Supplementary files, data, and references

Supplementary file 1

Supplementary methods

Sample collection

PB samples and SF samples were collected from patients at Keio University Hospital. All patients fulfilled the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for RA[49]. This study was approved by the Institutional Review Board of Keio University School of Medicine and conducted in compliance with the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participating individuals. There was a total of 8 cohorts in this study. The analysis contents for each cohort, corresponding to figure number, and time axis are shown in online supplementary Table S1.

Patient data and assessments

The following parameters were collected from medical charts: tender joint count, swollen joint count, Health Assessment Questionnaire Disability Index (HAQ-DI), patient global assessment using a visual analogue scale (PtGA), and physician global assessment using a visual analogue scale (PhGA). Antibody levels against cyclic citrullinated peptide (ACPA) were measured by ELISA (Mesacup-2 test, Medical and Biological Laboratories, Nagoya, Japan). Rheumatoid factor (RF) and matrix-metalloproteinase-3 (MMP-3) were measured by means of the latex agglutination test. C-reactive protein (CRP) was measured by a latex turbidimetric immunoassay. The erythrocyte sedimentation rate (ESR) was measured by Westergren methods. Disease activity was assessed by standard composite indices, such as DAS-28ESR (disease activity score-28 ESR), DAS-28CRP, SDAI (simplified disease activity index), CDAI (clinical disease activity index)[50].

Immunophenotyping

We performed immunophenotyping using only fresh samples to avoid unwanted changes by freezing cells. Peripheral blood was collected using heparin blood collection tubes (TERUMO, Tokyo, Japan) and 100 µl for each panel was mixed with fluorochrome-conjugated monoclonal antibodies against human cell surface antigens. After the mixture was incubated for 15 minutes, the cells were fixed and red blood cells were lysed using 1.5 ml of FACS Lysing Solution (BD Biosciences, Mountain View, CA, USA) for 10 minutes, washed with staining buffer (PBS with 0.5% BSA and 2 mM EDTA), and analysed using a FACSAria III flow cytometer (BD Biosciences). SF was collected at the time of arthrocentesis and incubated with 10 U/ml hyaluronidase for 15 minutes at 37 °C. Cells were washed with staining buffer, stained with antibodies for 15 minutes, and analysed using a FACSAria III flow cytometer. Because subpopulations of Tscm (e.g., Th1, Th17, and Tfh) are present in very small numbers in PB, $CD4^+$ T cells were enriched before staining to simultaneously analyse the developmental stages and polarities of $CD4^+$ T cells. Therefore, PBMCs were isolated using density gradient centrifugation (Lymphoprep Axis-Shield, Oslo, Norway) with

heparinized blood, and $CD4^+$ T cells were then isolated from these by negative selection ($CD4^+$ T Cell Isolation Kit, Miltenyi Biotec, Bergisch-Gladbach, Germany). $CD4^+$ T cells were stained and analysed using a FACSVerse flow cytometer (BD Biosciences). For the gating strategies (according to previously published methods[9,51] with minor modifications) and antibodies used in each panel, see online supplementary Figure S1-S2. We defined developmental stages as follows: Tn; $CCR7^+CD95^-CD45RA^+$ or $CD45RO^-$, Tscm; $CCR7^+CD95^+CD45RA^+$ or $CD45RO^-$, Tcm; $CCR7^+CD45RA^-$ or $CD45RO^+$, Tem; $CCR7^-CD45RA^-$ or $CD45RO^+$, Temra; $CCR7^-CD45RA^+$ or $CD45RO^-$, and we defined polarity as follows: Th1; $CXCR3^+CCR6^-CXCR5^-$, Th17; $CXCR3^-CCR6^+CXCR5^-$, Th1/17; $CXCR3^+CCR6^-CXCR5^-$, Tfh; $CXCR5^+$, Tfh1; $CXCR3^+CCR6^-CXCR5^+$, Tfh2; $CXCR3^-CCR6^-CXCR5^+$, Tfh17; $CXCR3^-CCR6^+CXCR5^+$, Tfh1/17; $CXCR3^+CCR6^+CXCR5^+$.

