

BRIEF REPORT

Synovial Tissue Heterogeneity in Japanese Patients With Rheumatoid Arthritis Elucidated Using a Cell-Type Deconvolution Approach

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Objective. Recent advances in single-cell RNA sequencing technology have improved our understanding of the immunological landscape of rheumatoid arthritis (RA). We aimed to stratify the synovium from East Asian patients with RA by immune cell compositions and gain insight into the inflammatory drivers of each synovial phenotype.

Methods. Synovial tissues were obtained from East Asian patients in Japan with RA (n = 41) undergoing articular surgery. The cellular composition was quantified by a deconvolution approach using a public single-cell–based reference. Inflammatory pathway activity was calculated by gene set variation analysis, and chromatin accessibility was evaluated using assay of transposase accessible chromatin–sequencing.

Results. We stratified RA synovium into three distinct subtypes based on the hierarchical clustering of cellular composition data. One subtype was characterized by abundant HLA-DRA^{high} synovial fibroblasts, autoimmune-associated B cells, GZMK⁺ GZMB⁺ CD8⁺ T cells, interleukin (IL)1-β⁺ monocytes, and plasmablasts. In addition, tumor necrosis factor (TNF)-α, interferons (IFNs), and IL-6 signaling were highly activated in this subtype, and the expression of various chemokines was significantly enhanced. Moreover, we found an open chromatin region overlapping with RA risk locus rs9405192 near the *IRF4* gene, suggesting the genetic background influences the development of this inflammatory synovial state. The other two subtypes were characterized by increased IFNs and IL-6 signaling, and expression of molecules associated with degeneration, respectively.

Conclusion. This study adds insights into the synovial heterogeneity in East Asian patients and shows a promising link with predominant inflammatory signals. Evaluating the site of inflammation has the potential to lead to appropriate drug selection that matches the individual pathology.

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INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by persisting synovitis, which causes articular destruction. In recent years, some pathological studies of inflammatory synovium in non-Asian patients with RA reported the presence of histological diversity and proposed a synovial classification based on the degree and type of infiltrating immune cells.^{1,2} Importantly, patient stratification using synovial information has the potential to be useful in predicting treatment responsiveness. For instance, randomized controlled studies that enrolled patients with RA who exhibited inadequate responses to tumor necrosis factor (TNF)- α inhibitors revealed that an interleukin (IL)-6 receptor inhibitor (tocilizumab) was significantly more effective than an anti-CD20 antibody (rituximab) in those with synovium with limited B cells, which was defined based on pathological findings and B cell-related gene expression.³

Recent developments in single-cell RNA sequencing (RNA-seq) technology have enabled unbiased analysis of synovial constituent cells, providing novel insights into the landscape of RA pathology. The Accelerating Medicines Partnership at the National Institutes of Health in the United States used an integrated approach of single-cell RNA-seq, mass cytometry, bulk RNA-seq, and flow cytometry of dissociated RA synovium, and has offered a synovial atlas consisting of 18 cell clusters (Supplementary Table 1, available on the *Arthritis & Rheumatology* website at <http://onlinelibrary.wiley.com/doi/10.1002/art.42642>).⁴ Stratifying the synovium based on these single-cell transcriptomic data may result in more elaborate synovial phenotyping.

Here, we performed bulk RNA-seq on synovium from East Asian patients with RA, and the cellular composition of these samples was quantified by a deconvolution approach with a public single-cell-based expression reference. RA synovium was stratified into three distinct subtypes based on cellular composition. One of these subtypes was characterized by abundant HLA-DRA^{high} synovial fibroblasts (SFs), autoimmune-associated B cells (ABCs), *GZMK*⁺ *GZMB*⁺ CD8⁺ T cells, IL1- β ⁺ monocytes, and plasmablasts. Gene set variation analysis revealed enhanced TNF- α , interferons (IFNs), and IL-6 signaling in this phenotype, and marked chemokine expression suggested a highly interactive inflammatory environment. Furthermore, analysis of open chromatin structure by bulk assay of transposase accessible chromatin-sequencing (ATAC-seq) suggested the involvement of genetic predisposition in the formation of this synovial environment.

