Single cell analysis of spondyloarthritis regulatory T cells identifies distinct synovial gene expression patterns and clonal fates

Davide Simone, Frank Penkava, Anna Ridley, Stephen Sansom, M. Hussein Al-Mossawi & Paul Bowness

Regulatory T cells (Tregs) play an important role in controlling inflammation and limiting autoimmunity, but their phenotypes at inflammatory sites in human disease are poorly understood. We here analyze the single-cell transcriptome of >16,000 Tregs obtained from peripheral blood and synovial fluid of two patients with HLA-B27+ ankylosing spondylitis and three patients with psoriatic arthritis, closely related forms of inflammatory spondyloarthritis. We identify multiple Treg clusters with distinct transcriptomic profiles, including, among others, a regulatory CD8+ subset expressing cytotoxic markers/genes, and a Th17-like RORC+ Treg subset characterized by IL-10 and LAG-3 expression. Synovial Tregs show upregulation of interferon signature and TNF receptor superfamily genes, and marked clonal expansion, consistent with tissue adaptation and antigen contact respectively. Individual synovial Treg clones map to different clusters indicating cell fate divergence. Finally, we demonstrate that LAG-3 directly inhibits IL-12/23 and TNF secretion by patient-derived monocytes, a mechanism with translational potential in SpA. Our detailed characterization of Tregs at an important inflammatory site illustrates the marked specialization of Treg subpopulations.
Regulatory T cells (Tregs) are specialized T lymphocytes that control immune responses during inflammatory and autoimmune processes. Although Tregs are characterized by expression of the master transcription factor FOXP3 and the interleukin (IL)-2 receptor α-chain CD25, they show considerable functional heterogeneity and utilize diverse suppressive mechanisms including secretion (or sequestration) of soluble mediators, direct cytotoxicity, and contact-dependent receptor inhibition. Integrating environmental signals, they can traffic to specific target organs and adopt organ-specific gene signatures and functions, while also maintaining plasticity within tissues. Low dimensional analyses based on phenotypical markers do not fully capture the increasingly apparent functional and transcriptional variety of Tregs, potentially overlooking functional cell states that may play a role in controlling inflammation. The phenotype and transcriptional profile of Tregs is yet to be fully delineated, especially at the single-cell level, at many sites of tissue inflammation in humans, including the synovial fluid (SF) in the course of inflammatory arthritis, representing an opportunity for the study of local regulatory mechanisms.

The spondyloarthritides (SpA) are a group of chronic immune-mediated arthritic conditions characterized by inflammation of spinal and other joints. The commonest forms of SpA, ankylosing spondylitis (AS) and psoriatic arthritis (PsA), together affect ~1% of the population, and are characterized by complex immune dysregulation, largely genetically predisposed but with likely common environmental triggers. Although the role of effector immunity, and of type 17 immunity in particular, is widely recognized in SpA, the impact and phenotype of Tregs is largely unknown.

Tregs undergo thymic selection and express a unique rearranged T-cell receptor (TCR) α-β chain pair. Although Tregs specific for exogenous antigens have been described, they are thought to recognize self-peptides more frequently than conventional T cells with a resulting skewed TCR repertoire. There is evidence that TCR engagement can shape the gene signature of Tregs, but detailed analysis of Tregs antigen specificity has proven challenging because of their relative rarity. Nevertheless, antigen-specific modulation by Tregs could constitute a potential advancement for cell-based therapy of autoimmune diseases. Thus, a deeper understanding of the role of antigens in human Treg biology is very important.

We here report single-cell RNA sequencing of (scRNA-seq) ~17,000 Tregs from the blood and inflamed joints of patients with AS and PsA, allowing us to define an atlas of Tregs in the context of active joint inflammation. We identify functionally distinct specialized Treg clusters with unique gene expression programs and describe specific changes in transcriptional profile occurring in SF Tregs, providing insight into Treg adaptation during inflammation. Furthermore, pairing gene expression analysis with TCR sequencing, we identify clonally expanded and likely antigen-driven Tregs in the SF, and show for the first time functional heterogeneity within individual Treg clones. Among the specialized Treg subpopulations, we describe two LAG-3 (lymphocyte activation gene-3)-expressing Treg subsets (with coexistent cytotoxic and Th17-like features) and show that LAG-3 can directly control inflammatory responses in myeloid cells from SpA patients.

