

# The pathogenic role of stem cell-like memory T cells in rheumatoid arthritis

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## Research article

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# Abstract

**Background:** Stem cell-like memory T cells (Tscm) are a subset of memory T cells that have the characteristics of stem cells. The role of Tscm cells in rheumatoid arthritis (RA) is not well characterized.

**Methods:** After measuring percentages of CD4<sup>+</sup> and CD8<sup>+</sup> Tscm cells within the peripheral blood and synovial mononuclear cell populations in RA and health controls (HCs), we confirmed the stem cell nature of Tscm cells from RA patients. The association of Tscm cells with disease activity was also analyzed. Next, the pathogenicity of Tscm cells was examined in RA patients by assessing T cell activation markers and cytokine secretion after stimulation with IL-6 and anti-CD3/CD28 beads. Finally, the transcriptomes of Tscm cells from RA patients were compared with those from HCs.

**Results:** The percentages of CD4<sup>+</sup> and CD8<sup>+</sup> Tscm cells among total T cells were significantly higher in RA patients than in HCs. Upon stimulation, Tscm cells from RA patients differentiated into daughter T cell subsets with self-renewal capacity. The percentage of CD4<sup>+</sup> Tscm cells correlated with expression of RA disease activity markers. Tscm cells from RA patients were more easily activated by IL-6 and anti-CD3/CD28 beads than those from HCs. Transcriptome analysis revealed that Tscm cells from RA patients showed patterns distinct from those of HCs.

**Conclusion:** The percentage of transcriptionally distinct and potentially pathogenic Tscm cells are higher in RA patients than in HCs; these cells may be a continuous source of pathogenic T cells, which perpetuate RA.

## Background

Stem cell-like memory T (Tscm) cells are the least differentiated type of memory T cell; these cells show stem cell characteristics (i.e., the capacity for self-renewal and differentiation into subsets of effector T cells) (1, 2). Originally, these cells were identified in mice; however, they have since been found in humans and non-human primates (3–5). Tscm cells play important roles in various conditions. For example, they provide long-term immunity to patients infected with yellow fever virus, and help CAR-T cells renew themselves in vivo (6, 7). They also act as a reservoir of pathogenicity; CD8<sup>+</sup> Tscm cell numbers are elevated in patients with immune thrombocytopenia (ITP) (8), and in those with acquired aplastic anemia (9). Recently, we reported the pathogenic role of Tscm cells in systemic lupus erythematosus (10).

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease characterized clinically by polyarthritis and pathologically by synovial hyperplasia and prominent infiltration by inflammatory cells (11). Although the pathophysiology of RA is still not fully understood (12), CD4<sup>+</sup> T cells are thought to play a critical role. In addition, inflammatory cytokines, including TNF, IL-6, and IL-1, are important; this was proven by the success of biologic agents that target these inflammatory cytokines (13–16). The recent success of JAK inhibitors (which block multiple cytokines simultaneously) as a treatment for RA supports the role of inflammatory cytokines (17, 18). However, it is still almost impossible to cure RA,

even with the best combinations of novel drugs (19). This suggests that there might be a hidden pathway underlying the pathogenicity of RA, which cannot be easily eradicated using current treatment tools.

Previously, we reported a pathogenic role for Tscm cells in systemic lupus erythematosus; in this case, Tscm cells generate pathogenic follicular helper T cells (10). Here, we examined the numbers of Tscm cells and their pathogenic features, together with the clinical implications and inherent transcriptional characteristics, in RA patients to identify a possible role for this cell type as a continuous source of pathogenic T cells.

## Methods

### Study population and clinical information

Ninety-eight RA patients and 73 healthy individuals were enrolled and peripheral blood mononuclear cells (PBMCs) and synovial fluid mononuclear cells (SFMCs) were isolated on a Ficoll-Hypaque (GE Healthcare, USA) gradient. All RA patients met the 2010 classification criteria of American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) (20). Laboratory investigations included erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), rheumatoid factor (1), anti-citrullinated protein antibody (ACPA). Disease activity was evaluated by measuring tender joint count (TJC; TJC 68 and TJC 28), swollen joint count (SJC; SJC 68 and SJC 28), ESR and CRP in each patient. The 28 Joints disease activity score (DAS28-ESR and DAS28-CRP) was available in 94 RA patients. Remission status was defined by ACR/EULAR Boolean criteria (21). Clinical characteristics and treatments for the enrolled patients are listed in Table 1.

This study was approved by the institutional review board of Seoul National University Hospital, and all samples were collected after obtaining written informed consent.

### Isolation of mononuclear cells from synovial tissue

Synovial tissue was obtained from knee joint replacement surgery. Tissue was chopped, treated with Type 2 collagenase (Worthington biochemical, USA) and incubated for more than 2 hours at 37°C. After incubation, culture media (RPMI-1640 (Welgene, Korea), 10% fetal bovine serum (Biowest, France), 1% penicillin/streptomycin (Gibco, USA)) was added, and cells were filtered and separated by Ficoll-Hypaque gradient (GE Healthcare).

### Immuno-phenotyping and cell sorting

For cell surface phenotyping, Fc receptors on PBMCs ( $1 \times 10^7$  cells/mL) were blocked with purified mouse anti-human IgG (BD Biosciences) and the cells were stained with adequate fluorescent antibodies.

Detailed flow cytometry gating strategy was described in Supplementary Figure 1. To measure the absolute number of CD4<sup>+</sup> or CD8<sup>+</sup> Tscm cells, Accucheck counting beads (Thermo Fisher, USA) were used.

Following antibodies were obtained from BD Biosciences (San Jose, USA): CD3 (clone SK7), CD4 (SK3), CD8 (SK1), CD45RO (UCHL1), CD62L (DREG-56), CD45RA (HI100), CD27 (M-T271), CXCR5 (RF8B2), ICOS (DX29), PD-1 (EH12.1)

Anti-CD28 (CD28.2) antibody was obtained from eBioscience (San Diego, USA) and antibodies specific for CD127 (A019D5), CD122 (Tu27), CD95 (DX2), CCR7 (G043H7) were obtained from BioLegend (San Diego, USA).

Stained cells were analyzed using a LSR Fortessa flow cytometer (BD Biosciences) and were sorted using a FACSAria instrument (BD Biosciences). All data were analyzed using FlowJo software (Treestar, USA).

### **Differentiation capacity of Tscm cells**

Sorted Tscm cells were stimulated for 6 days with anti-CD3/CD28-coated beads (Dynabeads, Thermo Fisher) at a 1:1 ratio. Differentiation capacity into other daughter T cell subsets (Tcm, Tem, and Temra and Tfh cells) were examined by staining CD3, CD4, CD45RO, CCR7, CD45RA, CXCR5, ICOS and PD-1.