PATIENTS AND METHODS

See online supplementary materials and methods, available on the *Arthritis & Rheumatology* website at <http://onlinelibrary.wiley.com/doi/10.1002/art.42642>. In short, synovial tissues were obtained from East Asian patients with RA ($n = 41$) undergoing articular surgery. We performed bulk-RNA sequencing and the cellular composition was quantified by a deconvolution approach using a public single-cell-based reference. Inflammatory pathway activity was calculated by gene set variation analysis, and chromatin accessibility was evaluated using ATAC-seq.

RESULTS

Three synovial phenotypes based on cell-type abundance. We collected synovial tissue from 41 East Asian patients with RA who had undergone joint surgery and obtained comprehensive gene expression information by bulk RNA-seq. These data were integrated with a public single-cell RNA-seq reference⁴ to estimate the proportion of subpopulations in each sample using a deconvolution approach.⁵ Furthermore, we estimated the activation state of disease-relevant inflammatory signaling,^{6,7} and performed bulk ATAC-seq to evaluate the chromatin accessibility of inflammatory drivers.

RA synovium was stratified into three distinct subtypes based on the hierarchical clustering of cellular composition data (Figure 1A). Synovial type 1 (ST1) was characterized by abundant HLA-DRA^{high} SFs, ABCs, *GZMK*⁺ *GZMB*⁺ CD8⁺ T cells, IL-1 β ⁺ monocytes, and plasmablasts. In ST2, IFN- γ activated monocytes, nuclear protein 1 (*NUPR1*)⁺ monocytes, and cytotoxic T cells were dominant, whereas there were higher proportions of naïve and memory B cells, and follicular helper T cells or peripheral helper T cells (Tfh/Tph) in ST3. The proportions of each cell population by synovial type are summarized in Figure 1B.

The association between synovial phenotypes and clinical characteristics. We next performed a comparative analysis of clinical characteristics among the synovial phenotypes (Figure 1C, Supplementary Table 2, <http://onlinelibrary.wiley.com/doi/10.1002/art.42642>). Intriguingly, none of these clinical characteristics (disease activity, duration, sex, age, affected joints, serological findings, and treatment history) were different between the synovial phenotypes, suggesting it is challenging to estimate local immunological kinetics of the synovium from clinical parameters.

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Data are available in a public, open access repository. The data sets generated during this study are available at the National Bioscience Database Center (NBDC) with the study accession code hum0373.