Results

Single-cell RNA expression profiling of Tregs from HLA-B27+ AS SF and blood reveals diverse Treg clusters. To characterize the transcriptional landscape of Tregs in patients with SpA, we used fluorescent-activated cell sorting (FACS) to isolate CD3+CD45RA−CD25+CD127low memory Tregs (see “Methods,” Fig. 1a, and Supplementary Fig. 1a) from the peripheral blood (PB) and SF of two patients with HLA-B27+ AS presenting with active knee arthritis. scRNA-seq including 5’ V(D)J) 10× Genomics technology allowed exploration of their immune TCR repertoire together with transcriptional definition. We did not include CD4 in the sorting strategy to allow us to capture all Tregs including previously described CD8+ Tregs. After careful quality control to remove doublets and low-quality cells (“Methods”), we obtained 13,397 single-cell Treg transcriptions from both PB and SF. Through sample integration and unsupervised clustering, we identified ten Treg clusters (Fig. 1b). All clusters were present in both patients (Supplementary Fig. 1b) and in both PB and SF. Although none of the clusters were exclusively found in one compartment, SF showed enrichment of Canonical, Cycling, Cytotoxic, CC4/Helios+, and interferon (IFN) signature clusters. Conversely CCR7+ and KLRB1+ Tregs were enriched in the blood (Fig. 1c). Notably, all clusters expressed the lineage-defining genes FOXP3 and IL2RA at comparable levels (Fig. 1d).

To characterize each cluster and to assist with the annotation, we performed multiple pairwise differential gene expression analyses (Fig. 1e, Supplementary Fig. 1c, and Supplementary Data 1). The largest cluster was characterized by high expression levels of canonical Treg genes including FOXP3, TIGIT, CD27, and TNFRSF18. The second biggest cluster (enriched in blood) expressed high levels of CCR7 and the transcription factors JUNB and TCFF. Other distinct cell clusters were characterized by specialized functional and lineage markers (e.g., KLRB1, which encodes CD161, or GZMA and GZMB, indicative of cytotoxic function) or cell state features (e.g., the cycling cluster or the MTRNR2L12+ cluster, whose eponymous marker is a mitochondrial dimer). Pathway analysis revealed putative functional pathways for each cluster (Supplementary Fig. 2a), including a specific cluster with strong enrichment in genes associated with IFN response.

We next analyzed, in the joint object including Tregs from PB and SF, the distribution of effector molecules, including co-inhibitory receptors, associated with different mechanisms of suppression across the various clusters. Figure 1f left hand panel shows that CTLA4 and TIGIT were highly expressed (up to 70% of cells) in multiple clusters; however, TIGIT was markedly downregulated in the KLRB1+ cluster relative to other clusters (log fold change = −1.02, p = 1.1 × 10−57). Other markers were expressed at lower levels but notably the cytotoxic Treg cluster expressed high levels not only of GMZB but also of PDCD1 and TGFBI. LAG3 (and to a lesser extent IL10) was preferentially expressed by the cytotoxic, KLRB1+ and cycling clusters (Fig. 1f, right hand panel). Spearman’s pairwise correlation analysis showed co-expression of LAG3 with IL10 (Supplementary Fig. 2c). Co-expression of ENTPD1 (CD39, which converts ATP to AMP) was also seen with CTLA4 with TIGIT (Supplementary Fig. 2c). Notably, we did not observe notable coexistence of inhibitory checkpoint markers, as commonly described for tumor-infiltrating lymphocytes. We then looked for a co-expression network in our Treg dataset, finding the tightest co-regulated gene pairs were FOXP3 and IL2RA, and TNFRSF18 and TNFRSF4 (Supplementary Fig. 2d). Overall, our data clearly demonstrate the existence of multiple Treg populations with distinct phenotype at a major site of tissue inflammation, with specific population enrichment and gene expression patterns.

Coordinated gene expression patterns characterize Th17-like and cytotoxic Treg subsets in AS joints. One cluster, which we designated “KLRB1+,” expressed not only KLRB1 (coding for CD161, a C-type lectin-like receptor, associated with Th17, MAIT, and NK cells), but also the Th17 transcription factor RORC (Fig. 2a), a broad Th17 gene module (Fig. 2b), and GPR25,
**Fig. 1** Single-cell RNA-sequencing analysis of Tregs from HLA-B27+ ankylosing spondylitis blood and synovial fluid reveals multiple distinct clusters.

**a** Experimental design of scRNA-seq of Tregs including gene expression and V(D)J TCR gene segment sequencing. PBMCs and synovial fluid mononuclear cells (SFMCs) from two patients with active HLA-B27+ AS were sampled during an arthritis flare. Figure generated using Biorender.

**b** Reduced dimensionality visualization (UMAP plot) and clustering of the transcriptome of 13,397 Tregs from peripheral blood and synovial fluid.

**c** Cell numbers from peripheral blood and synovial fluid and fractions composing each tissue. The asterisks indicate clusters enriched in either peripheral blood (CCR7+ and KLRB1+) or synovial fluid (all the others) (*p* < 0.05, z-test of two proportions).

**d** Expression (scaled log(UMI + 1)) of lineage-defining markers FOXP3 and IL2RA across various clusters, sorted by average expression from the highest-expressing cluster.

**e** Heatmap of row-wise z-score-normalized mean expression of selected marker genes, chosen among top differentially expressed for each cluster and other known markers.

