CD4+ tissue resident memory Th17 cells drive IL-17A-mediated joint pathology in Spondyloarthritis

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Objectives

Interleukin (IL)-17A is a key driver of Spondyloarthritis (SpA) joint pathology. We aimed to identify its cellular source in synovial tissue from patients with Axial SpA (AxSpA) and Psoriatic arthritis (PsA).

Methods

Synovial tissue from patients with SpA was profiled using single-cell RNA sequencing (scRNA-seq: AxSpA, n=5 and PsA, n=6) or spatial RNA profiling (PsA, n=4). CellPhoneDB was used to infer cell–cell communication. Tissue resident memory Th17 (TRM17)-like cells were generated *in vitro* using blood memory CD4+ T cells from SpA patients. An epigenetic inhibitor library, siRNA and clustered regularly interspaced short palindromic repeats (CRISPR) were used to identify epigenetic regulator(s) for TRM17.

Results

scRNA-seq showed that *IL17A* expression in SpA synovium was restricted to *CD4+CXCR6+* TRM17 cells. Cell-cell communication and single-cell spatial analysis support the interaction between TRM17 and *CLEC10A+* dendritic cells, which were activated in SpA. Both sublining and lining fibroblasts in SpA synovium exhibited an enhanced IL-17A response signature. TRM17-like cells generated *in vitro* phenocopied the TRM17 found in joint tissue and produced IL-17A in response to T cell receptor (TCR) stimulation but not cytokines. IL-23 enhanced TCR-mediated IL-17F and IFN-γ but not IL-17A production. Perturbation of BRD1 inhibited the generation of TRM17-like cells.

Conclusions

CD4+ TRM17 cells are the predominant source of IL-17A in SpA. The TCR stimulation is essential for the secretion of IL-17A by TRM17-like cells. The epigenetic regulator BRD1 contributes to the generation of TRM17. Depleting TRM17 cells in SpA is a therapeutic strategy with potential to induce long-term remission.

Key messages

- **What is already known on this topic**

Interleukin (IL)-17A plays a key role in the immunopathogenesis of Spondyloarthritis (SpA), but its cellular source in joint tissue has not been determined previously. The induction of CD4+ tissue resident memory Th17 (TRM17) cells following the clearance of pathogens has been described in skin, lung and kidney. Whether CD4+ TRM17 cells contribute to the pathology of SpA is not clear.

- **What this study adds**

I. CD4+ TRM17 cells are present in SpA synovial tissue and are the predominant source of *IL17A*
II. CD4+ TRM17 cells in SpA joints express *IL17A* without any *in vitro* exogenous stimulation
III. T cell receptor (TCR) rather than cytokine stimulation is essential for IL-17A production by TRM17-like cells
IV. The epigenetic regulator BRD1 contributes to the generation of TRM17-like cells.

- **How this study might affect research, practice or policy**

This study demonstrates for the first time that CD4+ TRM17 cells are the main source of IL-17A in SpA synovial tissue, making a strong argument that future research of IL-17A pathology in SpA joint should focus on CD4+ TRM17 cells rather than circulating Th17 cells, CD8+ T cells, innate-like lymphocytes or innate lymphoid cells. In addition, our data suggest that targeting TRM17 cells, the "factory" of IL-17A, has the potential to induce long-term remission, encouraging future efforts to develop new therapies to deplete TRM17 cells in SpA joints.

INTRODUCTION

Spondyloarthritis (SpA) is a group of common immune-mediated conditions that cause joint inflammation and pain. Axial SpA (AxSpA) and psoriatic arthritis (PsA) are two major forms of SpA, characterized by spine and skin involvement respectively. Despite symptomatic differences between axSpA and PsA, shared pathological drivers have been discovered including tumor necrosis factor and interleukin 17A (IL-17A). Drugs targeting IL-17A are effective in around 50% of SpA patients but require long-term administration.

IL-17A is known to promote tissue inflammation, at least in part through fibroblast activation. Among the different forms of IL-17 (A-F), IL-17A is the key driver in joint inflammation whereas a role of IL-17F in skin inflammation has been supported by recent trials[1]. IL-17A can be produced by a variety of immune cells, including conventional T cells, innate-like lymphocytes and innate lymphoid cells (ILCs). Whether a specific cell type dominates IL-17A production in SpA is debated[2]. Different studies have implicated all three subsets of innate-like lymphocytes (γδ T cells, iNKT cells, and MAIT cells) and CD8+ tissue-resident memory T cells as the sources of IL-17A in SpA joints[3–5]. Increasing evidence, however, is highlighting a role for CD4+ tissue-resident memory Th17 (TRM17) cells as key IL-17A producers in tissues such as the skin, lung and kidney[6–8]. Furthermore, CD4+ Th17 cells expressing CXCR6, an established marker for tissue residency, have been linked to brain inflammation in a murine model of multiple sclerosis [9]. It is therefore possible that a role for CD4+ TRM17 cells in SpA may have been missed in previous studies focused on synovial fluid samples and/or utilising *in vitro* restimulation to determine the source of IL-17 [3–5].

Notably, CXCR6+ Th17 cells found in the inflamed brain tissue exhibit a distinct epigenetic profile that is different from homeostatic Th17 cells with stem-like features[9]. Considering the established role of epigenetic modifiers in the regulation of T cell differentiation and function[10,11], it is plausible to predict that epigenetic regulators could be involved in the generation of CD4+ TRM17 cells.

Through profiling un-stimulated synovial tissue using single-cell RNA sequencing and spatial RNA profiling, we show that CD4+ CXCR6+ TRM17 cells are the predominant source of IL-17A in SpA. We also establish a model to generate TRM17-like cells *in vitro* and show that T cell receptor (TCR) engagement, rather than cytokines, is essential for these cells to produce IL-17A or IL-17F. IL-23 enhances the TCR-meditated secretion of IL-17F, but not IL-17A, by TRM17-like cells. Lastly, we show that the epigenetic regulator bromodomain-containing protein 1 (BRD1) contributes to the generation of TRM17-like cells.

METHODS

Patient Recruitment

Fresh synovial tissue was obtained at arthroplasty or synovial biopsy was collected from patients attending the Oxford University Hospitals (OUH), Queen Elizabeth Hospital and Shanghai Sixth People's Hospital with appropriate consent and ethical approvals. All patients included in this study met the criteria of the Assessment of Spondyloarthritis International Society (ASAS) for AxSpA or CASPAR criteria for PsA. The demographics of AxSpA and PsA patients recruited for this study are shown in **Table S1**.

Preparation of samples and libraries for scRNA-seq

Synovial tissue was obtained from surgical procedures. Cells were isolated from fresh tissue through enzyme digestion using 100 µg/ml Liberase TL and 100 µg/ml DNase in RPMI. The isolated cells were either directly subjected to the Chromium Single Cell Gene Expression Chip (10X Genomics) assay or sorted into live CD3+ and CD3- populations using Fluorescence-activated Cell Sorting (FACS). For FACS, the single cell suspension was first incubated with Human TruStain FcX solution (Biolegend) to block Fc receptors and then stained with anti-CD3 (clone OKT3) and Fixable Viability Dye eFluor 780 (eBioscience/ThermoFisher). Approximately 20,000 cells from each population were loaded onto a Chromium Next GEM Chip (10X Genomics), and scRNA-seq libraries were prepared according to the manufacturer's instructions. For samples directly applied to library preparation without sorting, around 20,000 cells from each sample were loaded. The resulting DNA libraries were sequenced at >50,000 reads per cell on a NovaSeq 6000 using PE150 mode.