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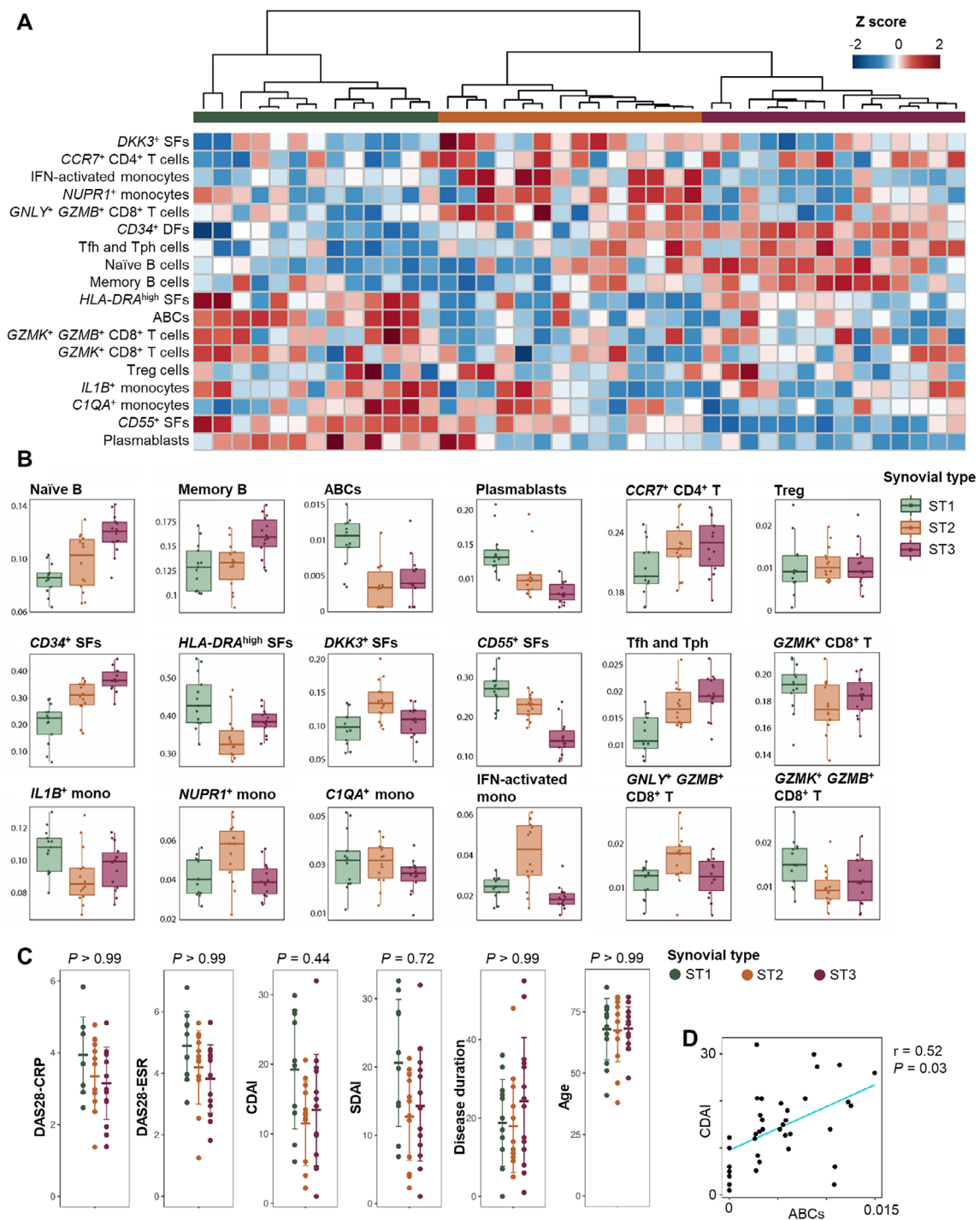


Figure 1. Synovial stratification based on estimated cell fractions and comparison of clinical metrics among synovial phenotypes. **A**, Hierarchical clustering of synovial samples according to normalized cellular proportions. Columns and rows denote each subject and cell population, respectively. **B**, The cellular proportion of subpopulations in each synovial subtype. Vertical axes represent percentages. **C**, DAS28-CRP, DAS28-ESR, CDAI, SDAI, disease duration, and age at surgery in each synovial phenotype. Each dot denotes individual samples, and bars represent mean $\pm$ SD. P values were assessed by Kruskal-Wallis tests followed by pairwise Wilcoxon tests with Bonferroni correction. **D**, Scatter plot depicting the proportion of ABCs and CDAI. Each dot and blue line denote individual samples and regression line, respectively. P values were determined by Spearman's rank correlation coefficient with Bonferroni correction considering other metrics in online Supplementary Figure 1, available on the *Arthritis & Rheumatology* website at <http://onlinelibrary.wiley.com/doi/10.1002/art.42642>. ABCs = autoimmunity-associated B cells; CDAI = clinical disease activity index; CRP = C-reactive protein; DAS28 = Disease Activity Score 28; ESR = erythrocyte sedimentation rate; mono = monocytes; SD = standard deviation; SDAI = simplified disease activity index; SFs = synovial fibroblasts; ST = synovial type; Tfh = follicular helper T cells; Tph = peripheral helper T cells; Treg = regulatory T cells; VAS = visual analog scale.