**f** Distribution of genes with known T-regulatory function across various clusters. Dotplot heatmap showing average scaled expression (color) and percentage of cells (dot size) expressing the genes, split into highly expressed (left panel) and cluster-specific (right panel).
encoding an orphan G protein-coupled receptor previously associated with AS. Interestingly, this cluster had lower expression of TIGIT and IKZF2 (the gene encoding the transcription factor Helios). We confirmed that these cells were mostly Helios− and TIGITlow (Fig. 2c and Supplementary Fig. 3a) by flow cytometry of blood samples from 14 SpA patients. This population shares features with a RORγt+ Helios− Treg subpopulation described in the mouse intestine.

A second distinct effector Treg subset, which we termed “cytotoxic,” expressed multiple genes associated with cytotoxic function, including granzymes A, K, B, and H, PRF1 (perforin), and GNLY (granulysin) (Fig. 2d). This cluster was comprised of two subpopulations, largely separated by the expression of CD4 and CD8A/CD8B (Fig. 2e), and by their distinct effector programs. The CD4+ sub-cluster was additionally enriched for IL10, MAF, and CTLA4. The CD8+ sub-cluster had a more...
marked cytotoxic profile that included NKG7, GNLY, and GZMB (Fig. 2f), with FOXP3 and IL2RA expression comparable to CD4+ Tregs (Supplementary Fig. 3b). The presence of a CD8+ Treg subset expressing Granzyme B and Granzyme K in the PB and in the SF of patients with SpA, representing up to 1.5% of all the CD3+CD25+CD127low cells, was confirmed by flow cytometry (Fig. 2g). A further subset of interest, predominantly seen in SF, expressed a gene signature indicative of exposure and response to type I and type II IFNs (Fig. 2h).

PsA SF (and blood) Tregs contain similar subset identities and gene signatures to AS Tregs. To confirm the findings and validate our observations in a second cohort, we carried out a second analysis of Tregs in PsA, a related SpA condition. We used a scRNA-seq dataset previously published by our group15 of FACS-sorted memory CD4+ (CD3+CD45RA−CD45T+), and CD8+ (CD3+CD45RA−CD8+) cells isolated from the SF and PB of three PsA patients (Fig. 3a). Tregs were identified (using unsupervised clustering) in this dataset as a distinct cluster characterized by significant upregulation of FOXP3, IL2RA, and IKZF2. The raw gene expression data matrix of the PsA Treg cluster comprising 3066 cells (951 from PB and 2115 from SF) was exported for in-depth downstream analysis (“Methods”).

Six clusters emerged from the analysis, the largest of which expressed conventional Treg markers (FOXP3, TIGIT, and TNFRSF18) and HLA class II associated genes, very similar to the canonical cluster in the AS dataset (Fig. 3b, c and Supplementary Fig. 4). Each cluster was found in all three patients and they were similarly distributed in PB and SF (Supplementary Fig. 4b, c). Other identified clusters (Supplementary Data 3), similarly observed in AS, included a CCR7+ cluster, a CCR4+Helios+ cluster, a KLRB1+ cluster, characterized by the expression of GPR25, ROBC, CCR6, IL6R, IL1R, and by the downregulation of TIGIT and IKZF2 (Fig. 3d), and a CD8+ cytotoxic cluster (Fig. 3e). The presence of CD8+ Tregs, clustering with the rest of the CD4+ Tregs rather than with CD8+ Teffs, suggested that shared transcriptional regulatory signatures prevailed over the expression of lineage markers such as CD4 and CD8. Both the KLRB1+ and cytotoxic clusters from the PsA patients exhibited gene expression profiles closely matching the analogous AS populations described in Fig. 2 and indeed the two datasets could be readily integrated into a single object including cells with shared transcriptional features from all AS and PsA patients (Fig. 3f and Supplementary Data 4).

The smaller clusters, indicating rarer phenotype (e.g., IFN signature or MTRNR2L12+) that we described in Fig. 1, were not observed in the PsA dataset, potentially due to a smaller sample size. A rare cluster, strongly characterized by expression of LGALSI (L-Galectin1), was detected in the SF and blood from all three PsA patients (Supplementary Fig. 4d). A distinct cluster characterized by cell cycle-related genes was not observed, because cycling Tregs clustered together with the other cycling T cells in the original PsA dataset and were thus not found in the downstream analysis. In summary, Tregs from PsA joints and blood closely match those identified in AS, with common subsets and matching gene expression signatures.