Cell sorting

To compare samples obtained from the same patient before and after treatment, PBMCs were frozen once at -80 °C using CELLBANKER 1 (TAKARA BIO, Shiga, Japan) and Mr. Frosty (Thermo Fisher Scientific, MA, USA). Frozen PBMCs were rapidly thawed at 37 °C using Anti-Aggregate Wash (Cellular Technology Limited, OH, USA), incubated for 30 minutes with complete RPMI medium (RPMI1640 supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 55 μM 2-mercaptoethanol (Thermo Fisher Scientific), 1 mM sodium pyruvate, 100 U/ml penicillin, 100 U/ml streptomycin, and 10% FBS (Thermo Fisher Scientific)), and then stained and sorted using a FACSAria III flow cytometer. To compare samples obtained from the HC, treatment-naïve RA, and treated RA groups, we isolated SF or, in the proliferation assay, PBMCs or SF mononuclear cells by density gradient centrifugation. In these experiments, $CD4^+$ or $CD8^+$ T cells were isolated by positive selection (MojoSort human CD4 or CD8 Nanobeads, Biolegend, CA, USA). The cells were stained and sorted using a FACSAria III flow cytometer. The gating strategies and antibodies used for sorting are shown in supplementary Figure S3.

RNA isolation

Total RNA was isolated from sorted cells using an RNeasy Micro Kit (Qiagen, CA, USA) according to the manufacturer's instructions. The quality of the RNA was assessed using an RNA 6000 Pico LabChip kit (Agilent Technologies, CA, USA). The RNA integrity number values were typically > 7.5 (median, 8.6).

Amplicon sequencing

AmpliSeq human-transcriptome libraries were constructed and sequenced using an Ion Proton platform (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, for library preparation, a barcoded cDNA library was first generated with a SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific) from 0.2 ng or 1 ng of total RNA obtained from each T cell subset in RA patients and HCs. The cDNA was then amplified using an Ion AmpliSeq Transcriptome Human Gene Expression Kit. Because we started with small amounts of input RNA, the purified cDNA libraries had to be amplified to obtain a sufficient quantity to prepare the sequencing libraries. The amplified libraries were then re-purified, diluted

to 75 pM and pooled equally into eighteen individual samples per pool. Pooled libraries were multiplexed and clonally amplified using an Ion OneTouch 2 System. They were then sequenced on Ion PI chips using an Ion Proton sequencing system. The data were first analysed using a Torrent Suite and ampliSeqRNA analysis plugin to generate count data.

Differential expression and gene enrichment analysis

Read count normalization was performed using the voom method. For quality control, we removed approximately 10% of samples that showed obviously low library quality. Some bias by library batch and library concentration were corrected using calcResiduals function of R pcbcStats package. The extraction of differentially expressed genes was conducted based on the empirical Bayes method in the R/Bioconductor limma package. The significance of overlap between the two gene sets was assessed with Fisher's exact test. DEGs were extracted by setting the criterion for statistical significance as a *P* value < 0.05 and an absolute fold change > 1.2. The significance of the overlap between the DEGs and gene sets was assessed with Fisher's exact test using the 'fisher test' function in the R/CRAN stats package. GO and enrichment analysis was conducted using the MSigDB GO, hallmark gene sets, and canonical pathways.

Principal component analysis, hierarchical clustering, and pathway mapping

Principal component analysis was performed using TMM normalized data with the "prcomp" function in the R/CRAN stats package. Visualization of scatter plots constructed using PC1 and PC2 from the PCA results was performed using the R/CRAN ggplot2 package. Clustering was performed using cosine distance and the Ward linkage method with the "Heatmap" function of the R/Bioconductor ComplexHeatmap package. Pathway mapping was performed using the KEGG Mapper analysis tool[52] (http://www.genome.jp/kegg/tool/map_pathway2.html).

T cell proliferation assay

$CD4^+$ Tn, Tcm, and Tem cells and $CD8^+$ Tn, Tcm, Tem, and Temra cells ($1x10^4$ cells each) were sorted as previously described (supplementary Figure S3). Cells were labelled with a 2 μM CellTrace Violet reagent (Thermo Fisher Scientific) for 7 minutes at 37 °C. The cells were then washed and activated using the same number of Dynabeads with Human T-Activator CD3/CD28 (VERITAS, Tokyo, Japan) in complete RPMI. After 5 days, the dead cells were stained with 7-aminoactinomycin D and analysed using a FACSVerse flow cytometer. The proliferation index was calculated using FlowJo software version 7.6.5 (FlowJo, OR, USA).

Antibodies

The following antibodies and reagents were used: anti-CD3 (APC/Cy7, UCHT1 or PE/Cy7, Hit3a), anti-CD4 (PE/Cy7, SK3, or HV500, OKT4), anti-CD8 (FITC, RPA-T8 or HV500, RPA-T8 or BV510, RPA-T8), anti-CD25 (PE, BC96), anti-CD45RA (BV421, HI100), anti-CD45RO (APCCy7, UCHL1), anti-CD95

(BV421, DX2), anti-IL-2Rβ (PE, TU27), anti-CD127 (FITC, A019D5), anti-CXCR5 (PerCP/Cy5.5, J252D4), anti-CCR7 (Alexa647, TG8/CCR7 or BV510, G043H7), and anti-HLA-DR (PerCP/Cy5.5, L243) from BioLegend; anti-CD4 (Horizon V500, RPA-T4), anti-CD8 (Horizon V500, RPA-T8), anti-CXCR3 (APC, 1C6/CXCR3), and anti-CCR6 (PE, 11A9) from BD Biosciences; and 7-aminoactinomycin D from Bay Bioscience (Kobe, Japan).