Computational analysis of scRNA-seq data

The paired reads obtained were mapped to the hg38 reference genome to generate gene expression matrices using CellRanger. For PsA samples (from the Cartography Consortium), the full data set was analysed in Panpipes [12] and T cell compartment was partitioned out for downstream analyses along with the AxSpA data. T cells of PsA samples and full dataset of AxSpA samples were then merged and analyzed using the Seurat R package (v4.0.5). Cells with low-quality profiles were excluded based on the number of detected genes, the percentage of mitochondrial RNA among total UMIs, and the total number of UMIs. The raw read counts were normalized using the NormalizeData function, and variable genes were identified for each sample. To identify cell types, we mapped our cells into a synovium reference map built by Fan Zhang et. al.[13] using Symphony algorithm. For the reclustering analysis of cellular subsets, a certain population were extracted and data from different sample were subject to FastMNN algorithms for integration. We then used FindNeighbors and FindCluster functions to cluster cells based on global transcriptional profile. For differential expression analysis, the FindMarkers function was used to test the normalized data with the default Wald test method. Unless specified, default parameters were used for each

function.

For cell-cell interaction inference, normalised expression matrix and cluster annotations were exported from the Seurat object as input for cellphonedb. Cellphonedb v5.0.0 was used as a ligand-receptor interaction database. 'statistical analysis' method from Cellphonedb tools was used to assess the strength and significance of interactions.

In vitro model for TRM17 cells

PBMCs were obtained by density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich) from the whole blood of patients with AxSpA or PsA attending OUH (Ethics 06/Q1606/139). Memory CD4+ T cells were isolated using an untouched negative isolation kit (Miltenyi Biotec). Isolated memory CD4+ T cells were stimulated with plate bound α-CD3 (5 µg/ml, Biolegend, cat: 300332), soluble α-CD28 (1 µg/ml, Biolegend, cat: 302934), and Th17 skewing cytokines (IL-1β and IL-23, 20 ng/ml) for six days as part of a Th17 expansion phase. Cells were cultured throughout in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS) and 1% GlutaMAX (Gibco) (R10) at 37°C in a humidified atmosphere containing 5% carbon dioxide. Cells were then cultured without TCR stimulation in the presence of TGF-β (2 ng/ml) and IL-7 (20 ng/ml) for a further six days to mimic a tissue residency phase. The phenotype of input (day 0) and output (day 12) cells were measured using flow cytometry following intracellular staining (post PMA/Ionomycin stimulation). Following the tissue residency phase, cells were restimulated in various combinations of TCR stimulation (anti-CD3 and anti-CD28 T cell activation beads) with or without IL-1β and/or IL-23 for a further three days, after which cell culture supernatant was collected for ELISA.

For experiments involving BRD1 knockout, resting isolated memory CD4+ T cells were transfected with Cas9 and guide RNA for BRD1 or AAVS control and were rested for a further three days to allow for complete protein degradation. These cells were then used as input for the TRM17 model as described above. To model antigen re-call, BRD1-KO cells were re-stimulated with anti-CD3 and anti-CD28 T cell activation beads (TAB, Miltenyi Biotec) and cytokines (IL-1β and IL-23, 20ng/ml, from PeproTech) for 3 days before harvesting supernatant for cytokine secretion analysis using ELISA.

BRD1 knockout

To precomplex Cas9/RNP, 6 ul 30 µM guide RNA (AAVS1 and BRD1 from IDT) and 2 µl 30 µM TrueCut Cas9 Protein v2 (catalog number A36499; Thermo Fisher Scientific) were gently mixed by pipetting up and down in a PCR strip and incubated at room temperature for 20 min.

For nucleofection, 200 µl complete R10 media per well of a 96-well plate was

prewarmed. 1 million CD4+ T cells were re-suspended in 20 µl primary cell nucleofection solution (P2 Primary Cell 96-well Nucleofector kit, Lonza) and mixed with 8 µl precomplexed Cas9/RNP. The cell/RNP mix was transferred to Nucleofection cuvette strips. Cells were electroporated using a 4D nucleofector (4D-Nucleofector Core Unit: Lonza, AAF-1002B; 4D-Nucleofector X Unit: AAF-1002X; Lonza) with the EH100 pulse condition. After nucleofection, cells were moved to prewarmed R10 media and cultured for 3 days before the TRM17 assay.

Patient and Public Involvement

We will actively engage the patients with SpA through the Oxford Patient Engagement Network for Arthritis and Musculoskeletal Conditions (OPEN ARMS, https://www.ndorms.ox.ac.uk/get-involved/open-arms-1/open-arms) and the Botnar AxSpA day. We will discuss with patients about the impact of findings from this study and co-develop a dissemination plan to maximize the potential benefits of our findings to patients.

Additional methods

Information for Th17 expansion assay for the screen of epigenetic inhibitor library, siRNA screen of the targets of OF-1, intracellular cytokine staining (ICS) and enzyme-linked immunosorbent assays (ELISA) are available in **supplementary methods**.

RESULTS

Tissue resident memory CD4+ T cells are the main source of *IL17A* in SpA joint tissue

To investigate the cellular origin of IL-17A in the inflamed joints of SpA patients, we analyzed synovial tissue from 5 AxSpA patients and 6 PsA patients. We conducted single-cell transcriptomic profiling of these tissues, capturing a total of 85,307 cells which were categorized into T cells, natural killer (NK), mast and innate lymphoid cells (ILCs), B and plasma cells, myeloid cells, stromal cells, and endothelial cells (Figure 1A). Within this cellular milieu we found almost all *IL17A*+ cells are localized within the *CD4*+ T cell subset (Figure 1B). Notably, the vast majority of the *IL17A*+ cells did not express *CD8A* (encoding the CD8 protein), *TRAV1-2* (encoding the T cell receptor α-variable 1-2 protein, abundant in mucosal-associated invariant T [MAIT] cells), or *TRDC* (encoding the delta constant region of the T cell receptor, characteristic of γδ T cells) (Figure 1C). As ILCs especially ILC3 cells are known to be capable of producing IL-17, we investigated further into ILCs via the re-clustering of the cluster that contains NK, mast and ILCs (Fig S1A). Albeit expressing high levels of *RORC* and *IL23R, IL7R*+*KIT*+ (ILCs) did not express detectable *IL17A* nor *IL17F* (Fig S1B). In line with previous findings [14], the expression of *IL17F* is not limited to T cells and extend to stroma and endothelial cells (Fig S2). This finding strongly argues that αβ CD4+ T cells, rather than γδ T, MAIT or CD8+ tissue-resident memory T cells, as the principal source of *IL17A* in the SpA synovium.

To further characterise these cells, we carried out comparative gene expression analysis and revealed 478 upregulated and 373 downregulated genes in *IL17A+CD4+* T cells compared to *IL17A-CD4+* T cells (Figure 1D). As expected, previously established gene markers for Th17 cells (*IL17A, RORC, IL23R and CCR6*) were significantly increased. Interestingly, *IL17A+* CD4+ T cells also expressed a higher level of *CXCR6*, a marker for tissue-resident memory T cells. Notably, CXCR6+ Th17 cells have recently been identified to drive the pathology in skin, kidney and brain inflammatory diseases [6,8,9]. To test if the *IL17A+* CD4 T cells in SpA joints are tissue resident memory T cells, we further assessed the expression of classical markers for tissue-resident and circulating T cells (Figure 1E). Compared to other T cell subsets, *IL17A+* CD4+ T cells demonstrated higher expression levels of *CD69* and *ITGA1* (encoding CD49a protein) in addition to *CXCR6*. Notably, the increase of tissue residency markers was accompanied by the reduced expression of circulating markers (*S1PR1*, *SELL*, and *CCR7*), supporting their classification as tissue-resident memory (TRM) cells. We did not observe an enrichment of *ITGAE* (encoding CD103) on these cells, consistent with the restriction of CD103 to mucosal TRM cells[15] and the lack of its expression in TRM17 cells found in kidneys of patients with ANCA-associated glomerulonephritis[8].