SF Tregs upregulate inhibitory markers and show evidence of exposure to TNF and IFNs. We next compared the normalized expression of each detected transcript between the SF and PB Tregs in the AS dataset. Four hundred and one genes were differentially expressed between the two groups, of which 366 were enriched in SF (Fig. 4a and Supplementary Data 2). FACS comparison of SpA patient SF and blood confirmed higher cell surface expression of FOXP3 and CD25 (IL2RA), and also increased numbers of Tregs in the joints (Supplementary Fig. 5a, b). The tumor necrosis factor (TNF) receptor superfamily genes TNFRSF4 (OX40) and TNFRSF18 (GITR), and the chemokine CCL5 (RANTES) were also among the genes upregulated in SF. Annotation of the SF-enriched genes using the Reactome resource showed that SF Tregs’ enriched genes were involved in type I and II IFN responses, but also in TCR signaling (Fig. 4b). Interestingly, TNXIP, encoding a Thioredoxin-interacting protein that regulates redox reactions, was the strongest downregulated gene in the SF. Among the genes upregulated in PB, CCR6 and CCR7 were the most striking and consistent with their role in trafficking to the organ and lymphoid structures16,17. Analysis of individual clusters showed frequent recurrence of the same gene pathways (including IFN response pathways) across multiple Treg subsets (Supplementary Fig. 5c).

Effector genes, such as TIGIT, CTLA4, ENTPD1, TNFRSF4, and TNFRSF18, were upregulated in the SF (Fig. 4c), suggesting an activated phenotype of synovial Tregs, whereas the expression of genes belonging to the Treg core set was maintained (Supplementary Fig. 5d, e), showing that at least at RNA level, these cells remain committed to a suppressive functional program.

A similar analysis of the PsA dataset (shown in Supplementary Fig. 5g) revealed very similar genes upregulated in SF, with almost identical pathways observed including most strongly IFN and, to a lesser extent, TCR signaling.

Thus, both AS and PsA synovial Tregs show transcriptional responses related to inflammatory cytokines (particularly IFN, but also TNF), and evidence of TCR signaling (with enhanced expression genes related to suppressive function) consistent with local response to both cytokines and antigen.

Clonally expanded Tregs with identical TCRα/β sequences and distinct transcriptional features are expanded in SpA (AS and PsA) SF. The observation of the upregulated TCR signaling pathway genes in synovial Tregs suggested a possible role for cognate antigen in synovial Treg activation. To further explore this,
Fig. 3 Parallel single-cell RNAseq analysis of psoriatic arthritis blood and synovial fluid Tregs confirms regulatory subset identities and gene signatures. a Experimental workflow. The Treg dataset was obtained from ref. 15 identifying the cluster representing Tregs. Figure generated using Biorender. b UMAP visualization of 3066 Tregs from peripheral blood and synovial fluid obtained from three PsA patients. c PsA Treg clusters and their differentially expressed genes. Shown is a heatmap of row-wise z-score-normalized mean expression of selected marker genes, chosen between top differentially expressed for each cluster. d, e Differentially expressed genes in KLRB1+ cluster (d) and CD8+ cluster (e) identified in the PsA dataset compared to the remaining Tregs. Blue dots indicate p < 0.05, Wilcoxon’s rank-sum test with Bonferroni correction. f UMAP plot depicting integrated AS and PsA Treg datasets demonstrating common and overlapping identities.
we next determined clonal diversity in our two Treg datasets making use of the novel 5′ chemistry on the 10x Chromium platform to map the TCR αβ variable region in our data. Given the large dataset size, we only performed clonal enrichment analysis on clones with at least three cells present in either blood or SF. Cells were defined as belonging to the same clone if they had identical TCR α- and β-nucleotide sequences (“Methods”). Based on this assumption, we identified 13 statistically enriched clones in AS01 and 52 clones in AS02. The majority of the TCR clonotypes and the totality of those larger than 100 cells were enriched in the SF (Fig. 5a), and exclusively found in the CD4+ compartment. Next, we explored the gene expression of the SF-enriched clones compared to the PB-enriched clones (Fig. 5b and Supplementary Data 5). The upregulation of TIGIT, TNFRSF18, and LGALS1 largely recapitulates the SF signature described in Fig. 4, with the additional detection of cycling markers (TUBB and TUBA1B) and CD177. Given the prevalent localization of the Treg clones in the SF rather than the PB, in order to identify the specific transcriptional features of expanded clones, we next compared the differential gene expression of the top five SF clones vs. the remaining SF cells (Fig. 5C). Among the top upregulated genes (apart from TCR α- and β-chain variable gene transcripts, not shown), were CD177 (whose product is the glycosyl-phosphatidylinositol anchored glycoproteins NB1), SIRPG (encoding SIRPβ2), and the alarmins S100A6 and S100A4 (together with cell cycle markers). Interestingly, KLRB1, CCR6, and CCR7 were all downregulated in the enriched Treg clones (Supplementary Data 6).

Despite the smaller size of the PsA dataset, we were able to identify the presence of one markedly expanded TCR clonotype in the SF of one patient (Fig. 5f and Supplementary Data 7). No cells with the identical TCR were found in the PB. On the Uniform Manifold Approximation and Projection (UMAP) plot, the majority of the clone-sharing cells localized close to each other, prevalently in the canonical Treg cluster, although cells were found in other clusters (with the exception of the CD8+ and the KLRB1+ clusters) (Fig. 5g). The expanded PsA clone highly expressed TNFRSF9, encoding for the costimulatory molecule CD137 (also known as 4-1BB), CCR8, IL1R1, and IL1R2 (Fig. 5h).