The correlation analysis between each cell subset proportion and clinical parameters revealed a significant positive correlation between ABCs, one of the ST1 hallmarks, and the disease activity index represented by the Clinical Disease Activity Index (CDAI; $P = 0.03$, Figure 1D, Supplementary Figure 1, <http://onlinelibrary.wiley.com/doi/10.1002/art.42642>). Correlations between ABCs in peripheral blood and RA disease activity have been previously reported,⁸ but our data suggest that synovial ABCs might be one of the hubs of inflammation, especially in the ST1 phenotype.

Diversity of inflammatory signaling by synovial phenotypes. Differences in the cellular composition in the synovium may link to predominant inflammatory signaling and mediator expression for each synovial phenotype. The approach to elucidate these core immune processes may provide further insight into therapeutic targets that match the individual's pathological condition, and the factors that lead to the destructive clinical course in patients.

Firstly, to reveal disease-relevant inflammatory signaling activity in each synovial phenotype, we performed gene set enrichment analysis using gene set variation analysis (GSVA) software.⁷ We defined each cytokine activity score based on gene expression data of SFs stimulated with one of eight cytokines (TNF- α , IL-1 β , IL-6, IFN- γ , IFN- α , IL-17, IL-18, or transforming growth factor (TGF)- β) in a previous report from our laboratory.⁶ We found that TNF- α , IFN, and IL-6 scores were significantly higher in ST1 than in ST3 (Figure 2A). These results indicated that ST1 is an inflammatory synovial state. Previous single-cell analysis of the synovium has elucidated the major sources of cytokines in the joints, and it has been reported that IFN- γ is secreted by $GZMK^+ GZMB^+$ T cells, IL-1 β by IL-1 β^+ monocytes, and IL-6 by HLA-DRA^{high} SFs, whereas TNF- α is secreted by several populations, mainly T cells but also ABCs.⁴ We have previously reported that activated SFs in a synergistic proinflammatory environment highly express pathogenic genes, including *IL6* and *MMP1*, as a consequence of dynamic chromatin structural remodeling.⁶ In fact, in vitro analysis showed that IL-6 and MMP-1 expression in the human rheumatoid SF cell line is markedly enhanced when cocultured with a combination of TNF- α , IL-1 β , and IFN- γ , compared with single cytokine stimulation at the transcript and protein level (Supplementary Figure 2, <http://onlinelibrary.wiley.com/doi/10.1002/art.42642>). In the pathophysiology of ST1, the complex microenvironment composed of ABCs, IL-1 β^+ monocytes, and $GZMK^+ GZMB^+$ CD8⁺ T cells may induce SFs activation and IL-6 and MMP-1 expression. In contrast, ST3 was presumed to be less affected by these inflammatory signaling pathways, suggesting the existence of other drivers leading to arthroplasty.

Core immunoinflammatory mediators by synovial phenotypes. Next, we compared the gene expression of immunoinflammatory mediators to elucidate key molecules that form the basis of the pathology of each synovial phenotype

(Figure 2B). ST1 was characterized by marked expression of chemokines (eg, *CXCL13*, *CCL19*) and IFN pathway-related molecules (eg, *IRF4*, *IFI27*). These results suggest that ST1 represents a synovium lined with a highly cell-to-cell interactive inflammatory environment. In contrast, certain genes that are reported to be closely related to osteogenesis (*BMP4*, *BMP7*) and the pathology of osteoarthritis (OA) (eg, *FGF2*, *ANG*)⁹ were highly expressed in ST3. Especially, *BMP4* is reported to stimulate the expression of *TNFSF11* in chondrocytes and osteoblasts.¹⁰ Therefore, ST3 was considered to involve alterations in bone metabolism.