### **Intracellular transcription factor staining**

Transcription factors associated with helper T subsets were stained. Fc receptors were blocked and cells were stained for CD3, CD4, CCR7, CD45RA. After surface-staining, cells were permeabilized with fixation/permeabilization solution (eBiosciences) and stained for transcription factors: T-bet (4B10), GATA3 (16E10A23) (Biolegend), ROR $\gamma$ t (Q21-559) (BD Biosciences), FoxP3 (PCH101) (eBiosciences).

### **IL-6 ELISA analysis**

Plasma from RA patients and HCs were collected and stored at below -80°C. IL-6 levels in plasma were measured using the Quantikine high sensitivity human IL-6 ELISA kit (enzyme-linked immunosorbent assay) (R&D systems, USA). All procedures were followed according to the instructions of the manufacturer. Standards were measured in triplicate and samples were measured in duplicate.

### **Assays for activation markers and cytokines (Cytometric Beads Assay)**

Sorted Tscm cells ( $4 \times 10^4$  cells) were stimulated for overnight with 50 ng/mL of recombinant human IL-6 protein (PeproTech, USA) and additionally stimulated with anti-CD3/CD28-coated beads (Thermo Fisher) if applicable. Next day, culture supernatants were stored for the cytokine assay and cells were stained for activation markers including CD69, CD25 (IL-2 receptor alpha) and CD154 (CD40 ligand).

Cytokine assay was proceeded with Cytometric Beads Assay (CBA). IFN- $\gamma$ , IL-4, IL-17A, and TNF cytokine levels were measured according to the manufacturer's protocol (LEGENDplex (Biolegend)) using LSR $\text{™}$  flow cytometer (BD biosciences).

## RNA-sequencing

RNA was extracted from FACS-sorted Tscm cells using RNeasy mini kit or RNeasy Plus Micro kit (both Qiagen, Germany). RNA-seq libraries were produced using the NEXTflex Rapid Directional mRNA-Seq Kit Bundles (Bioo Scientific, USA) and sequenced on the Illumina HiSeq 2500 platform (San Diego, USA). The sequence reads were analyzed by alignment to the Human Ensembl Archive Release 90 using STAR (22) with ENCODE options and quantification using RSEM (23).

## Statistical analysis

Data are expressed as the mean  $\pm$  SEM. For continuous variables, Student's t-test (unpaired, 2-tailed) was used when the sample size was  $> 30$ , while the Mann-Whitney U test was used when the sample size was  $< 30$ . All graphs were depicted using the Prism software (GraphPad software, Inc., USA). For RNA transcriptome analysis, differentially expressed genes were identified by DESeq2 (24) with Wald test and Likelihood ratio test corrected by Benjamini-Hochberg procedure (FDR  $< 0.05$ ) and one-way ANOVA ( $P < 0.01$ ) corrected by FDR  $< 0.05$ . The enrichment analysis of Gene Ontology terms and pathway were performed using Metascape (25) and volcano plots were drawn by R package EnhancedVolcano (Ver. 1.01). All statistical analyses were performed using SPSS (IBM, USA), otherwise stated.

## Results

The percentage of CD4 $^+$  or CD8 $^+$  Tscm cells is higher in RA patients than in HCs

We compared the percentages of Tscm cells within the PBMC populations isolated from 51 RA patients and 57 HCs (data from HCs were obtained in a previous study) (10). The flow cytometry gating strategy used to measure the percentages of Tscm cells is presented in Fig. 1A. The percentages of CD4 $^+$  or CD8 $^+$  Tscm cells among the total CD4 $^+$  or CD8 $^+$  T cell populations were significantly higher in RA patients than in HCs (CD4 $^+$  T cells:  $0.9\% \pm 0.1\%$  (RA) vs.  $0.4\% \pm 0.0\%$  (HC),  $p < 0.001$ ; CD8 $^+$  T cells:  $1.3\% \pm 0.2\%$  (RA) vs.

0.8% ± 0.1% (HC),  $p < 0.01$ ) (Fig. 1B). However, the absolute Tscm cell count in RA patients was comparable with that in HCs (Supplementary Fig. 1).

The synovium is the target of pathogenic factors in RA, and T lymphocytes play a pivotal role in disease pathogenesis. To determine whether Tscm cells are present in synovial tissue as well as in the PBMC population, we measured the proportion of Tscm cells in three samples of synovial fluid mononuclear cells (SFMCs) and one sample of synovial tissue. Tscm cells were present in both the CD4<sup>+</sup> and CD8<sup>+</sup> T cell compartments of SFMCs and synovial tissue (CD4<sup>+</sup> T cells: 0.8% ± 0.7%; CD8<sup>+</sup> T cells: 0.4% ± 0.1%) (Fig. 1C).

CD4<sup>+</sup> Tscm cells from RA patients have stem cell-like properties and differentiate into other subsets of T cells.

Stem cell-like properties endow the capacity for self-renew and the ability to differentiate into daughter cell subsets (2). To determine whether Tscm cells from RA patients also have these abilities, we stimulated FACS-sorted Tscm cells with anti-CD3/CD28-coated beads for 6 days. After stimulation, Tscm cells from RA patients differentiated into central memory T (Tcm, CCR7<sup>+</sup> CD45RA<sup>-</sup>), effector memory T (Tem, CCR7<sup>-</sup> CD45RA<sup>-</sup>), and terminal effector memory T (Temra, CCR7<sup>-</sup> CD45RA<sup>+</sup>) cells (Fig. 2A). The differentiation pattern in cells from RA patients was similar to that of cells from HCs (Fig. 2A, graph). The differentiated naïve-like T (Tnaïve, CCR7<sup>+</sup> CD45RA<sup>+</sup>) cell population comprised mostly Tscm cells (Fig. 2B); this is in accordance with a previous study showing that Tscm cells can self-renew and differentiate into Tcm and Tem cells (2). Examination of transcription factors T-bet (for Th1 (helper T cells)), GATA3 (for Th2 cells), RORγt (for Th17 cells), and FoxP3 (for Treg (regulatory T) cells) expressed by Tem cells differentiated from CD4<sup>+</sup> Tscm cells revealed that expression of these transcription factors was similar between the two groups (Fig. 2C).