These data show that expanded Treg clones are enriched in the SF, and that sister Treg clones selectively enter different Treg
clusters to adopt distinct gene expression modules within the inflamed joint.

A program of genes including the checkpoint inhibitor LAG3 is upregulated by a population of synovial KLRB1+ Tregs. We next wished to further investigate the expression of the checkpoint inhibitor LAG3, which we confirmed to be largely confined to the SF cytotoxic and KLRB1+ subsets in AS (Fig. 4), but also in PsA (Fig. 6a). Examination of individual Tregs expressing LAG3 confirmed the increase in the SF (Fig. 6b). LAG3-expressing Tregs presented an associated suppressive module including the coinhibitory receptor Tim-3 (HAVCR2), IL10, and its associated transcription factor MAF (Fig. 6c). We...
Fig. 5 Clonally expanded Tregs with identical TCRα/β sequences and distinct transcriptional features are expanded in the synovial fluid and are present in multiple Treg clusters. a Individual clonal expansion across peripheral blood (PB) and synovial fluid (SF) for two AS patients. Triangles represent CD4 clonotypes. Circles represent CD8 clonotypes. Scatter graphs only display clones where at least three cells are present in either PB or SF. Data points colored red show significantly expanded clonotypes (adjusted p-value ≤ 0.05). Two-sided Fisher’s exact test, Benjamini–Hochberg correction. 

b Differential gene expression of SF-enriched compared with PB-enriched clones. Genes with log fold change >0.25 (and < −0.25) are shown. Genes with adjusted p-value < 0.05 (Wilcoxon’s rank-sum test with Bonferroni correction) are colored in blue. TCR variable chain genes have been removed (full data in Supplementary Data 7). 

c Differential gene expression of the top five SF-enriched clonotypes (ranked by p-value, two-sided Fisher’s exact test, Benjamini–Hochberg correction) compared to the remaining, non-clonal SF Tregs. 

d Cluster assignment of Treg clones enriched in AS synovial fluid. 

e UMAP plots of AS Tregs showing the top five expanded AS clones in red, together with clonal numbers in PB and SF. 

f Distribution of the single expanded PsA clone on the PsA UMAP plot. 

g Distribution of the PsA clone to each PsA cluster. 

h Differential gene expression of PsA clone vs. non-clonal SF Tregs.
confirmed by FACS that CD161+ Tregs express LAG-3 upon activation more frequently than CD161− Tregs (Fig. 6d–f). Thus, LAG-3 is preferentially expressed on specialized Treg subsets as part of a suppressive module increased in the SF and LAG-3 protein is upregulated on the surface of AS CD161+ Tregs.

LAG-3 suppresses TNF and IL-12/23 production and costimulatory molecule expression by SpA monocytes. We next sought to explore whether LAG-3 binding played a functional role in the context of inflammation. As LAG-3 is a natural ligand of HLA class II, we explored its regulatory role towards inflammatory antigen-presenting cells. We tested the functional effects of LAG-3 on cytokine secretion from isolated SpA patient-derived CD14+ monocytes stimulated with lipopolysaccharide (LPS). A LAG-3 fusion protein with a human Fc portion (LAG-3-Fc) consistently inhibited monocyte production of TNF and IL-12/23 p40 (Fig. 6g, h and Supplementary Data 8). Blockade of HLA-II expression in CD161+ and CD161− Foxp3+ cells, exemplary flow cytometry plot. f Percentages of LAG-3+ cells in CD161+ vs. CD161− Foxp3+ CD4+ cells, and in CD8+ vs. CD4+ Foxp3+ cells, from AS PBMCs after activation, n = 8. * p < 0.05 unpaired non-parametric t-test. Boxplots show mean and standard error. g Representative plots of IL-12/23 subunit p40 and TNF expression in CD14+ monocytes in control conditions (left) and in the presence of LAG-3-Fc (right). h Cytokine production determined by flow cytometric intracellular staining (% of live monocytes), or by concentration in the culture supernatant (for IL-6) after LPS activation in the presence of LAG-3 (and control conditions). 1 Costimulatory monocyte surface markers changes after culture with LAG-3 fusion protein. For each marker, a representative stain from one individual (with geometric mean fluorescence intensity for each condition) is shown. Boxplots show data points (n = 5–7 AS monocytes) with minimum and maximum values and IQR (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, one-way ANOVA).
One study in particular described a very similar phenotype to the one we observed, characterized by markers such as CC4L4 and LAG3, and a stable expression of FOXP3 [42]. The chemokines CC4L4 and CC5 (also known as RANTES, among the top upregulated genes in the SF), are chemottractants for effector T cells that express their ligand CCR5 [43], possibly to regulate their function. Consistently, we previously observed upregulated CCR5 expression on effector CD8+ T cells in PsA SF [13]. The production and release of cytotoxic peptides by Tregs has been described previously: perforin- and granzyme B-expressing Tregs are able to kill autologous antigen-presenting cells [31], and enhanced CD8+ cytotoxicity was recently described in the SF of AS patients [32].