Focusing on these characteristic mediators of each synovial phenotype and chromatin structure in disease-susceptible regions revealed by genome-wide association studies (GWAS), accessibility of some single nucleotide polymorphisms (SNPs) was found to differ between synovial phenotypes. The *IRF4* region is a representative example of differential open chromatin structures between synovial phenotypes (Figure 2C). The RA risk locus rs9405192,¹¹ which is reported in East Asian patients, overlapped with the open chromatin peak detected in ST1 and ST2, whereas the chromatin structure in this region is closed in ST3 (false discovery rate <0.05). To the best of our knowledge, it has not been previously reported that rs9405192 has a regulatory effect on any gene in immune cells or tissues, but the possibility that the nuclear factor (NF)- κ B complex binds in the vicinity of this SNP has been suggested.¹² In ST1 and ST2, where the peaks exist in this region, inflammatory joint destruction may have progressed through *IRF4* expression, underlining the genetic risk of RA. In contrast, in ST2 and ST3, the chromatin structure in the region of the bone mineral density GWAS SNP rs10130587,¹³ which is present in the intergenic region of *BMP4*, was open (false discovery rate <0.05, Supplementary Figure 3, <http://onlinelibrary.wiley.com/doi/10.1002/art.42642>).

Figure 3 summarizes the assumed pathogenesis of each synovial phenotype. ST1, in which IL-1 β -producing monocytes, TNF- α -producing ABCs, and IFN- γ -producing $GZMK^+ GZMB^+$ CD8⁺ T cells are dominant, is the synovial phenotype with the strongest inflammatory aspect. IL-6-producing HLA-DRA^{high} SFs can be expanded in a local environment composed of these synergistic inflammatory cytokines. ST2 is characterized by activated IFNs and IL-6 signaling against the background of IFN- γ activated monocytes, *NUPR1*⁺ monocytes, and *GNLY*⁺ $GZMB^+$ CD8⁺ T cells infiltration. In ST3, the presence of B cells and Tfh/Tph cells suggests the persistence of strong T-B cell interaction, but inflammatory signaling is relatively poor and degeneration-related molecules are highly expressed.

DISCUSSION

In this study, we performed a cell-type deconvolution approach to gain insight into the synovial tissue heterogeneity in East Asian patients with RA. Our analysis sheds light on the