To further confirm these findings, we carried out spatial transcriptome analysis of joint synovium tissue samples from 4 PsA patients using CosMx Spatial Molecular Imaging technique (Figure 1F and G). We scanned 80 fields of view (FoVs) with a multiplex panel of 1000 genes (CosMx™ Human Universal Cell Characterization RNA Panel). We annotated cells with high-quality data based on canonical markers, and identified adipocyte, B, endothelial, sub-lining fibroblast, lining fibroblast, myeloid, CLEC10A+ myeloid, plasma, *IL17A+* T, CD4+ T, CD8+ T, and mural cells (Figure 1F, G, and H). In line with the findings in our scRNA-seq analysis (Figure 1 A-E), *IL17A+* T cells are *CD4+* with features of TRM cells (Figure 1 H). Taken together, the scRNA-seq and spatial transcriptome analysis of synovium tissue strongly argue that TRM17 cells are the main source of *IL17A* in SpA joints.

Both sublining and lining fibroblasts in SpA joint tissue exhibit an enhanced IL-17 response signature

The induction of a pro-inflammatory state in fibroblasts has been postulated as a critical mechanism underlying IL-17-mediated chronic inflammation. Within the synovium, sub-lining and lining fibroblasts are two distinct subsets that together constitute synovial fibroblasts. Through spatial transcriptomic mapping of SpA synovium, we observed that *IL17A+* T cells were predominantly localized in the sublining regions (24 of 27 *IL17A+* T cells) (Figure 1F and G). This prompted us to investigate whether sublining and lining fibroblasts from SpA synovium have been influenced by TRM17. To this end, we integrated a publicly available dataset of fibroblasts from healthy

synovium[16] with our dataset of SpA synovial fibroblasts, resulting in a combined dataset of 11,173 healthy and 27,975 SpA fibroblasts (Figure 2A). Consistent with previous studies, *THY1+PRG4-* sublining and *THY1-PRG4+* lining fibroblasts were discovered as two major subsets (Figure 2A and Figure S3). An IL-17 signalling score (indicative of response to IL-17) was then calculated using a set of genes induced by IL-17 in human synovial fibroblasts[17]. We found both sub-lining and lining fibroblast subsets in SpA exhibited an enhanced IL-17 response signature compared to their healthy counterparts (Figure 2B). Notably, established IL-17-induced effector cytokines and chemokines, such as *CXCL1*, *CXCL8*, *IL6*, and *CCL20*, were consistently upregulated in both sub-lining and lining fibroblast subsets within the SpA synovium. Thus, despite enrichment of TRM17 in sublining synovium, both sublining and lining fibroblasts show evidence of IL-17-induced activation.

We then asked if the enhanced IL-17 signature in SpA synovium fibroblasts is contributed by certain subset(s) within the sub-lining and lining fibroblast populations. To this end, we conducted a more detailed analysis of cell states within these two populations separately (Figure 2C and F). In the sub-lining fibroblasts, among eight identified subsets, a cluster (S1) marked by the expression of *CD74* and *CHI3L2* exhibited the highest IL-17 signalling score (Figure 2D). This subset was also characterized by the upregulation of pro-angiogenic genes, including *SEMA3A* and *VEGFC* (Figure 2E). In the lining fibroblast compartment, the L4 cluster displayed the strongest IL-17 signalling signature (Figure 2G) and was enriched in the expression of genes associated with oxidative stress and inflammatory responses, such as *SOD2*, *MT2A*, *RCAN1*, and *TNFAIP6* (Figure 2H).

CLEC10A+ dendritic cells are potential antigen presenting cells for TRM17 cells

Tissue-resident memory T cells typically require antigen presenting cells (APCs) for their generation and effector function. It has been well established that myeloid cells especially dendritic cells (DCs) are the key APCs to support T cell function. To identify the myeloid cellular subset that serves as APC for TRM17 cells, we extracted and re-clustered the myeloid cells from SpA tissue (Figure 1A) and identified ten transcriptionally distinct myeloid subsets: plasmacytoid dendritic cells (pDC), *CLEC9A+* DC, *CLEC10A+* DC, *TNF+* macrophage cluster 1 (M1), *TNIP3+* macrophage cluster 2 (M2), *EGR2+* macrophage cluster 3 (M3), *GIMAP+* macrophage cluster 4 (M4), *C9+* macrophage cluster 5 (M5), *PRODH2+* macrophage cluster 6 (M6), and *LYVE1+* macrophage cluster 7 (M7) (Figure 3A). We then searched for potential APCs for TRM17 using cell-cell communication analysis for all ten myeloid subsets and *IL17A+* T cells (Figure 3B). The strongest interaction with *IL17A+* cells was observed with *CLEC10A+* DCs, which expressed high levels of ligands for CD28 (*CD80* and *CD86*), produced a chemokine (*CCL20*) known to be chemotactic for Th17 cells through CCR6 and were enriched for the key cytokine for the generation of tissue

resident memory T cells (*TGFB1*, encoding TGF-β).

Activated T cells have been shown to act on myeloid cells and induce their activation [18] [19]. Thus, if *CLEC10A*+ DC cells have served as the APC for TRM17 cells, they should have received reciprocal signals from these cells and show signs of activation. To look for evidence of *CLEC10A*+ DC activation in SpA synovium, we carried out a differential gene expression analysis which revealed 541 upregulated and 90 downregulated genes in *CLEC10A*+ DCs in SpA versus healthy synovium (Figure 3C). Notably, genes associated with an inflammatory response (*RELB*, *IRF4*, and *NFKB1*) were significantly increased. Pathway enrichment analysis of these upregulated genes using the MSigDB database revealed significant enrichment in several pro-inflammatory pathways, including TNFα signalling, IL-2/STAT5 signalling, IFNγ response, and IL-6/JAK/STAT3 signalling (Figure 3D). Lastly, we observed direct physical cell interaction with *CLEC10A*+ DC in some TRM17 cells using the single-cell spatial data (Figure 3E). Taken together, these data suggest that *CLEC10A*+ dendritic cells are activated in SpA joints and are the potential APCs for TRM17.

In vitro generated TRM17-like cells are dependent on T cell receptor stimulation, but not IL-23, for IL-17A production.

We then asked what signals induce the production of IL-17A by TRM17 cells. Due to the difficulties in obtaining adequate number of TRM17 from synovial tissue for functional studies, we developed an *in vitro* model to induce TRM17-like cells. It has been shown that following immunization effector Th17 cells can give rise to lung TRM17 cells which are maintained in the tissue by the survival cytokine IL-7 following the clearance of antigens[7]. Accordingly, we generated a two-phase *in vitro* model to generate TRM17-like cells (Figure 4A). In phase I, circulating blood memory CD4+ T cells from patients with SpA were stimulated with anti-CD3 antibody, anti-CD28 antibody and Th17 expansion cytokines (IL-1β and IL-23) to generate effector Th17 cells. These cells were then cultured with TGF- β (to model the tissue niche) and IL-7 (to provide the survival signal) in the absence of T cell receptor stimulation in phase II. Using this model, we were able to induce IL-17A+CXCR6+ TRM17-like cells which were largely absent in the blood memory compartment (Figure 4B and C).

A better efficacy of IL-23 blockade has been observed in skin compared to joints [20–23]. This drove us to investigate the role of IL-23 in IL-17A production by TRM17-like cells. Figure 4D shows that T cell receptor engagement using anti-CD3 and anti-CD28 T cell activation beads (TAB) rather than cytokine stimulation (IL-1β and IL-23) induce IL-17A production in restimulated TRM17-like cells. Furthermore, the addition of IL-23 to either TAB or TAB + IL-1β conditions did not significantly enhance the production of IL-17A. Interestingly, despite the dependence on the presence of TAB, the secretion of IL-17F and IFN-γ was significantly increased by IL-23. This effect strongly argues that the lack of effect on IL-17A by IL-23 is not due to the pre-existence of IL-23 in the cell culture media. In line with this, IL-23 blockade did not reduce the

secretion of IL-17A either in TAB alone or TAB+IL-1β conditions (Figure 4E).