To better understand whether Tscm cells from RA patients differentiate into Tfh (follicular helper T) cells, stimulated Tscm cells were stained for Tfh-associated markers. After stimulation with anti-CD3/CD28, Tscm cells from RA patients differentiated into Tfh cells (designated CD3<sup>+</sup> CD4<sup>+</sup> CXCR5<sup>+</sup> PD-1<sup>+</sup> ICOS<sup>+</sup>) (Supplementary Fig. 2A). However, the percentage of differentiated Tfh cells among the CD4<sup>+</sup> T cell population was no higher in RA patients than in HCs. This was confirmed by results showing that the percentage of Bcl-6<sup>+</sup> cells among CD4<sup>+</sup> T cells was similar between RA patients and HCs (Supplementary Fig. 2B).

In addition to the capacity for differentiation, Tscm cells renewed themselves in response to anti-CD3/CD28 stimulation (Fig. 2D). Tscm cells (CD4<sup>+</sup> CFSE<sup>low</sup> CCR7<sup>+</sup> CD45RO<sup>-</sup> CD62L<sup>+</sup> CD95<sup>+</sup>) were detected consistently within the population of proliferated Tscm cells (denoted by low level of CFSE), in both RA patients and HCs (Fig. 2E).

Clinical implications of Tscm cells in RA patients.

We compared the numbers of Tscm cells in RA patients with active disease with those in patients in remission. The percentage of Tscm cells in RA patients in remission was significantly lower than that in RA patients with active disease (CD4<sup>+</sup> T cells: 0.26% ± 0.05% (RA remission) vs. 0.9% ± 0.1% (active RA), p = 0.034; CD8<sup>+</sup> T cells: 0.04% ± 0.1% (RA remission) vs. 1.3% ± 0.2% (active RA), p = 0.023) (Fig. 3A). When we analyzed the association between Tscm cells and expression of disease activity markers in RA patients, we found that the percentages of CD4<sup>+</sup> Tscm cells among the CD4<sup>+</sup> T population correlated with the tender joint count (TJC), swollen joint count (SJC), disease activity score 28 (DAS28)-ESR, and DAS28-CRP scores (Fig. 3B, C). However, the percentage of CD8<sup>+</sup> Tscm among the CD8<sup>+</sup> T cell population was not related to markers of disease activity (Fig. 3B, D).

Tscm cells from RA patients are more easily activated in response to key inflammatory cytokines in vitro.

IL-6 is one of the most important cytokines in the pathogenesis of RA; it activates various leukocytes and osteoclasts and mediates B cell differentiation to generate autoantibodies (11). Here, we found that IL-6 levels in the plasma of RA patients were higher than those in the plasma of HCs (Fig. 4A). To evaluate the effect of IL-6 on Tscm cells, we compared the activation status of Tscm cells after stimulation with IL-6 and/or anti-CD3/CD28 beads. Expression of surface activation markers CD69, CD25 (IL-2 receptor alpha), and CD154 (CD40 ligand) was highest in the presence of both anti-CD3/CD28 and IL-6. However, activation of Tscm cells from RA patients was further enhanced (compared with those from HCs) when stimulated with TCR and IL-6 (CD69, p = 0.148 for stimulation with anti-CD3/CD28 beads, and p = 0.023 for stimulation with both anti-CD3/CD28 beads and IL-6; CD25, p = 0.055 for stimulation with anti-CD3/CD28 beads, and p = 0.078 for stimulation with both anti-CD3/CD28 beads and IL-6; CD154, p = 0.055 for stimulation with anti-CD3/CD28 beads, and p = 0.016 for stimulation with both anti-CD3/CD28 beads and IL-6) (Fig. 4B, C). Thus, IL-6 (the key inflammatory cytokine in the pathogenesis of RA) acts synergistically with the TCR to strongly activate Tscm cells in RA patients.

Next, we measured the concentration of cytokines in culture supernatants of activated Tscm cells using a cytometric beads assay. The following cytokines were measured: IFN-γ (for Th1 cells); IL-4 (for Th2 cells); and IL-17A (for Th17 cells). Tscm cells from RA patients produced more cytokines when stimulated with IL-6 and anti-CD3/CD28 beads than when stimulated only with anti-CD3/CD28 beads (TNF, p = 0.013; IL-4, p = 0.002; IFN-γ, p = 0.002; IL-17A, p = 0.019) (Fig. 4D). Tscm cells from RA patients secreted more TNF than those from HCs when stimulated with anti-CD3/CD28 beads (p = 0.014). In addition, more IL-17A and IL-4 was released by Tscm cells from RA patients than by those from HCs when stimulated with anti-CD3/CD28 beads, regardless of the presence of IL-6 (IL-17A, p = 0.029 for stimulation with anti-CD3/CD28, and p = 0.049 for stimulation with both anti-CD3/CD28 beads and IL-6; IL-4, p = 0.037 for stimulation with anti-CD3/CD28 beads, and p = 0.027 for stimulation with both anti-CD3/CD28 beads and IL-6).

Transcriptome analysis of Tscm cells revealed that RA Tscm cells are distinct from HC Tscm cells.

Finally, we examined RNA transcriptome patterns to detect inherent differences between Tscm cells from RA patients and those from HCs. We compared RNA transcription patterns among CD4<sup>+</sup> Tscm cells from active RA patients (n = 3), RA patients in remission (n = 3), and HCs (n = 2). First, we compared the transcriptome patterns of Tscm cells from RA patients overall (active RA patients plus RA patients in remission) with those of cells from HCs to identify RA-specific transcripts. We identified 332 differentially expressed genes (DEGs) (False Discovery Rate (FDR) < 0.05, -fold change > 2), among which 120 DEGs were upregulated in RA patients and 212 were upregulated in HCs (Fig. 5A). Enrichment analysis revealed that the GO terms for upregulated DEGs in RA were classified as 'Protein glycosylation', 'Transcription', 'Positive regulation of MAP kinase cascade', 'Positive regulation of mitotic nuclear division', and 'Defense response to virus' (Fig. 5B). When a gene expression heatmap of active RA signatures and HC signatures was constructed, 153 genes showed higher expression in those with active RA (Supplementary Fig. 3). These 153 genes were classified into the GO terms 'Cellular response to cytokine stimulus', 'Leukocyte activation', and 'Regulation of fibroblast proliferation' (Fig. 5C).

Next, we compared the transcriptome of Tscm cells from active RA patients with those of cells from RA patients in remission. Only 15 DEGs were identified (FDR < 0.05, -fold change > 2). We found that many genes (n = 38) showed the highest expression in active RA, moderate expression in RA patients in remission, and the lowest expression in HCs (Fig. 5D). Principal Component Analysis (PCA) revealed that Tscm cells from all three groups showed distinct gene expression profiles; the profile of Tscm cells from RA patients in remission fell in-between those of Tscm cells from patients with active RA and those from HCs (Fig. 5E). These results suggest that Tscm cells from patients with active RA are distinct from those from HCs and RA patients in remission, although Tscm cells from RA patients in remission showed some differences from those from HCs.