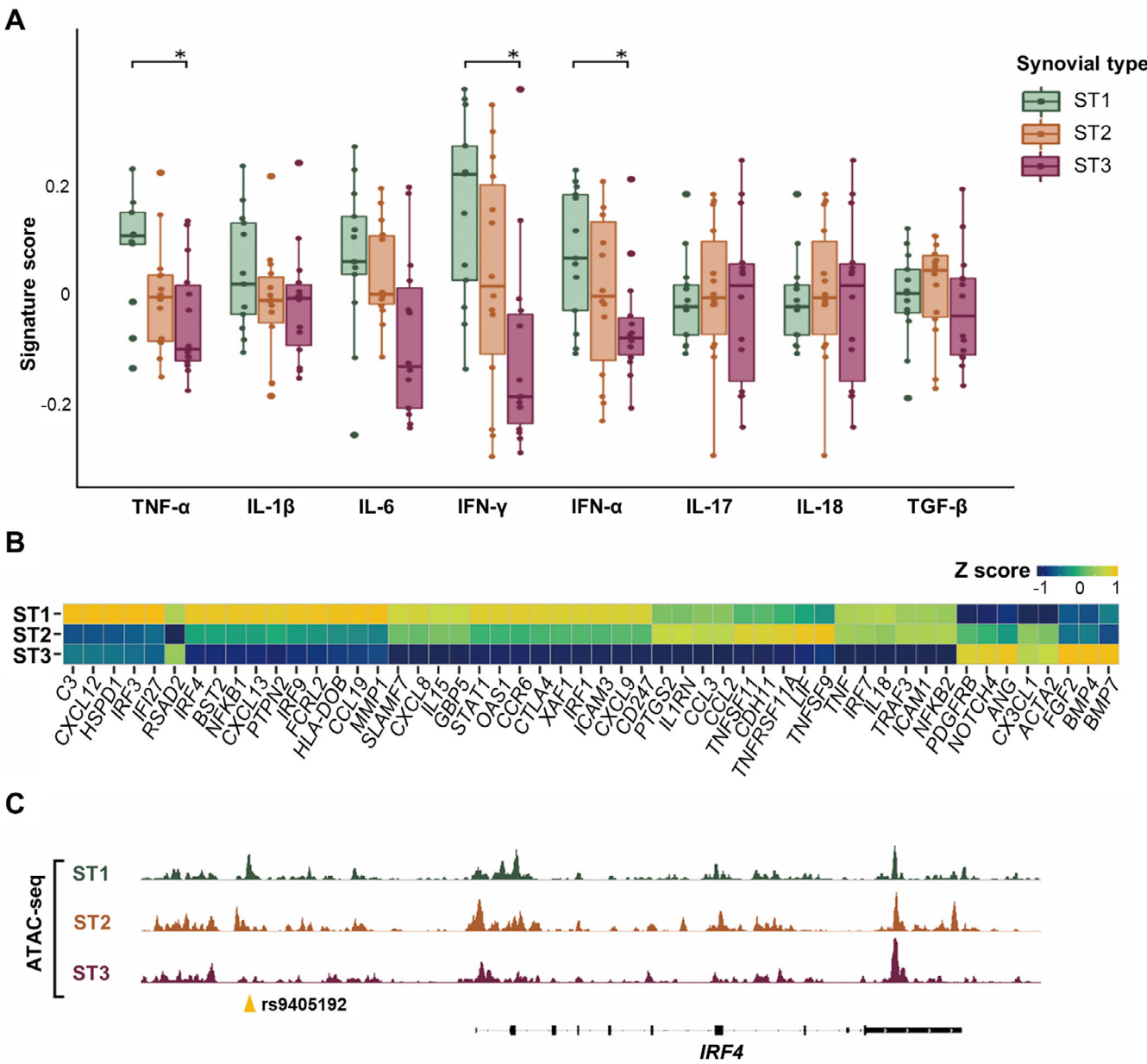


Figure 2. The activity of proinflammatory cytokine signaling and the epigenomic status of immunoinflammatory mediators that constitute the synovial local environment. **A**, Our laboratory previously reported the transcriptomic perturbation of SFs to various inflammatory stimuli,⁶ from which we obtained differentially expressed genes (DEGs) between stimulation and control. To elucidate the activity of eight cytokines (TNF- α , IL-1 β , IL-6, IFN- γ , IFN- α , IL-17, IL-18, and TGF- β), we defined cytokine activity scores normalized by the enrichment score by applying the “top 1000 DEGs” to GSEA software.⁷ Boxes represent the interquartile range; horizontal lines in boxes represent the median; whiskers represent the distribution, and dots represent individual samples. *P* values were determined by Kruskal-Wallis tests followed by pairwise Wilcoxon tests with Bonferroni correction (**P* < 0.05). **B**, Transcript abundance of immunoinflammatory mediators in each synovial phenotype. **C**, Organization of transcriptional regulatory regions around *IRF4* and the positional relationship of RA risk loci (yellow triangle, rs9405192). Data were visualized using the Integrative Genomics Viewer (IGV). ATAC-seq = assay of transposase accessible chromatin–sequencing; IFN = interferon; IL = interleukin; RA = rheumatoid arthritis; SFs = synovial fibroblasts; ST = synovial type; TGF = transforming growth factor; TNF = tumor necrosis factor.

diversity of the immunological basis of synovitis with advanced destruction and the genetic background involved.

Synovial heterogeneity and its prognostic ability to predict treatment outcomes have been assessed mainly in White patients with RA at a relatively early stage of the disease. Humby et al

stratified 144 samples of treatment-naïve early RA synovium into three pathology groups: lymph-myeloid, diffuse-myeloid, and pauci-immune patterns.¹ In this report, the expression of lymphoid-associated genes correlated with osteoclast-targeting genes predicting radiographic joint damage progression at