Identification of BRD1 as a novel epigenetic regulator of Th17/TRM17 cells

The unique epigenetic profile of CXCR6+Th17 cells reported in the murine multiple sclerosis model [9] drove us to hypothesize that epigenetic regulators could contribute to the generation of TRM17 cells. To test this hypothesis, we screened a library of 38 epigenetic inhibitors using cells from SpA patients. Due to the low-throughput nature of the TRM17 model, we performed the initial screen using a Th17 expansion assay and discovered three inhibitors (Bromosporine, PFI-1 and OF-1) that suppress Th17 responses in both intracellular cytokine staining (ICS) and IL-17 enzyme-linked immunosorbent assays (ELISA) (Figure 5A). We deprioritized the first two inhibitors as Bromosporine is a pan-bromodomain inhibitor with multiple targets and the target of PFI-1, BET Bromodomain, has already been shown to regulate Th17 cells[24,25]. In contrast OF-1, an inhibitor for the bromodomain and PHD finger-containing protein (BRPF) family [26], has not been implicated previously in Th17 response and thus was chosen for further investigation. To this end, we first used siRNA to screen all three members in the BRPF family and found both BRPF1 and BRD1 silencing inhibited IL-17A production (Figure 5B). Further transcriptome analysis showed that knockdown of BRD1, but not BRPF1, reduced both Th17 marker genes (*IL17A, IL17F, RORC and IL23R*) and TRM markers (*CXCR6 and ITGA1*) (Figure 5C). Taken together these data suggest a potential role of BRD1 in TRM17 cell generation.

We then used our TRM17 in vitro model to investigate the role of BRD1 in the generation of TRM17-like cells. As the transient nature of siRNA-mediated gene suppression is not suitable for the 12 days TRM17 model, we deployed clustered regularly interspaced short palindromic repeats (CRISPR) technology to stably knockout BRD1 (Figure 5D). The BRD1-KO CD4+ T cells were rested for three days (allowing the degradation of endogenous BRD1 protein) before being used as the input for TRM17 generation model. Figure 5E shows that, in comparison to AAVS control guide RNA, BRD1 knockout significantly inhibited the generation of CXCR6+IL-17A+ TRM17-like cells (Figure 5E). No changes in cell viability or proliferation were observed. Additionally, the restimulation of TMR17-like cells induced a lower level of IL-17A secretion in BRD1-knockout cells than the AAVS control cells (Figure 5F). Similar results were observed for IL-17F (Figure S4). In contrast to IL-17A and IL17-F, the percentage of IFN-γ+CXCR6+ cells and the secretion IFN-γ were not affected by BRD1 KO (Figure 5E and F).

Discussion

In this study, we show that CXCR6+ tissue resident memory CD4+ T cells are the main source of IL-17A in SpA joint tissue. Despite the predominant localization of TRM17

cells in the sublining regions, both sublining and lining fibroblasts in SpA joints exhibit an enhanced IL-17 response signature suggesting the diffusion of IL-17A within the tissue. We also show that T cell receptor stimulation rather than cytokine is essential for the production of IL-17A by *in vitro* induced TRM17-like cells. Lastly, we identify BRD1 as a novel Th17 regulator that contributes to the generation of TRM17-like cells in vitro.

In contrast to previous studies, we found that CD4+ TRM17 cells rather than γδ T cells, CD8+ tissue-resident memory T cells or MAIT cells are the main sources of IL-17 in SpA joints[3–5]. This discrepancy is likely due to differences in the materials studied (synovial tissue rather than synovial fluid) and the methods of IL-17 detection (non-stimulated single-cell RNA-seq rather than PMA/ionomycin-stimulated cells for intracellular IL-17 staining). We believe that our findings from *ex vivo* isolated and non-stimulated synovial tissue more accurately reflect the true biology in SpA joints.

Importantly, we found the *in vitro* generated TRM17-like cells produced IL-17A in response to T cell receptor (TCR) engagement rather than cytokine stimulation. Intriguingly, IL-23 enhanced the TCR stimulation induced production of IL-17F, but not IL-17A, suggesting that cytokine stimulation has a greater influence on IL-17F production. In line with this, IL-17F has been shown to be the dominant IL-17 isoform produced by cytokine-activated innate lymphocytes (MAIT cells, γδ T cells and ILC3s) [27,28] indicating a closer association of IL-17F with the innate response in lymphocytes.

In summary, we demonstrate for the first time that the CD4+ TRM17 population is the main source of IL-17A in SpA synovial tissue, making a strong argument that future research into IL-17A pathology in SpA should focus on TRM17 rather than circulating Th17 cells, CD8+ T cells or innate-like lymphocytes. In addition, our data suggest that targeting TRM17, the “factory” of IL-17A, has the potential to induce long-term remission, encouraging future efforts to develop new therapies to deplete TRM17 cells in SpA.

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Competing interests

This work has been partially funded by J&J to the Cartography Consortium. LC has received research support from Novartis. PB has received research support from Regeneron, Benevolent AI, Novartis and GSK. H.H.U. has received research support or consultancy fees from J&J, UCB Pharma, Eli Lilly, BMS Celgene, GSK.

Research Ethics Approval

Venous blood was obtained under protocols approved by the Oxford Research Ethics committee (ethics reference number 06/Q1606/139). Synovial tissue were obtained under protocols approved by the Oxford Research Ethics committee (ethics reference number 06/Q1606/139), South Birmingham Research Ethics committee (ethics reference number 14/WM/1109) and Ethics Committee of Shanghai Sixth People's Hospital (2024-KY-132).

Data availability

Data are available upon reasonable request.

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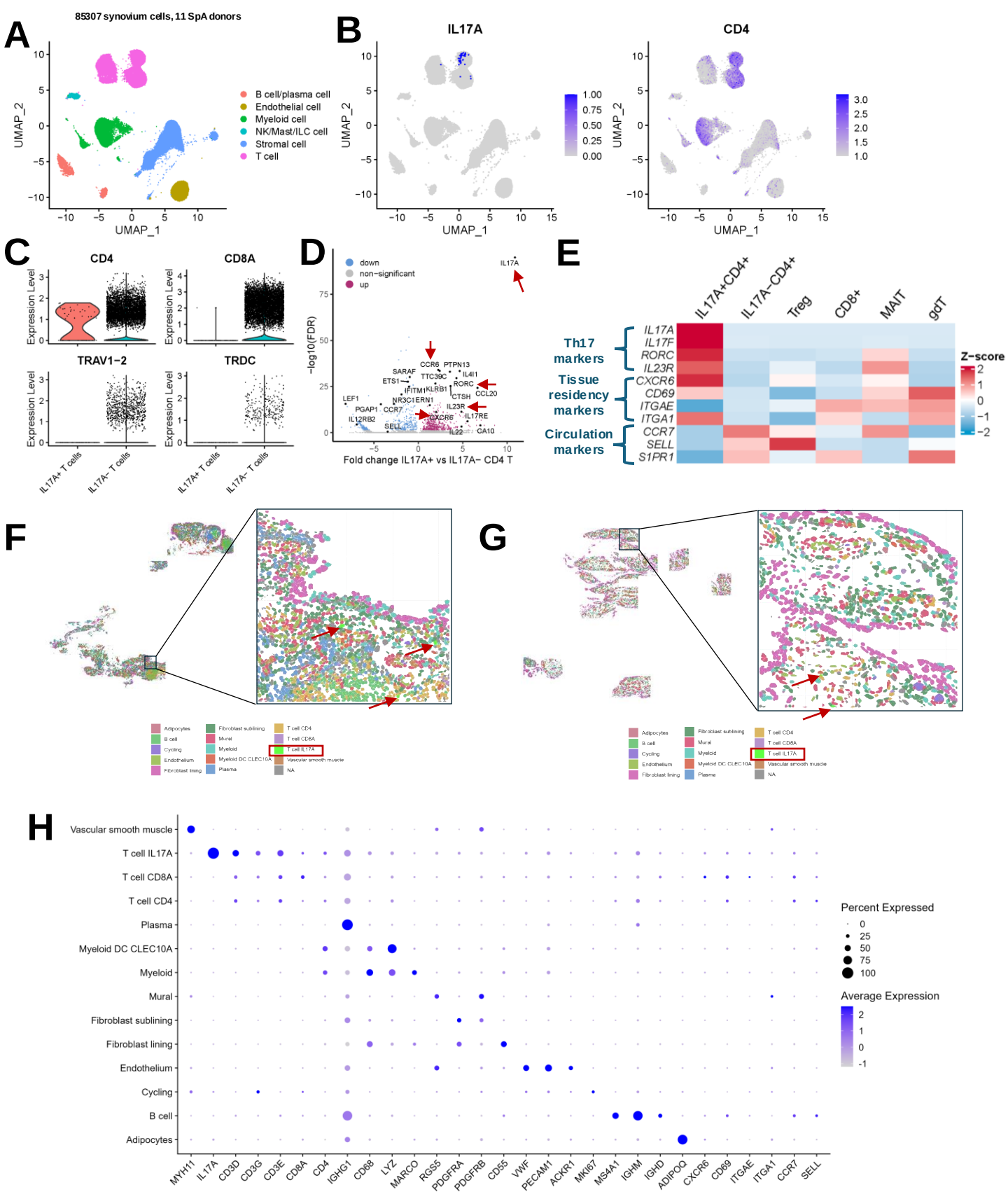