## Discussion

This study demonstrates a role for Tscm cells in the pathogenesis of RA. We showed that the percentages of Tscm cells among the CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations were significantly higher in RA patients than in HCs. In addition, the percentages of Tscm cells among the CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations in RA patients in remission were significantly lower than those in active RA patients. The percentage of CD4<sup>+</sup> Tscm cells correlated with RA disease activity. When stimulated with IL-6 and anti-CD3/CD28 beads, Tscm cells from RA patients were more easily activated than those from HCs. Finally, the RNA transcriptome of Tscm cells from RA patients was clearly different from that of HCs. The transcriptome of Tscm cells from RA patients in remission was similar (but not identical) to that of cells from HCs.

CD4<sup>+</sup> T cells play important roles in the pathogenesis of RA. Inflammatory cytokines secreted by T cells recruit and activate various inflammatory cells and induce proliferation of synoviocytes; this results in the pannus formation, which can ultimately destroy joints (26). Our study shows that Tscm cells, which are present in both peripheral blood and synovial tissues, differentiate into naïve, memory, and effector T

cells. When effector memory T cells differentiated from Tscm cells were stained for transcription factors characteristic of helper T cells, we found expression of T-bet and ROR $\gamma$ t, but minimal expression of GATA3 (Fig. 2C). Th1 and Th17 subsets are thought to drive the pathogenesis of RA (11). In addition, Tscm cells differentiated into Tfh cells under appropriate conditions. Tfh cell numbers increase in patients with new onset RA (27), and Tfh cells from RA patients have a critical role in the chronic inflammation in the joints and are associated with disease activity (28). These results suggest that Tscm cells from RA patients contribute to the pathogenesis of RA by continuously providing pathogenic T cell subsets with self-renewal capacity.

We also found that the percentage of Tscm cells among total T cells was significantly lower in patients in remission than in those with active disease (Fig. 3A). Moreover, the percentage of CD4<sup>+</sup> Tscm cells among total CD4<sup>+</sup> T cells correlated with the TJC (TJC68, TJC28), SJC (SJC68, SJC28), DAS28-ESR, and DAS28-CRP (Fig. 3B, C). Our findings suggest that RA disease activity is maintained by memory/effector T cells supplied by Tscm cells. It is interesting that disease activity correlated with CD4<sup>+</sup> Tscm cells, but not with CD8<sup>+</sup> Tscm cells. This is consistent with the fact that CD4<sup>+</sup> T cells play a clear and defined role in the pathogenesis of RA, whereas the role of CD8<sup>+</sup> T cells is unclear (29).

When Tscm cells from RA patients were stimulated with IL-6, they expressed activation markers CD69, CD25, and CD154 (Fig. 4). Tscm cells from RA patients were more easily stimulated *in vitro*. Elevated levels of IL-6 in the plasma of RA patients can result in a microenvironment in which Tscm cells are more easily stimulated (Fig. 4A). We showed that the activated Tscm cells secreted TNF and IL-17A in response to IL-6 (Fig. 4D). IL-17A, acting synergistically with TNF, can induce activation of synovial fibroblasts, chondrocytes, and osteoclasts in patients with RA (11). Also, synovial T cells stimulate FLS (fibroblast-like synoviocytes) in the presence of IL-17; activated FLS then produce inflammatory cytokines (30, 31). Thus, we might infer that Tscm cells in a pro-RA microenvironment (e.g., high levels of IL-6) differentiate into pathogenic cells.

Recently, a study revealed that CD4<sup>+</sup> Tscm cells were expanded in CD4<sup>+</sup> T lymphocytes of RA patients and citrullinated vimentin (Vim<sub>cit</sub>)-specific CD4<sup>+</sup> Tscm cells were increased in active RA patients (32). These findings confirmed our current study results in terms of expansion of CD4<sup>+</sup> Tscm cells and CD8<sup>+</sup> Tscm cells in RA patients even though statistical significance (in case of CD8<sup>+</sup> Tscm cells) was not reached in the previous report. In addition, we can infer that Vim<sub>cit</sub>-specific CD4<sup>+</sup> Tscm cells can secrete disease-prone inflammatory cytokines under IL-6 and/or TCR-activated environment of RA patients.

Transcriptome analysis revealed that upregulated transcriptome profiles for 'Protein glycosylation', 'Transcription', 'Positive regulation of MAP kinase cascade', and 'Positive regulation of mitotic nuclear division' are related to RA pathogenesis. From a functional perspective, GO terms 'Cellular response to cytokine stimulus', 'Leukocyte activation', and 'Regulation of fibroblast proliferation' were more prominent in RA patients. All of these genes and their associated pathways are related to the pathogenesis of RA, which suggests that the Tscm cells already harbor pathogenetic signature of RA. Interestingly, the

transcriptome of RA patients in remission was more similar to that of HCs than to that of RA patients with active disease, although there were some differences. These results are consistent with the clinical finding that clinical remission in RA is different from cure (33). These findings also suggest that the transcriptome features of Tscm cells could be a biomarker of an RA cure in the future.

## Conclusions

Tscm cells are critical players in the pathogenesis of RA; these cells have an inherent ability to self-renew and differentiate into the pathogenic effector, memory, and follicular helper T cells. Tscm cells in an RA environment may sustain disease by continuously supplying pathogenic T cells, thereby preventing cure, even in patients treated with the latest drugs.

## Declarations

### Supplementary information

**Additional file 1 : Figure S1 Flow cytometry panel for the detection of Tscm cells.** Absolute number of CD4<sup>+</sup> or CD8<sup>+</sup> Tscm cells among CD4<sup>+</sup> or CD8<sup>+</sup> T cells. Age were matched between RA patients and HCs.

### **Additional file 2 : Figure S2 Tscm cells from RA patients differentiate into follicular**

**helper T cells.** (A) Tscm cells from 10 RA patients and 7 healthy controls (HCs) were

analyzed for their ability to differentiation into follicular helper T (Tfh) cells after stimulation

with anti-CD3/CD28 beads for 6 days. Tfh cells were designated as CD3<sup>+</sup>, CD4<sup>+</sup>, CXCR5<sup>+</sup>,

PD-1<sup>+</sup>, ICOS<sup>+</sup> cells. Graph about the proportion of Tfh cells within the CD4<sup>+</sup> T cells are also

shown. (B) The percentage of Bcl-6 among CD4<sup>+</sup> T cells after stimulation.

**Additional file 3 : Figure S3 Heat map of the comparison between active RA and HCs.** (A) Heatmap of 153 genes extracted by criteria which expression was enhanced in Tscm cells of active RA than those of HCs.