Among the other clusters, upregulated genes include MALATI, linked to a suppressive function by promoting the expression of MAF and IL10 [33], MTRNR2L12 (humanin), shown to inhibit apoptosis [34] and to have neuroprotective functions [35], and ANXA1 (annexin-1), associated with resolution of inflammation [36].

We also observed a synovial Treg signature consistent with exposure to IFNs, including IGF1, MX1, and IFN6. This signature was manifested in an individual subset defined by this phenotype and in global clusters seen across all synovial Tregs. IFNs are classically produced in response to viral infections, but might in SpA reflect intracellular bacteria or bacterial products. We also found evidence of TNF exposure: TNF receptor superfamily (TNFRSF) genes were in fact among the top upregulated genes in SF Tregs. Activation of GITR (TNFRSF18), OX40 (TNFRSF4), and TNFR-RII (TNFRSF1B) in Tregs, via nuclear factor-κB, is known to provide crucial survival and stability signals [36], and maintain FOXP3 demethylation [37]. These changes could provide environmental adaptation and survival in a TNF-rich environment such as the SF, while preserving, or even enhancing, their suppressive function. In parallel, expression of these genes may constitute a (nonspecific) tissue module or signature, as shown in a recent scRNA-seq analysis of murine non-lymphoid tissue Tregs [38].

Use of the 5′-scRNA-seq technology here allowed us to study the individual TCR α-β pairs of over 16,000 AS and PsA Tregs. We observed selective expansions of individual sister clones in the joints of both AS patients and one of the PsA patients studied (noting the smaller numbers of PsA Tregs may have limited our ability to detect statistically significant expansions). Individual sister clones with identical TCR α-β nucleotide sequences were detected within different Treg clusters, showing that entry into these populations is not fixed or determined at an early developmental stage, and that single clones can have divergent fates. This divergence of fate has been described previously for effector T cells [39] but, to our knowledge, not previously been described for Tregs. Moreover, it is also non-stochastic, as evidenced by selective enhancement within specific subsets and the virtual absence of clones within the KLRB1+ subset.

Antigen contact via the TCR is an incompletely understood feature of Treg biology, and identification and characterization of antigen-specific Tregs in humans has proven difficult, in part because of their relative rarity in blood. We show that the TCR repertoire of SF Treg cells is diverse and distinct from PB Treg cells, with multiple significant clonal expansions, which are unique to the synovial compartment. This suggests that synovial Tregs may have expanded upon contact with an antigen (possibly of self or microbial origin) after an encounter in another body site or directly in the joint. Significantly expanded clones showed increased expression of markers including CD177, a ligand of PECAM1 associated with neutrophil function but recently observed in breast cancer-infiltrating Tregs [40], and SIRPγ, whose product mediates adhesion to antigen-presenting cell by binding to CD47 potentially optimizing antigen presentation [41]. In the PsA clone, we observed upregulation of genes such as CCR8, IL1R1/2, MAGEH, and LAYN. These markers have been associated to highly suppressive features [42,43] or strongly characterizing intratumoral Tregs in two independent studies [44,45]. Finally, TNFRSF9, encoding for the costimulatory molecule CD137 (also known as 4-1BB), has been reported to identify antigen-specific Tregs in immune-driven conditions [46,47].

Intrigued by the selective and marked upregulation of LAG3 expression in two SF Treg clusters, we explored its functional role in modulating inflammatory responses. LAG-3 (also known as CD223) is a transmembrane protein, which shares 20% homology at the amino acid level with CD44, and it binds a common ligand, the HLA class II molecule. Expressed on a number of T-cell subsets (including Tregs), because of its expression by tumor-infiltrating lymphocytes [48] often in association with exhaustion markers, LAG-3 has become an important potential target for immunotherapy [49]. Although being studied as a new possible checkpoint inhibitor to treat solid tumors, its role and its mechanism of action on Tregs are not clear. LAG-3 is expressed by Tregs upon activation and conditional LAG3-knockout Tregs exhibit reduced functionality [50], suggesting it might confer suppressive activity. Although the first reports studying LAG-3 binding to myeloid cells observed dendritic cell (DC) activation [51], one following report described downregulation of CD86 on bone marrow-derived DCs following major histocompatibility complex class II binding by LAG-3-expressing Tregs. LAG-3 on Tregs was also highlighted in an innate-like cell 3-driven experimental inflammatory model of colitis, where LAG-3+ Tregs specifically targeted CX3CR1+ macrophages, decreasing their production of IL-23 and IL-1β, ameliorating disease [52]. Interestingly, CX3CR1+ macrophages are increased in many inflamed tissues in AS, including the intestine [53]. Together, these lines of evidence indicate that LAG-3 might represent a suppressive mechanism in gut Tregs, which have the potential to traffic to other sites, perhaps in response to Th17-driven inflammation. Our description of enhanced LAG-3 expression in SpA SF (Tregs) is we believe the first in human inflammatory arthritis.

