^+ cells as a percentage of total cells) over the entire tissue; and

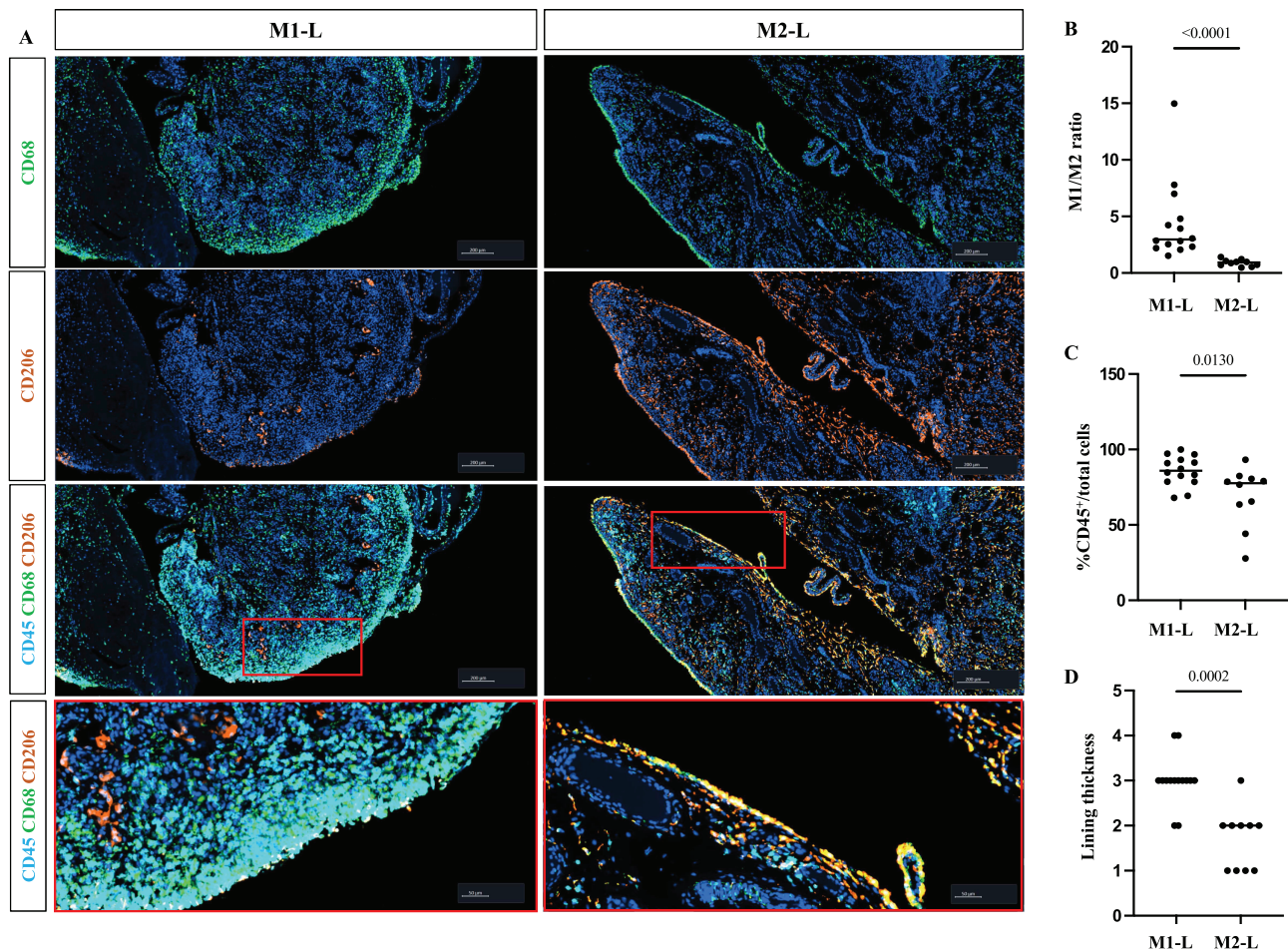


Figure 3. Macrophage composition and thickness of lining in JIA synovial biopsies. (A) Representative images from two synovial biopsies, stained with a three-plex fluorescence IHC panel (CD45, CD68, and CD206 in cyan, green, and orange, respectively; nuclei in dark blue). Left panel, biopsy with M1-like macrophage rich lining (M1-L); right panel, biopsy with M2-like macrophage rich lining (M2-L). Bottom panels, magnifications of red-boxed areas. (B) M1/M2 macrophage ratio (number of $\text{CD45}^+\text{CD68}^+\text{CD206}^-$ cells divided by number of $\text{CD45}^+\text{CD68}^+\text{CD206}^+$ cells) over the entire surface of M1-L versus M2-L synovial biopsies. (C) Immune cells (CD45^+ cells as a percentage of total nuclei) over the entire surface of M1-L versus M2-L synovial biopsies. (D) Lining thickness (visualized using CD68 staining, on a scale of 1 to 4) in M1-L versus M2-L synovial biopsies. Horizontal bars, medians. Significant P values (Mann-Whitney test) are indicated on graphs. IHC, immunohistochemistry; JIA, juvenile idiopathic arthritis; M1-L, M1-like macrophage predominant lining; M2-L, M2-like macrophage predominant lining.