Figure 1. Tissue resident memory CD4+ T cells are the main source of IL17A in SpA joint tissue. (A) UMAP visualization of major cell types of cells from SpA synovial tissue. (B) UMAP visualization of normalized expression of IL17A and CD4. (C) Violin plot of normalized expression of CD4, CD8A, TRAV1-2, TRDC in IL-17A$^+$ and IL-17A$^-$ T cells. (D) Volcano plot of differentially expressed gene in IL17A$^+$ T cells compared to IL17A$^-$ T cells (FDR < 0.05). (E) Heatmap of normalized and scaled expression of Th17, tissue residency, and circulation signature markers in different subsets of T cells. (F-G) CosMx images showing spatial distribution of IL17A+ T cells and other cell populations in synovial tissue of two PsA patients. (H) Expression of cell type, tissue residency, circulation marker genes by IL17A+ T cells and other cell populations identified from CosMx data.

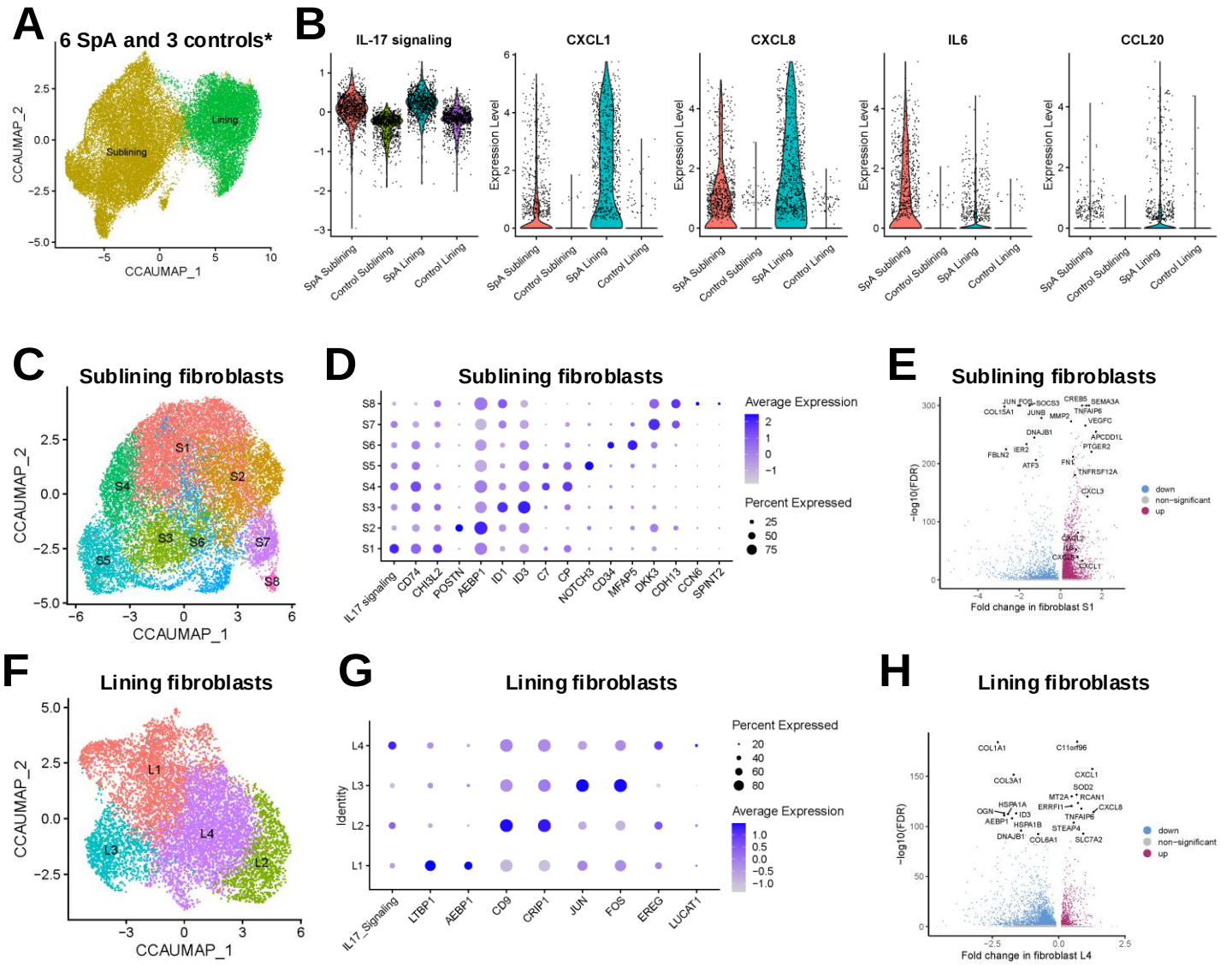


Figure 2. An enhanced IL-17 response signature is present in both sublining and lining fibroblasts from SpA synovium. (A) UMAP visualization of fibroblast subsets of combined cells from SpA and healthy synovial tissues. (B) Violin plot of IL-17 signalling score and normalized expression of four selected IL-17 signalling genes in different fibroblast subsets in SpA and healthy synovium. The data for control synovium (n=3) were sourced from GSE216651. (C) UMAP visualization of subsets of sub-lining fibroblast cells from SpA synovium. (D) Relative expression of maker genes of different sub-lining fibroblast subsets from SpA synovium. (E) Volcano plot of differentially expressed genes in S1 sub-lining fibroblast cells compared to other sub-lining fibroblast cells in SpA synovium (FDR < 0.05). (F) UMAP visualization of subsets of lining fibroblast cells from SpA synovium. (G) Relative expression of maker genes of different lining fibroblast subsets from SpA synovium. (H) Volcano plot of differentially expressed gene in L4 lining fibroblast cells compared to other lining fibroblast cells in SpA synovium (FDR < 0.05).