Our data confirm the co-expression of LAG3 and IL10, suggesting they are part of a coordinated suppressive program specific for tissue immunity. Interestingly, IL-27 has been reported to promote simultaneous LAG-3 expression and IL-10 production in murine and human T cells [54,57]. Supporting a potential application of the LAG-3-mediated suppression in inflammatory arthritis, its natural target, the HLA class II complex, is highly expressed on inflammatory synovial monocytes [58], DCs [59], and macrophages [60]. Although we have not directly confirmed the inhibitory capacity of LAG-3+ Tregs, we have shown that LAG-3 inhibits production of TNF and IL-12/23, and induces down-regulation of activation markers CD40, CD80, and CD86 on myeloid cells. TNF and IL-12/23 are key inflammatory cytokines whose inhibition has been shown to be of therapeutic efficacy in SpA; hence, our data support a LAG-3-based therapeutic approach for SpA and related inflammatory-mediated diseases. The inhibitory mechanism we describe could additionally have a role in limiting Th17 responses.

Although we do not know whether these populations are specific to synovial Tregs (or indeed SpA joints), we identify specialized subsets including one expressing KLRB1 and LAG3 (which we show can suppress SpA monocyte inflammatory responses) akin to an intestinal mouse Treg subtype and a cytotoxic Treg population that includes a CD8+ subset. Although previous studies have demonstrated a functional role of CD8 Tregs in tissue immunity [61-63], a weakness of our current study is that we have not directly shown suppressive function of these cells: future studies should address whether and how different Treg subsets exert their suppressive function in the synovial...
environment and whether these mechanisms can be harnessed therapeutically. Another limitation of our study is the limited number of patients studied with scRNA-seq. We mitigated this by studying a large number of cells and confirmed our findings across two independent datasets and additionally verified the key findings using FACS analysis of additional patients.

In conclusion, we present a large human Treg dataset in the context of inflammation, which shows distinct Treg subsets and identifies a broad transcriptional profile upregulated across all synovial Tregs. TCR analysis shows that sister clones can specifically enter different subsets and provides evidence of Treg clonal expansion, which may be driven by antigen. Our in-depth characterization of Treg subsets shows specific and coordinated expression of LAG-3 on certain Treg subsets. Demonstration of LAG-3 function allows us to identify potential therapeutic approaches, both for autoimmune diseases (mimicking Treg functions) or malignancy (by inhibiting Treg functions).

Methods
Participant recruitment and ethical approval. Patients with AS and PsA were recruited during routine clinical care following written informed consent in accordance with the protocols approved by the Central Oxford–Christ Church Research Ethics Committee (IFIA, Immune Function in Inflammatory Arthritis: ethical reference 06/Q1606/139). All patients (Supplementary Table 1) fulfilled the disease classification criteria (respectively ASAS and CASPAR)45 and were naïve to biologic disease-modifying antirheumatic drugs (DMARD) and not on any conventional DMARD at the time of the sample. All patients with AS were HLA-B27 positive with evidence of active axial and peripheral joint involvement. Patients with PsA had large joint peripheral oligoarthritis, although none were HLA-B27 positive. SF samples were obtained during knee joint aspiration performed for therapeutic reasons.