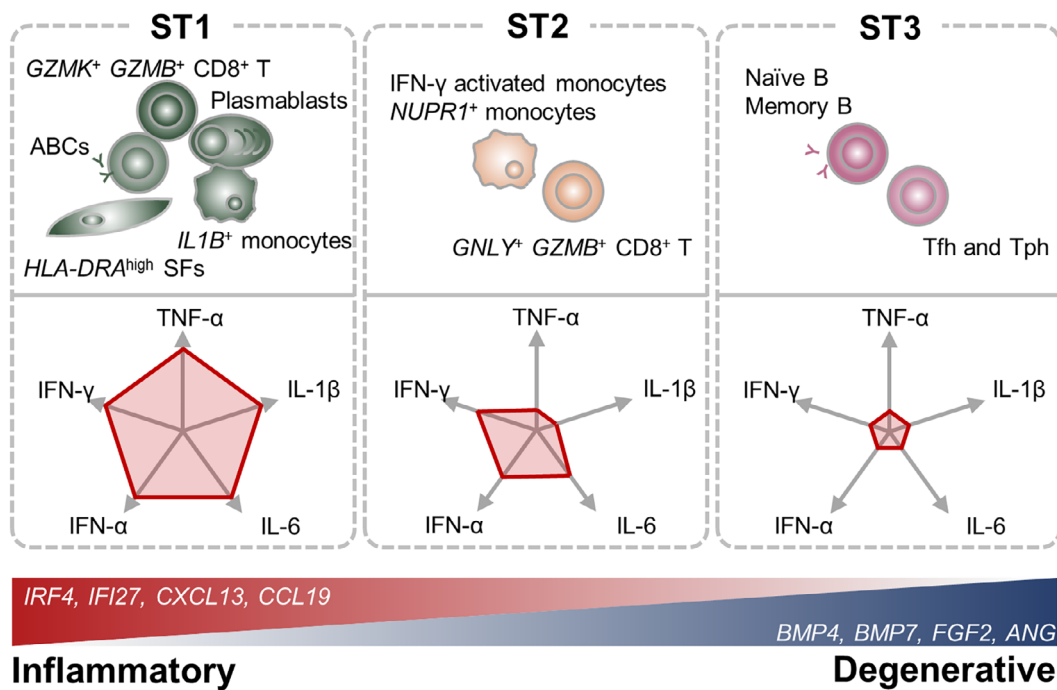


Figure 3. A graphical summary of the present study. We conducted a multilayer analysis on synovium from East Asian patients with rheumatoid arthritis (RA) with advanced destruction. A cell-type deconvolution approach with a public single-cell-based expression reference was used to stratify the RA synovium into three distinct subtypes. The ST1 subtype was considered to have the most inflammatory and interactive synovial environment, with the involvement of genetic predisposition, including *IRF4*, in the formation of this phenotype. ABCs = autoimmune-associated B cells; IFN = interferon; IL = interleukin; SFs = synovial fibroblasts; ST = synovial type; Tfh = follicular helper T cells; TGF = transforming growth factor; TNF = tumor necrosis factor; Tph = peripheral helper T cells.

12 months. Using microarray-based gene expression profiling, Dennis et al divided 69 RA synovium samples into myeloid, lymphoid, low inflammatory, and fibroid clusters.² They observed that a baseline synovial myeloid gene signature, but not a lymphoid gene signature, had higher expression levels in patients with good clinical responses to anti-TNF- α therapy at 16 weeks compared with those having poor responses. Although our study differs from these previous reports in the target ethnicity and clinical stages of RA, the stratified synovial types may reflect some commonalities. That is, abundant lymphocyte and myeloid cell infiltration is predicted in ST1, and ST3 is a low inflammatory type. These commonalities between the data sets could be core features of RA that are preserved across patient backgrounds, including ethnic differences. Most of the previous studies on gene expression profiling in synovium were focused on the White population, which may have been influenced by a genetic predisposition specific to the White population. The present study shows that the results of the combined analysis of gene expression of synovium in East Asian patients and scRNA-seq reference data from non-Asian ethnicities resulted in data that were consistent with previous reports. Moreover, by adopting the deconvolution approach that refers to synovial single-cell RNA-seq data, we added deep insights into the proportion of unbiasedly defined cell populations.