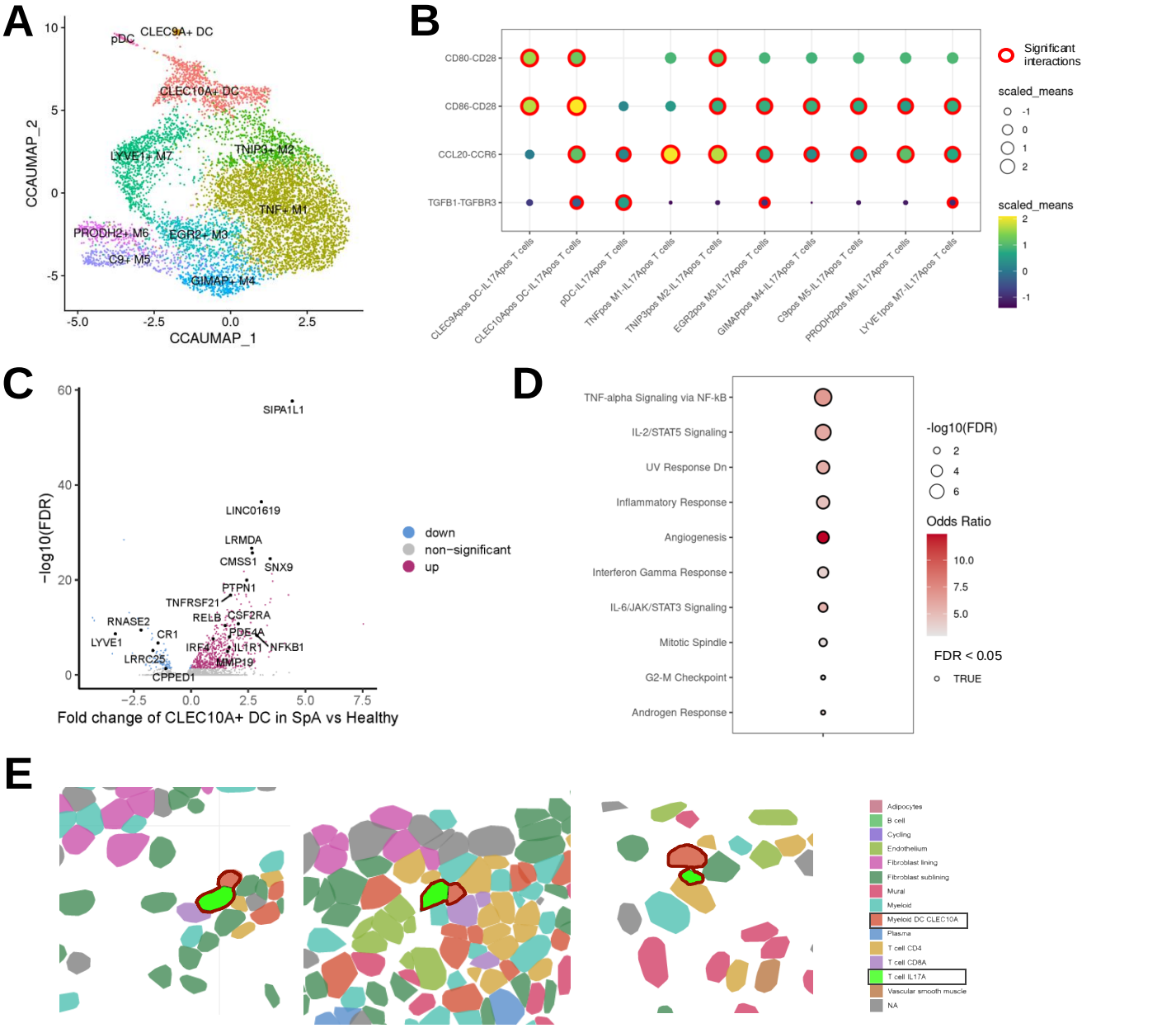


Figure 3. Cell-cell communication and single-cell spatial analysis support the interaction between CLEC10A+ dendritic cells and TRM17. (A) UMAP visualization of subsets of myeloid cells from SpA synovial tissues. (B) Cell-cell interactions between different myeloid subsets and IL17A+ T cells in SpA synovium inferred by CellPhoneDB. (C) Volcano plot of differentially expressed gene in CLEC10A+ DC cells in SpA synovium compared to healthy synovium (FDR < 0.05). (D) Enriched pathway in CLEC10A+ DC cells (All up-regulated genes were used for enrichment analysis using MSigDB database). (E) Three representative CosMx foci view of SpA synovium showing interaction between CLEC10A+ DC cells and IL17A+ cells (highlighted in red outline).

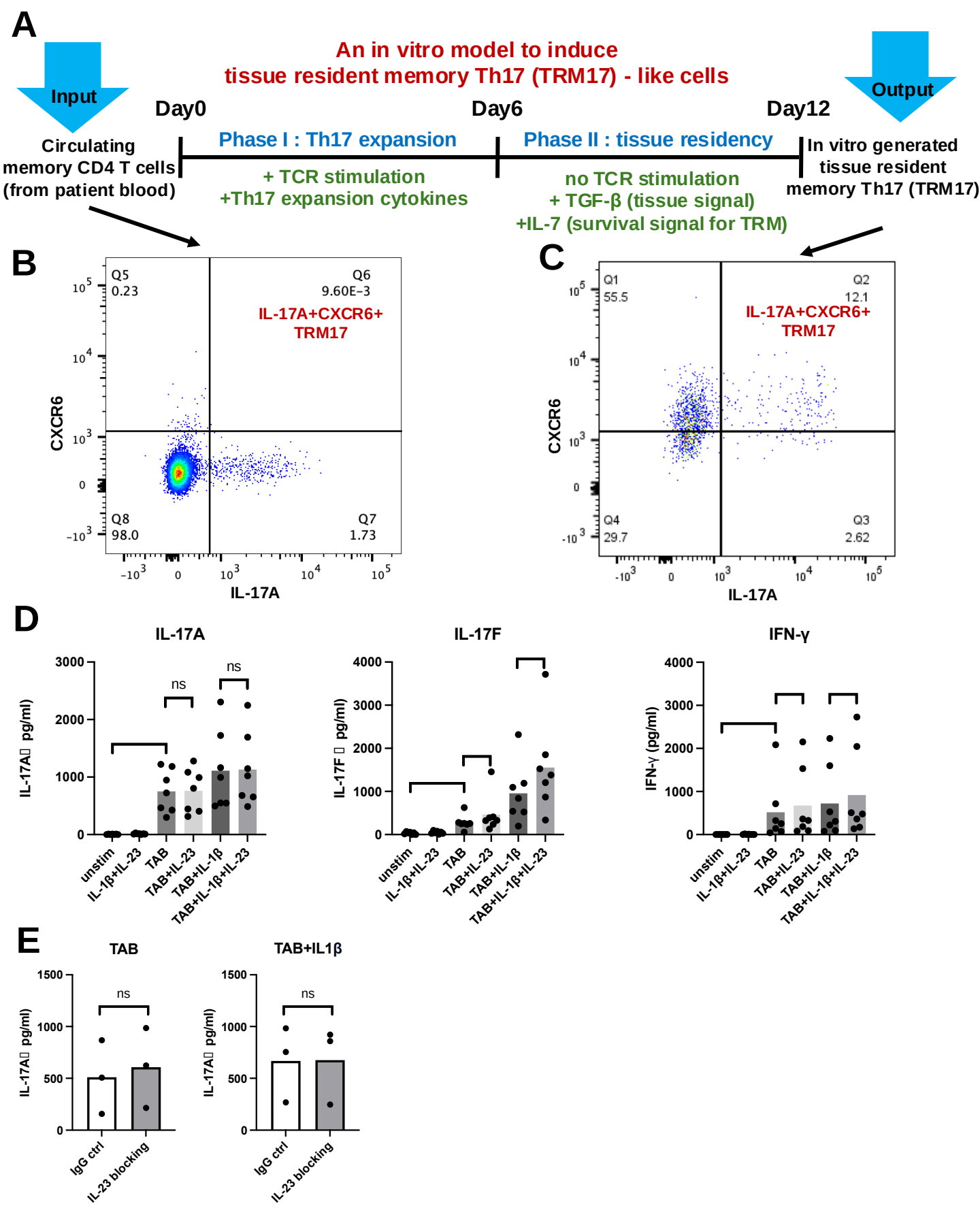


Figure 4. T cell receptor stimulation is the primary driver of IL-17A production by *in vitro* generated TRM17 - like cells. (A) Schematic representation of the in vitro TRM17 model. Assessment of IL-17A+CXCR6+ TRM17 in circulating memory CD4+ T cells (B) and induced TMR17 cells (C). (D) The secretion of IL-17A, IFN-γ and IL-17F by TRM-like cells stimulated with vehicle control, cytokines (IL-1β and IL-23), T-cell activation beads (TAB) or the combinations of cytokine and TAB. Data from 7 SpA patients are shown. (E) The levels of cytokines IL-17A produced by TRM17-like cells treated with 10 µg/ml IL-23 blocking antibody or IgG control in the presence of TAB or TAB and IL-1β. The P-value was assessed using the paired two-tailed Student's t-test (* $P \leq 0.05$).