This study was carried out in accordance with the Declaration of Helsinki with written informed consent from subjects. The protocol was approved by the institutional review board of Seoul National University Hospital.

## **Consent for publication**

Not applicable

## **Availability of data and materials**

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

## **Competing interests**

EBL has acted as a consultant for Pfizer and received a research grant from GC Pharma, South Korea.

## **Funding**

None

## **Author contributions**

All authors participated in drafting the article or revising it critically, and all authors approved the final version to be submitted. EBL and YJL had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of data analysis. Study conception and design. EBL, YJL. Acquisition of data. EBL, YJL. Analysis and interpretation of data. YJL, EHP, JWP, KCJ, and EBL.

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## Tables

Table 1. Clinical characteristics of RA patients

Age, years, mean $\pm$ SD	61.0 $\pm$ 11.2
Female, n (%)	80 (84.2)
RA duration, years, median $\pm$ IQR	8.7 [4.0-14.8]
ESR (mm/hour), median $\pm$ IQR	33 [16.0-54.0]
CRP (mg/dL), median $\pm$ IQR	0.39 [0.1-2.3]
DAS28-ESR, median $\pm$ IQR	4.3 [3.4-5.1]
DAS28-CRP, median $\pm$ IQR	3.0 [2.2-3.6]
RF positive, n (%)	81 (85.3)
ACPA positive, n (%) (total n=63)	46 (73.0)
Treatment, n (%)	
Corticosteroids	59 (62.1)
Corticosteroid dose (prednisolone equivalent), mg/day	2.5 [0.0 - 5.0]
Methotrexate (mg/wk)	63 (66.3)
Hydroxychloroquine (mg/day)	23 (24.2)
Leflunomide (mg/day)	20 (21.1)
Sulfasalazine (mg/day)	13 (13.7)
NSAIDs	64 (67.4)
Etanercept	5 (5.3)
Abatacept	2 (2.1)
Xeljanz	9 (9.5)

ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; IQR, interquartile range; RF, Rheumatoid factor; ACPA, Anti-citrullinated protein antibody; NSAIDs, Nonsteroidal anti-inflammatory drugs