(3) significantly greater thickness of the synovial lining (Figure 3B–D). They also showed higher CD8⁺ T cell (but not CD4⁺ T cell or B cell) infiltration (Supplementary Figure 5A–D); strikingly, 9 out of 10 samples with an abundant layer of CD8⁺ T cells adjacent to the lining (Supplementary Figure 4) belonged to the M1-L group. As in the series analyzed by transcriptomic profiling, the two main patterns observed among JIA synovia were as follows: (1) higher M1-like macrophage and CD8⁺ T cell infiltration with thicker, M1-like macrophage rich synovial lining versus (2) lower immune cell infiltration, higher M2:M1-like macrophage ratios, and thinner, M2-like macrophage rich synovial lining. These were not significantly associated with clinical parameters of disease severity or outcome (data not shown).

DISCUSSION

We performed transcriptomic (bulk RNASeq) and spatial (multiplex fluorescence IHC) analysis of two large, overlapping series of synovial biopsies from patients with JIA and found striking similarities with the alterations in synovia from patients with early RA. This was somewhat unexpected because most samples were from patients with oligoarticular JIA, phenotypically very different from RA. This may suggest that the clinical spectrum of JIA is not mirrored in transcriptomic and immunohistological synovial findings. As in early untreated RA, JIA synovia fall along a continuum (rather than into discrete groups) based on gene expression, with two broad sample clusters: (1) those showing a relative up-regulation of inflammatory and adaptive immune (primarily B and plasma cell) gene signatures (GC1) and (2) those showing a relative upregulation of lipid metabolism/PPAR-related gene signatures (GC2), associated with M2-like or synovial tissue resident macrophages.³ Samples in the latter group indeed have higher inferred M2-like:M1-like macrophage ratios (data not shown) and lower inferred adaptive immune cell proportions, based on the bulk RNASeq data. Although few samples ($n = 13$) yielded sufficient material for both transcriptomic and IHC analyses, several cell populations (CD8⁺ T cells and M1-like and M2-like macrophages; Supplementary Figure 6) assessed in these samples by the two techniques were significantly correlated; this despite the differences between the two approaches (direct quantification of cells in a single section vs inference of relative proportions of cells from bulk RNASeq on heterogeneous tissue). Similar to transcriptomic profiling, multiplex fluorescence IHC highlights two broad categories of JIA samples: (1) synovia with higher immune (in particular, M1-like macrophage and CD8⁺ T cell) infiltration and thicker, M1-like macrophage rich synovial lining, and (2) synovia with higher M2-like macrophages in both lining and sub-lining, which showed lower levels of global (CD45⁺) immune cell infiltration and lining hypertrophy. This may suggest that the presence of CD206⁺ macrophages in the synovial lining is an important indicator, perhaps even a mediator, of synovial tissue immuno-homeostasis. Indeed, in adult synovia,

abundance of CD206⁺ tissue-resident macrophages in the lining (as opposed to classical monocyte-derived inflammatory macrophages) is a feature of normal synovia and of those with less active RA.^{3,12,13} Although we could not highlight such an association in the current series (likely because of group size and clinical and demographic heterogeneity; Supplementary Table 1), a possible immuno-modulatory effect of the balance between classical inflammatory versus nonclassical CD206⁺ macrophages in JIA synovia would be of great interest to investigate.

RNASeq data suggest that B cells are major drivers of inter-patient variability in the JIA synovial transcriptome. As previously described,⁸ we find these cells organized in lymphoid aggregates (with CD4⁺ and CD8⁺ cells) in a significant fraction of JIA synovia, with the number of such aggregates correlating well with B cell infiltration. Several functions have been attributed to B cells in JIA pathophysiology, including autoantibody production, antigen presentation, inflammatory cytokine production, and T cell co-stimulation.¹⁴ Interestingly, some samples display limited B cell infiltration (as assessed by fluorescence IHC and/or bulk RNA-Seq), raising the question of alternative inflammatory mechanisms in these patients. In RA, paucity of synovial B cells has been suggested to represent a biomarker of rituximab nonresponse (a treatment never tested in a trial setting in JIA), although recent studies have yielded conflicting results.^{6,15} In early RA, we found clinical disease activity (in particular, swollen joint counts) to be the main clinical correlate of the synovial transcriptome and inflammatory burden.³ In the current JIA cohort, we could not show any such relationship between transcriptomic or cellular profile and clinical characteristics. This is likely because of several factors, including (1) limited group sizes; (2) a preponderance of oligoarthritis (one to four affected joints), limiting our power to assess for associations within or between other JIA subtypes; and most importantly, (3) a patient population heterogeneous for multiple features known to modify synovial tissue (such as ongoing therapy and disease duration at the time of biopsy). Nevertheless, the identification of two broad “types” of synovia, using two very different techniques in this heterogeneous series, bodes well for the delineation of clinically meaningful groups in larger studies performed in early, untreated JIA.

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 Limaye 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.

Study conception and design. Triaille, Tilman, Lauwerys, Limaye.

Acquisition of data. Triaille, Tilman, Baert, Sokolova, Lorient, Nzeusseu-Toukap, Meric de Bellefon, Galant, Boulanger, Fonseca, Bouzin, Lauwerys, Limaye.

Analysis and interpretation of data. Triaille, Tilman, Lorient, Bouzin, Durez, Lauwerys, Limaye.

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