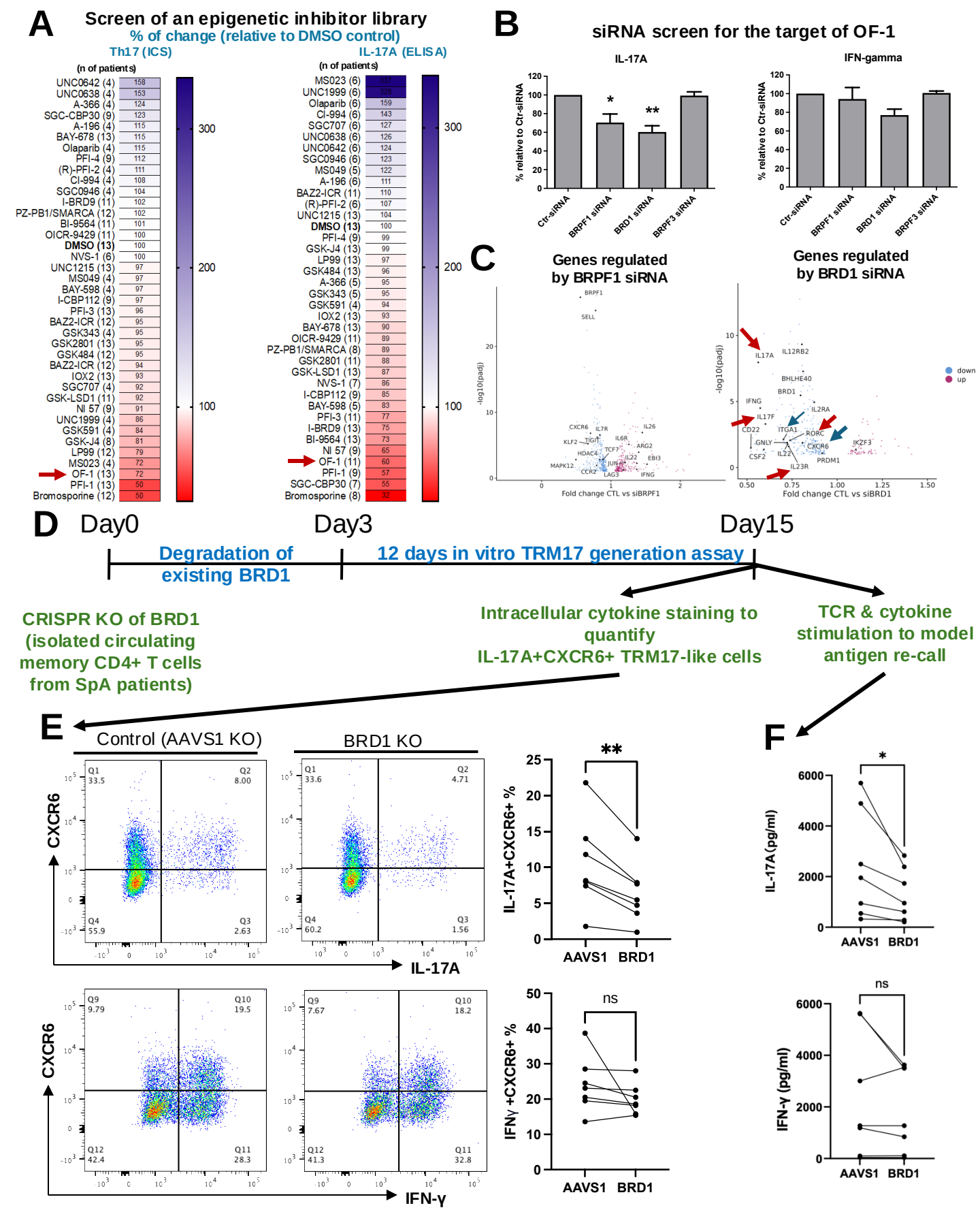


Figure 5. The epigenetic regulator BRD1 plays a role in TRM17 generation. Legend shown in next page

Figure 5. The epigenetic regulator BRD1 plays a role in TRM17 generation. A library of epigenetic inhibitors was screened using CD4+ T cells from SpA patients. Depending on the amount of blood obtained, a range of 4-13 donors were recruited for different inhibitors. The changes of Th17 frequency (based on IL-17A intracellular cytokine staining) and the level of IL-17A secretion (ELISA) relative to vehicle control (DMSO) are shown (A). (B) The respective influence of siRNAs for BRPF1, BRD1 and BRPF3 on IL-17A secretion by CD4+ T cells stimulated with T cell activation beads (TAB) and Th17-promoting cytokines (IL-1β and IL-23) (data from 3 SpA patients). (C) The transcriptome changes induced by the siRNA silencing of BRPF1 and BRD1 (data from 6 SpA patients). (D) Schematic representation of BRD1 knockout for the in vitro TRM17 model. (E and F) Effect of BRD1 knockout on the generation of TRM17-like cells and their IL-17A production upon antigen re-call (data from 7 SpA patients). The P-value was assessed using the paired two-tailed Student's t-test (* $P \leq 0.05$; ** $P \leq 0.01$).

Table S1. Patient Characteristics

Clinical data	SpA patients recruited for scRNA-seq	SpA patients recruited for spatial transcriptomics	SpA patients recruited for cellular assays
Number	11	4	12
Female/Male/Undetermined	6/4 /1	1/3/0	6/6/0
Age mean	54.8	51	48.1
CRP mean (mg/L)	17.03	15.75	5.7
BASDAI mean*	3.36	N/A	6.8
Swollen joint count 66 **	3.8	8.5	2.5
Tender joint counts 66 **	10	14.25	11.6

*BASDAI were only measure for AxSpA patients. **Swollen/Tender joint count were only measured for psoriatic arthrits patients.