## Abbreviations

ACPA : Anti-citrullinated protein antibody

ANOVA : Analysis of variance

CAR-T : Chimeric antigen receptor-T

CFSE : Carboxyfluorescein succinimidyl ester

CRP : C-reactive protein

DAS28 : The 28 joints disease activity score

DEG : Differentially expressed genes

ESR : Erythrocyte sedimentation rate

FDR : False discovery rate

FLS : Fibroblast-like synoviocytes

GO : Gene ontology

HC : Healthy controls

IL : Interleukin

ITP : Immune thrombocytopenia

JAK : Janus kinase

MAP : Mitogen-activated protein

PBMC : Peripheral blood mononuclear cells

PCA : Principal component analysis

RA : Rheumatoid arthritis

RF : Rheumatoid factor

SFMC : Synovial fluid mononuclear cells

SJC : Swollen joint count

TCR : T cell receptor

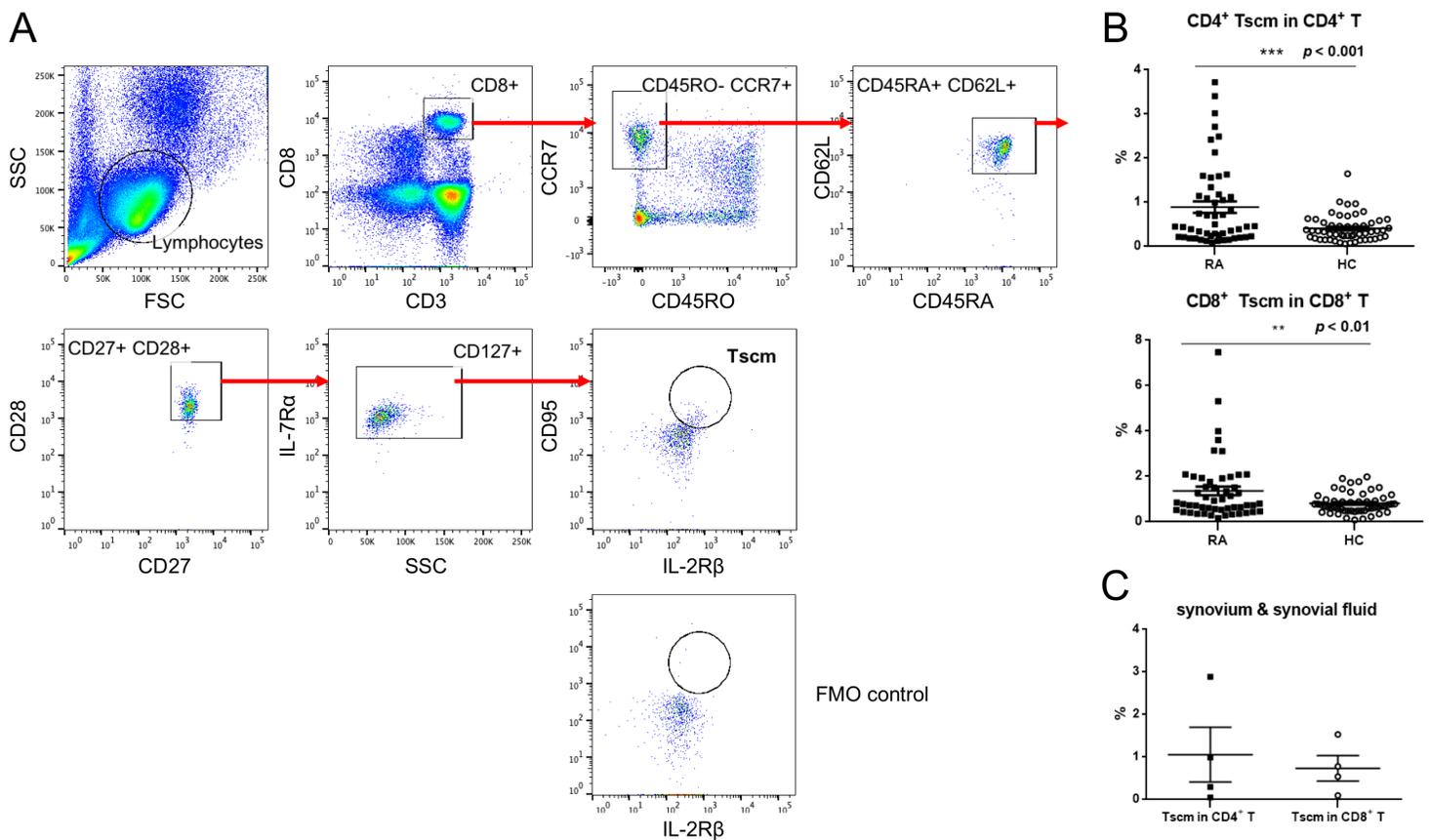
Th : helper T

TJC : Tender joint count

TNF : Tumor necrosis factor

Tscm : Stem cell like-memory T cells

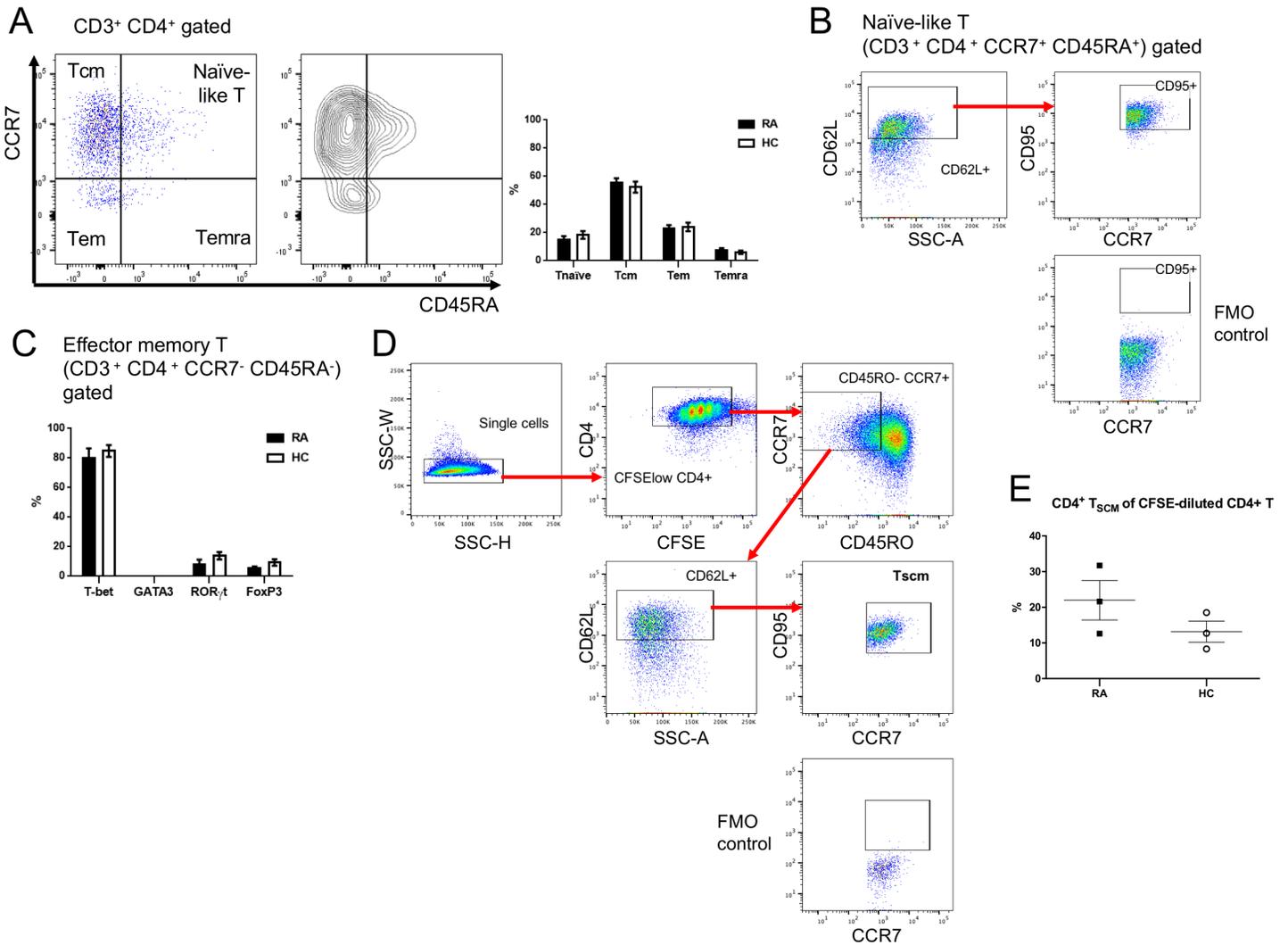
## Figures



**Figure 1**

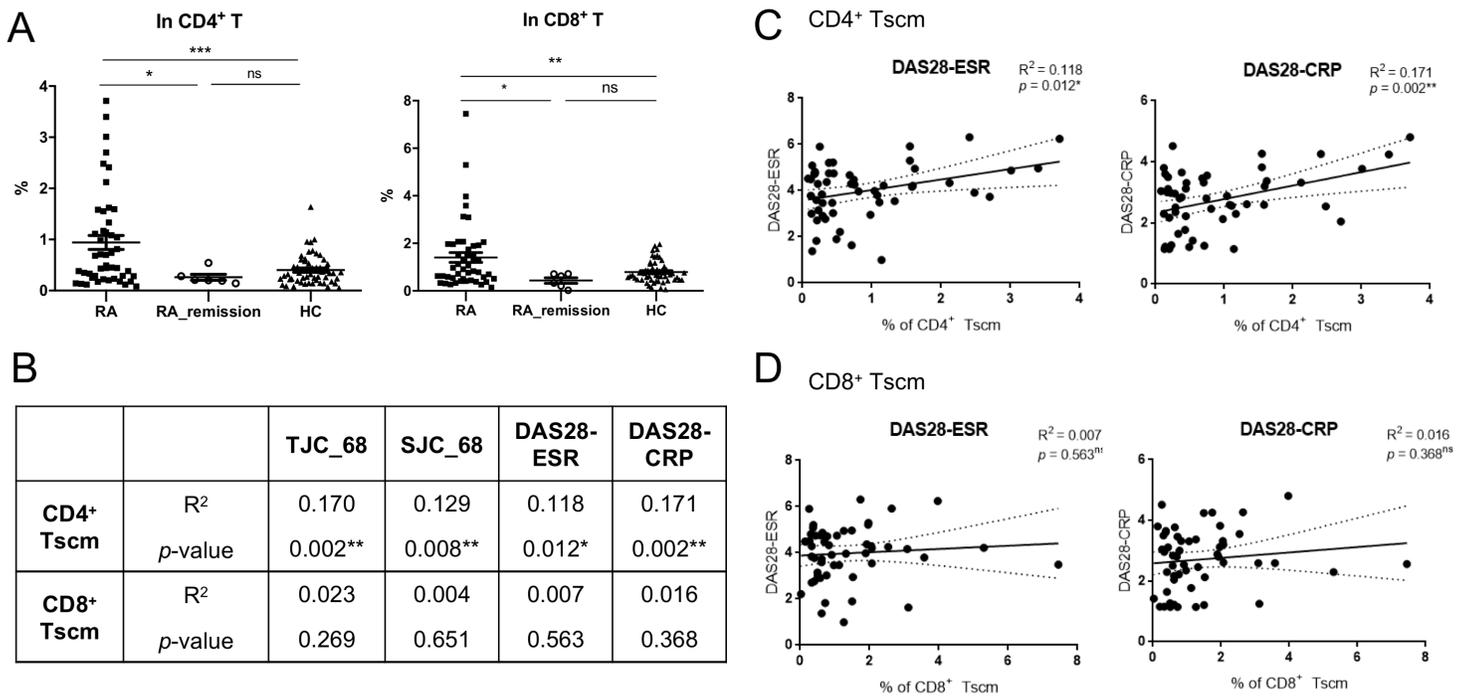
Increased ratio of Tscm cells among the CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations. (A) Flow cytometry panel used to detect Tscm cells from RA patients. Tscm cells were designated as CD3<sup>+</sup>, CD4<sup>+</sup> cells, or as CD8<sup>+</sup>, CD45RO<sup>-</sup>, CCR7<sup>+</sup>, CD45RA<sup>+</sup>, CD62L<sup>+</sup>, CD27<sup>+</sup>, CD28<sup>+</sup>, CD127 (IL-7R $\alpha$ )<sup>+</sup>, CD122<sup>+</sup>, CD95<sup>hi</sup> cells. The FMO control refers to the 'full color minus one' isotype control. (B) Percentages of Tscm cells among total