Furthermore, there were apparent differences in core inflammatory signaling molecules between synovial types, demonstrating the need to optimize therapeutic strategies according to the immunological properties of the synovium. For instance, ST1 is characterized by activation of TNF- α , interferons, and IL-6 signaling and infiltration of the cells producing these cytokines (ie, ABCs, $GZMK^+$ $GZMB^+$ $CD8^+$ T cells, $IL-1\beta^+$ monocytes, and $HLA-DRA^{high}$ SFs). In addition to humoral factors, ST1 is also characterized by enhanced chemokine expression levels (eg, *CXCL13*, *CCL19*), previously reported to be upregulated in synovium with pathological ectopic lymphoid tissue (ELT) formation.¹⁴ In such an orchestrated inflammatory environment, it might be challenging for existing therapeutic agents targeting single immune modulators (eg, TNF- α inhibitors, IL-6 receptor inhibitors, or CTLA-4 antagonists) to completely inhibit multiple inflammatory aspects. In ST2, IFNs and IL-6 signaling constitute the core of the synovial environment, and therapeutic approaches that inhibit downstream Janus kinase–signal transducers and activators of transcription signaling might be effective. ST3 possesses strong degenerative properties and would require a completely different therapeutic approach than ST1 and ST2, such as suppressing osteochondral destruction through targeting noninflammatory pathways or regenerating joint structures.

To date, GWAS studies have reported more than 100 RA-susceptible polymorphisms, some of which have been elucidated to regulate the expression of disease-relevant genes. However, information on the relationship between these risk SNPs and synovial phenotypes is scarce. Our analysis revealed that the open chromatin structure at rs9405192 in the *IRF4* region was significantly different between synovial phenotypes, and this locus was accessible in ST1 and ST2, and the expression of *IRF4* was highest in ST1. *IRF4* belongs to the IRF family of transcription factors and plays a crucial role in the development and function of various immune cells. For instance, it has been demonstrated that *IRF4* is a pivotal factor in plasmablast and ABC expansion through interaction with the switch associated protein-70 and defensin alpha 6 proteins.¹⁵ Although the precise mechanisms of how rs9405192 is associated with RA susceptibility have not been fully elucidated, dysregulation of *IRF4*-mediated signaling might influence the construction of the pathological synovial environment of ST1 through its genetic background.

This study includes some limitations. First, cell-type deconvolution provides relative proportions. For example, Tfh/Tph cells were dominant in ST3, but this does not necessarily mean that the absolute amount of Tfh/Tph was high in ST3. Tfh/Tph and ABCs have been reported to be mutually active in the ELT of the synovium, and it is possible that even in ST1 there is a certain level of Tfh/Tph cells, even though this population is not dominant. In the single-cell RNA-seq analysis of RA synovium,⁴ we detected Tfh/Tph cells as the major CXCL13-expressing cells. High expression of CXCL13 in ST1, as shown in Figure 2B, suggests the presence of activated Tfh/Tph in ST1, but not ST3. Second, this study did not integrate analyses using other modalities, such as pathological evaluation or flow cytometry. Lastly, because the present study included long-standing RA patients with joint destruction, the effect of secondary OA on gene expression cannot be fully isolated. Nevertheless, there are commonalities between the results of this study and previous reports on synovial tissue heterogeneity of early RA patients,^{1,2} suggesting the possibility that synovial features present early in the onset of RA may influence the late phenotype.

Overall, our findings shed light on the utility of the cell-type deconvolution approach in synovial phenotyping, and reveal the diversity of synovium with advanced destruction in East Asian patients with RA. Furthermore, we found promising links between this diversity and predominant inflammatory signals. Evaluating the site of inflammation has the potential to lead to personalized medicine that allows appropriate drug selection matching the individual's pathological condition.

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. Haruka Tsuchiya and Keishi Fujio 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. Nakajima, Tsuchiya, Ota, Okamura, Fujio.

Acquisition of data. Ogawa, Yamada, Yoshida, Maeda, Shirai, Kasai, Hirose, Ninagawa, Fujieda, Iwasaki, Aizaki, Kajiyama, Matsushita, Tamura, Mimura, Ohmura, Morinobu, Atsumi, S. Tanaka, Okamura.

Analysis and interpretation of data. Nakajima, Tsuchiya, Ota, Kawakami, Y. Tanaka, Takeuchi, Fujio.

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