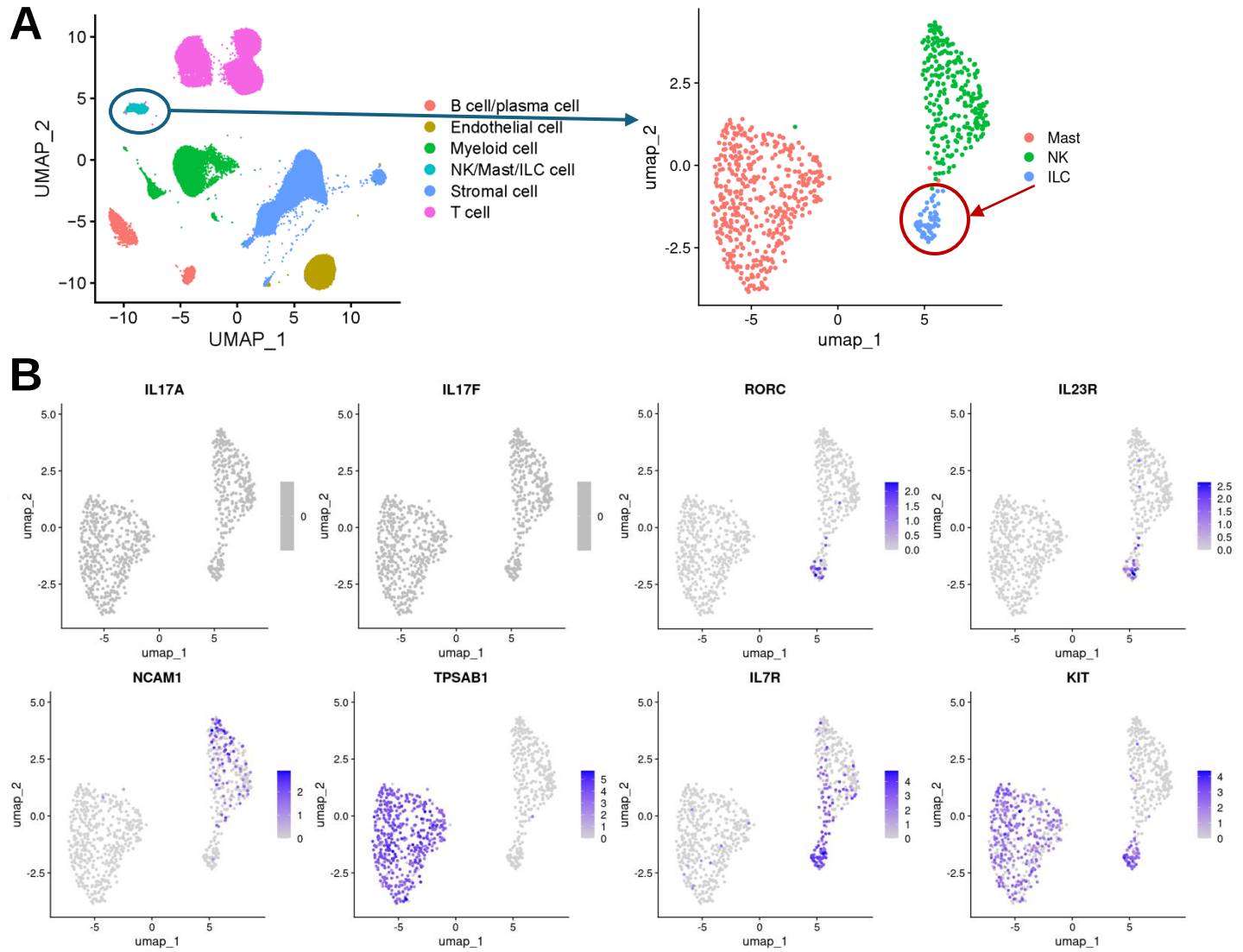


Figure S1. SpA joint tissue ILCs (NK and Mast cells) do not produce detectable *IL17*. (A) UMAP visualization of re-clustered cells from NK/Mast/ILC population from cells shown in Figure 1A. (B) UMAP visualization of normalized expression of selected marker genes.

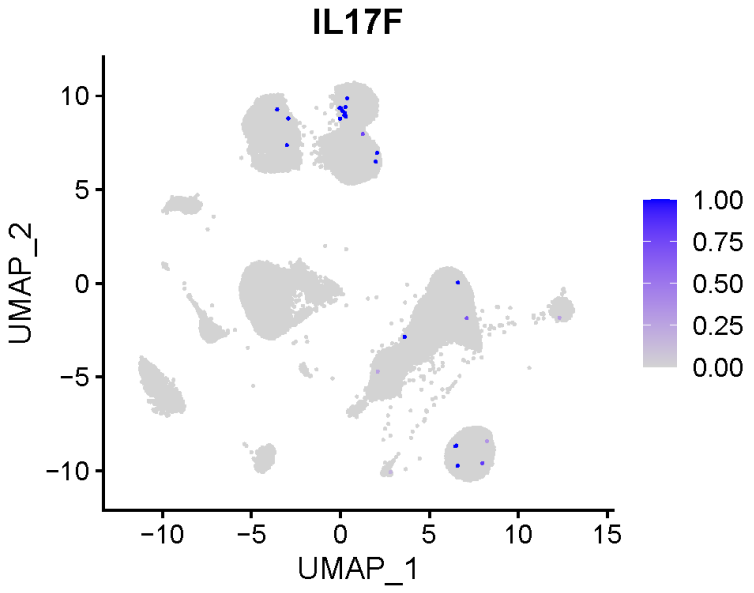


Figure S2. Multiple SpA joint tissue cell types express IL17F. UMAP visualization of normalized expression of *IL17F* by SpA synovial tissue cells.

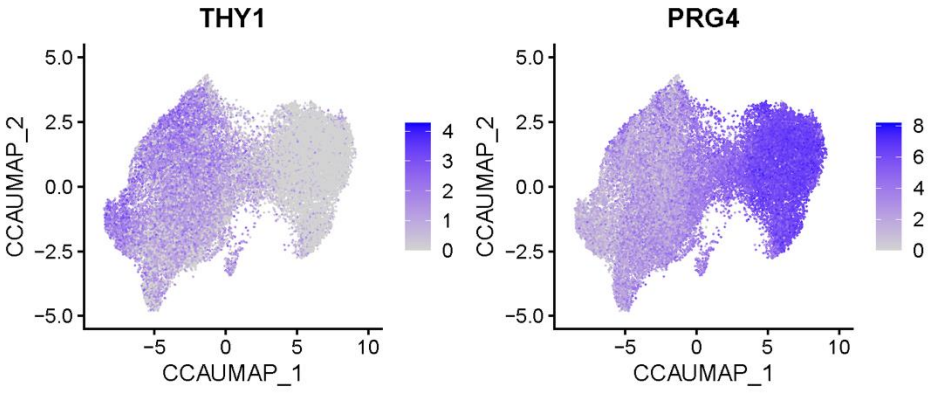
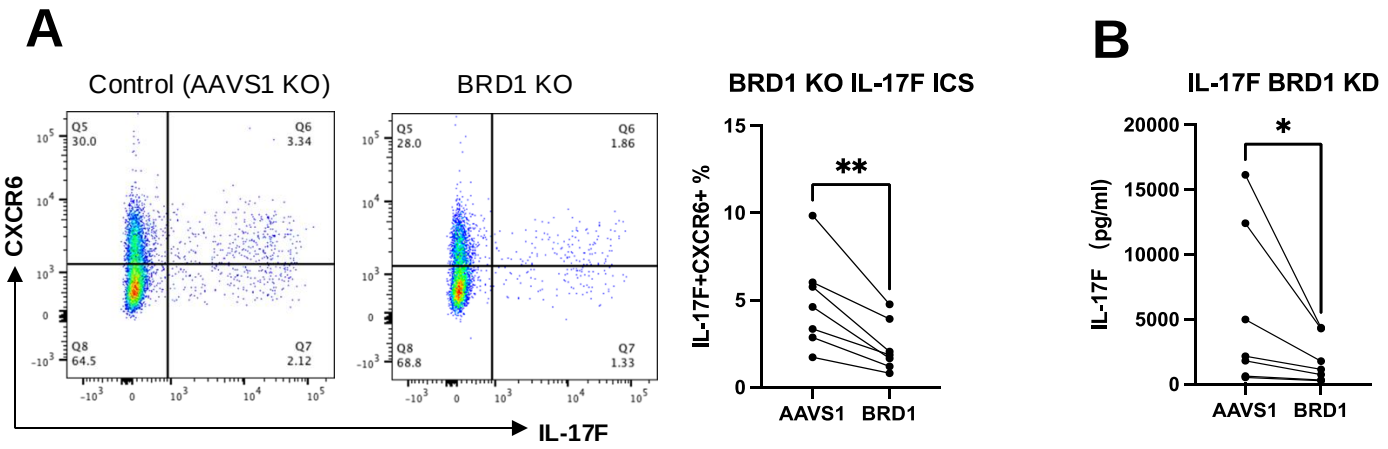


Figure S3. UMAP visualization of normalized expression of THY1 (marker for sub-lining fibroblast) and PRG4 (lining fibroblast).



Supplementary Figure 4. Inhibition of IL-17F+CXCR6+ cell frequency and IL-17F production in TRM17-like cells by BRD1 KO. Effect of BRD1 knockout on the generation of TRM17-like cells and their IL-17F production upon antigen re-call (data from 7 SpA patients). The changes of Th17 frequency (based on IL-17F intracellular cytokine staining) are shown (A) The P-value was assessed using the paired two-tailed Student's t-test (* $P \leq 0.05$; ** $P \leq 0.01$). The level of IL-17F secretion (ELISA) relative to vehicle control (DMSO) is shown (B). The P-value was assessed using the Wilcoxon test (* $P \leq 0.05$).