CD4+ and CD8+ T cells. (C) Graph showing the percentages of CD4+ and CD8+ Tscm cells among total CD4+ and CD8+ T cells in synovial fluid or synovial tissue. The bars represent the mean  $\pm$  SEM. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  (two-tailed Student's t-test).



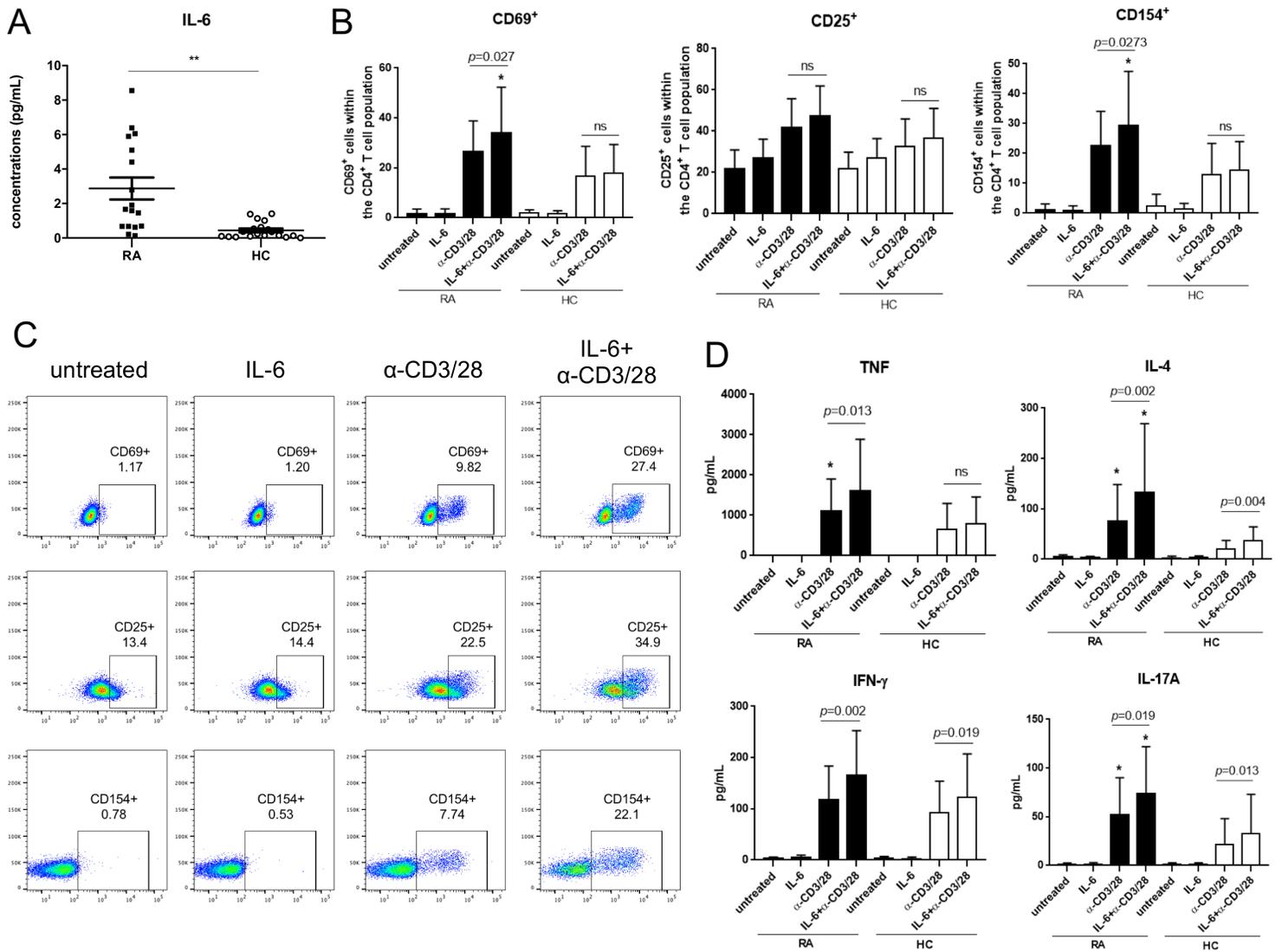
**Figure 2**

Tscm cells from RA patients differentiate into other T cells. (A) The differentiation capacity of Tscm cells isolated from 11 RA patients and 10 HCs was examined by detecting expression of CCR7 and CD45RA after stimulation with anti-CD3/CD28 beads for 6 days. A summary of the CD4<sup>+</sup> Tscm differentiation pattern is shown. (B) Flow cytometry panel to detect differentiated naïve-like T (CD3<sup>+</sup> CD4<sup>+</sup> CCR7<sup>+</sup> CD45RA<sup>+</sup>) cells after stimulation. (C) Transcription factor expression by differentiated effector memory T cells (CD3<sup>+</sup> CD4<sup>+</sup> CCR7<sup>-</sup> CD45RA<sup>-</sup>). (D) Self-renewal capacity of Tscm cells from RA patients. This ability was examined by detecting the percentages of CFSE-containing cells after stimulation with anti-CD3/CD28 beads for 6 days. (E) Graph showing the percentage of self-renewing Tscm cells.