Statistics

Continuous data are presented as the median and interquartile range (IQR) or as a number with a percentage value, as appropriate. The Wilcoxon rank sum test was used to examine the differences between continuous variables. Correlations between two continuous variables were analysed using the Spearman's rank correlation coefficient. Fisher's exact test was used to compare proportions in categorical data between groups. Repeated measures comparisons were conducted using the Wilcoxon signed-rank test (two-tailed). Univariate regression were performed using Wilcoxon test for disease status and Spearman's rank correlation coefficient for age. Multiple regression were performed using disease status and age. We considered a *P*-value < 0.05 to be significant. All statistical analyses without transcriptome analyses were performed with JMP 13 (SAS Institute, NC, USA). In transcriptome analysis, the significance of overlap between the two gene sets was assessed with Fisher's exact test using the 'fisher test' function in the R/CRAN stats package.

Supplementary file 2

Supplementary discussion

In this study, we performed the gene expression analyses using two experimental procedures for reducing individual variations. The first analysis was to compare before and after treatment of the same patient. In this method, inter-individual differences could be excluded by first comparing intra-individual change between before and after treatment. Actually, the results of clustering and PCA showed a difference for each individual rather than the influence of treatment (Figure 4A-B), indicating the importance of reducing the inter-individual variation.

The second procedure was that we sorted cells into each developmental stage before gene expression analysis. Since gene expression differs by developmental stage[16,51], the results might be affected by the proportion of cells in each developmental stage when whole $CD4^+$ and $CD8^+$ T cells were directly analysed for gene expression. Although this procedure could not exclude inter-individual variations other than the cell population of each developmental stage, it enabled us to compare clinical samples in various states (HC, untreated RA, stable RA after treatment, and SF) side by side. Because the comprehensive analysis was performed on a sufficiently large scale, we were able to convincingly identify the characteristics of T cells in RA. As a result of our two stratified analyses, we arrived at three interesting findings apart from the pathway analysis findings.

First, among the various axes used to evaluate T cells, the most influential elements were developmental stage and anatomical location, whereas disease and treatment status had less impact, as is clearly shown in Figure 5A-B. This indicates that it may be better to stratify T cells by developmental stage and anatomical location to reduce inter-individual variation; this will be helpful for emphasizing the effects of the disease, which may otherwise be undetectable due to inter-individual variation. Second, the pathways that were differentially regulated in SF from RA patients (in comparison to PB from RA patients) displayed higher statistical significance than the pathways that were differentially regulated in PB from RA patients (compared to PB from HC), as shown in the *P* values displayed in Figure 4C-D and Figure 5I-J, indicating that analysing samples from lesion sites is more likely to emphasize the features of the disease. Third, some of the pathways that were enriched in SF from RA patients (compared to PB from RA patients) were also found to be enriched in PB from RA patients (compared to HC) as well as in PB samples from TCZ-treated RA patients (compared to untreated RA patients), indicating that disease-specific changes in lesions can be evaluated in periphery to some extent. Therefore, in cases where it is difficult to obtain local samples, it may be possible to infer disease-specific changes from PB. However, this may only be applicable for RA because it is a systemic autoimmune disease. Whether this finding would also hold true in an organ-specific autoimmune disease, such as thyroid disease, will require further study.

Supplementary data

Supplementary data 1-8 were deposited to figshare.
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Supplementary data 1. DEGs between pre- and post-TCZ treatment.
Supplementary data 2. Enrichment pathways in DEGs between pre- and post-TCZ treatment
Supplementary data 3. The results of GO/Enrichment analysis of clustered genes of supplementary Figure S8.
Supplementary data 4. DEGs between synovial fluid of RA and peripheral blood of HC.
Supplementary data 5. Enrichment pathways in DEGs between synovial fluid of RA and peripheral blood of HC.
Supplementary data 6. DEGs between synovial fluid of RA and peripheral blood of untreated RA.
Supplementary data 7. Enrichment pathways in DEGs between synovial fluid of RA and peripheral blood of untreated RA.
Supplementary data 8. Enrichment pathways in DEGs between peripheral blood of untreated RA and HC and DEGs between peripheral blood of treated RA and untreated RA.

Supplementary references

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