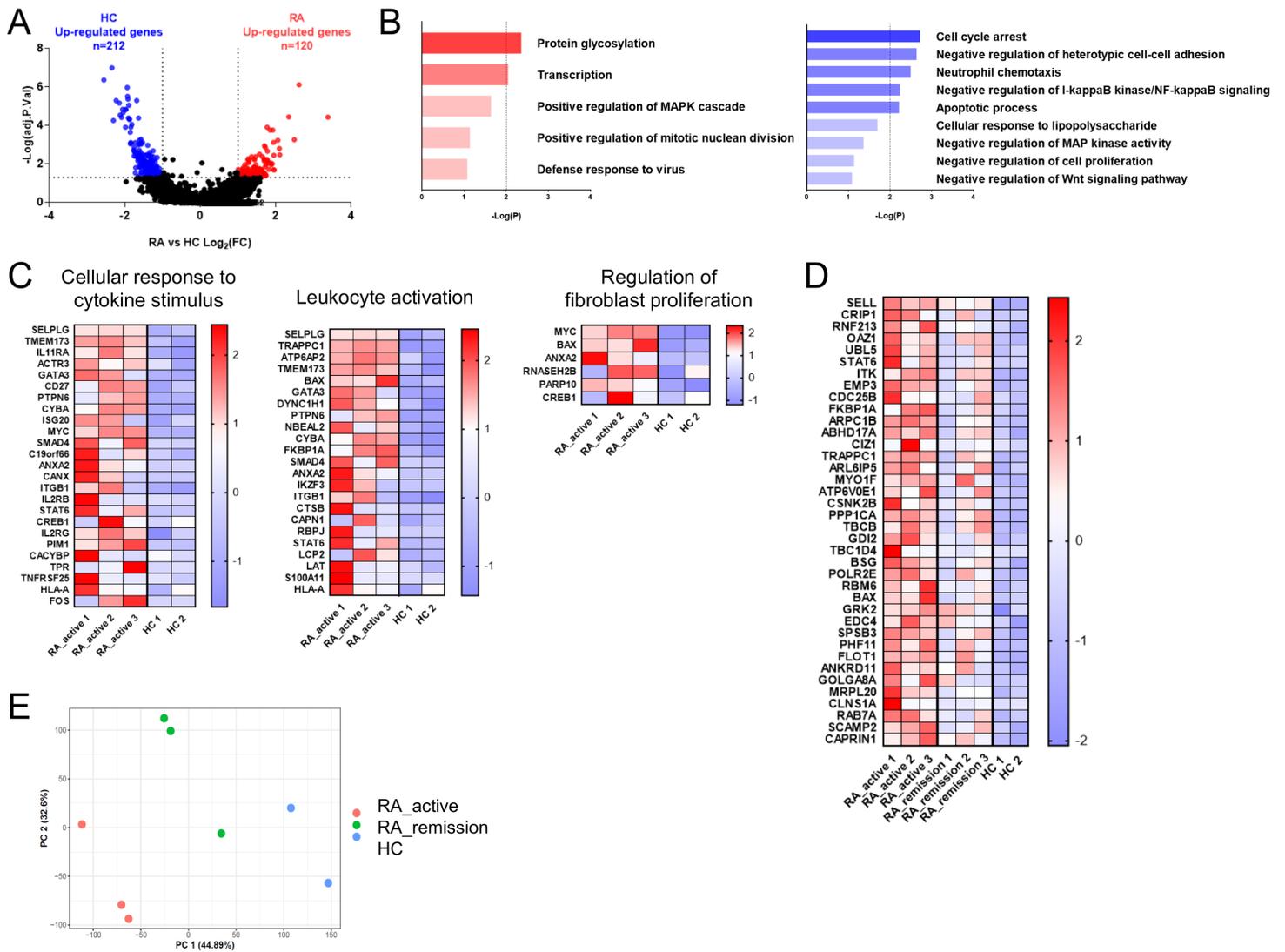
**Figure 3**

The percentage of CD4<sup>+</sup> Tscm cells correlates with markers of disease activity. (A) Percentage of Tscm cells among total CD4<sup>+</sup> and CD8<sup>+</sup> T cells from RA patients in remission, RA patients with active disease, and HCs. (B) Tables showing the correlation between the percentage of Tscm cells among total CD4<sup>+</sup> and CD8<sup>+</sup> T cells and markers of RA disease activity. (C) Graph showing the correlation between the percentage of CD4<sup>+</sup> Tscm cells among total CD4<sup>+</sup> T cells and the DAS28-ESR and DAS28-CRP. (D) Graph showing the correlation between the percentage of CD8<sup>+</sup> Tscm cells among total CD8<sup>+</sup> T cells and the DAS28-ESR and DAS28-CRP scores.



**Figure 4**

Tscm cells from RA patients are more easily activated by IL-6 in vitro. (A) IL-6 levels in the plasma of 17 RA patients and 19 HCs were measured by ELISA. (B) Expression of CD69, CD25, and CD154 by CD4+ Tscm cells from 10 RA patients and eight HCs after stimulation (or not) with IL-6, anti-CD3/CD28 beads, or IL-6 plus anti-CD3/CD28 beads. (C) Flow cytometry to detect activation markers expressed by CD4+ Tscm cells after stimulation. (D) After stimulation (or not), the concentrations of cytokines secreted into the culture supernatant were measured in a cytometric beads assay. ‘\*’ denotes statistically significant ( $p < 0.05$ ) differences between RA Tscm cells and HC Tscm cells under each condition. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  (Mann-Whitney U test).



**Figure 5**

Transcriptome analysis of Tscm cells from RA in remission, those with active disease, and HCs. (A) Volcano plot. Red dots denote genes upregulated in RA ( $\text{FDR} < 0.05$ ,  $\text{Log}_2\text{Foldchange} > 1$ ), and blue dots denote genes upregulated in HC ( $\text{FDR} < 0.05$ ,  $\text{Log}_2\text{Foldchange} < -1$ ). (B) Enrichment analysis of upregulated genes (left graph) and downregulated genes (right graph) in RA. (C) Heatmap of genes classified according to their functions. (D) Heat map comparing genes expressed by patients with active RA, those in remission, and HCs. (E) Principal component analysis. Pink dots represent Tscm cells from patients with active RA, green dots represent Tscm cells from RA patients in remission, and blue dots represent Tscm cells from HCs.

## Supplementary Files

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