Cell isolation and flow cytometry. SF mononuclear cell (SMNC) and PBMC were freshly isolated within 30 min of sample collection by density-gradient centrifugation using Lymphoprep (Sigma). Flow cytometric analysis was performed by washing cells twice using FACS buffer (phosphate-buffered saline (PBS) with the addition of 1% fetal bovine serum) in 96-well U-bottom plates (Corning) or round-bottom polystyrene tubes (BD Biosciences). Cells (0.2–0.5 x 10^6) per well or tube were stained. Staining buffer was prepared by adding fluorochrome-conjugated antibodies and fixable dyes (list in Supplementary Table 2) to FACS buffer and used to resuspend cell pellets for staining mixing thoroughly. Cells were then incubated for 20 min at 4 °C in the dark. Where surface staining included an antibody for LAG-3, cells were cultured overnight with plate-bound anti-CD3 (OKT3, 1 µg/ml, Biolegend) and soluble anti-CD28 (CD28.2, 1 µg/ml, Biolegend) for 37 °C. After staining, cells were washed twice with 200 µl FACS buffer and resuspended in 200 µl fixing buffer (PBS with the addition of 3% paraformaldehyde) before acquisition. When staining for intracellular proteins, after completing the staining for surface markers as described above, cells were first permeabilized by resuspending them in a fixation/permeabilization solution (Cytofix/Cyperm, BD Biosciences) at room temperature for 30 min, then washed twice in 200 µl PermWash buffer (BD Biosciences), and finally stained for intracellular proteins before being suspended in fixing buffer for the acquisition. For the detection of intracellular cytokines in monocytes, Bredfeldin A (GolgiPlug, BD Biosciences) was added before staining. When staining T cells for Foxp3 or Helios, a variation of the intracellular staining protocol was adopted, using, instead of Cytofix/Cyperm and PermWash, the equivalent products in the FOBX3/TF Staining Buffer Set (ThermoFisher). Sample acquisition was performed on a BD LSRII Fortessa flow cytometer. Calibration and setup was performed with BD FACSDiva C8RT Beads (BioLegend). Compensation was performed using BD CytoComp eBeads and the VIbbTools plugin (VIbbTools) during primary cells. Results were analyzed using FlowJo software (v. 10.6.2, Treestar). Dimensionality reduction of flow cytometry data and t-SNE plot generation was obtained using the “t-SNE” FlowJo plugin.

Fluorescence-activated cell sorting for scRNA-seq. After isolation by density centrifugation, PBMC and SFMC were immediately stained with fluorescein-conjugated antibodies in RNAse-free PBS, 2 mM EDTA, and then FACS-sorted prior to droplet-based scRNA-seq. AS samples were stained with the following antibodies: CD3-PerCP-Cy5.5 (OKT3), CD8a-PE (RPA-T4), CD45RA-PE/Dazzle (HI100), CD25-PE (BC96), and CD127-PE/CY7 (A019D5) (all from Biolegend, and used at 1 : 50 dilution) and Fixable Viability Dye eFluor520 (eBioscience, dilution 1 : 250) to exclude dead cells. Cells were sorted on a Sony SI800Z. Memory Tregs were sorted as in Supplementary Fig. 1A (CD49α/β (negative) CD3D/CD25 (-) CD127low). Cells were then collected in a collection buffer (Phenol Red-ve RPMI + 4% bovine serum albumin + Hepes 25 mM). After sorting, cells were stained separately with Fc blocker (concentration 1 : 20) (TruStain FCX, Biolegend), rested for 15 min, then washed, then resuspended in buffer to be further stained with the Cytofix/Cyperm tagged TotalSeq–CD251 Hashtag antibody. Cells were again washed twice with FACS buffer then kept on ice until loaded onto the Chromium controller. For sample AS02, PBMC and SFMC were not processed fresh but thawed after being cryopreserved in liquid nitrogen. For PsA samples, T cells were sorted and prepared for sequencing as previously described15.

10x Genomics single-cell RNA library preparation. Cells were counted and loaded into the chromium controller (10x Genomics) chip following the standard protocol for the chromium single-cell 5’ Kit (10x Genomics). The total time taken from sample retrieval to upload on the chromium chip was 4 h. A cell concentration was used to obtain an expected number of captured cells, ~15,000 cells per sample. All subsequent steps were performed based on the standard manufactured protocol. Libraries were pooled and sequenced on HiSeq 4000 lanes to obtain a read depth of ~30,000–40,000 reads per cell for PB and SF gene expression libraries of both patients, and 6000 or 20,000 reads per cell for V(D)J-enriched T-cell libraries from both PB and SF for patients AS01 and AS02, respectively (Supplementary Table 3). Chromium 10x V(DJ) single-cell sequencing data were mapped to the software package Cell Ranger (v.2.1 for the PsA samples and v.3.1 for the AS samples) against the GRCh38 reference provided by 10x Genomics with that release.

Dataset integration. The analytical strategy used to integrate data from different tissue samples and across different experiments uses the Seurat v.3 pipeline66. QC-filtered matrices from all patients were individually normalized using the “SCTransform” function before running the “SelectIntegrationFeatures” function to determine the top 3000 variable genes. TCR genes were then excluded from these variable features and the matrices integrated according to the standard Seurat version 3 SCT integration pipeline (“PrepSCTIntegration,” “IntegrateData,” “IntegrateBatch”)66. TCR genes were excluded from variable features to prevent downstream clustering based on clonality, which can differ between patient cohorts, and potentially distort clustering based on cell phenotype. A Treg cluster identified using the “CTRL” function in CellRanger (10x Genomics, v. 2.1 for PsA samples, and v. 3.0.2 for AS samples). All five samples had similar coverage in terms of unique mRNA molecules and genes represented, altogether surveying a total of 33,694 genes (sequencing metrics detailed in Supplementary Table F1). The generated consensus annotation file for each patient and sample type (blood or SF) were then used to construct clonality tables and input files for further downstream analysis using the jsoilite (v. 1.6.1) package.

Dataset integration. The analytical strategy used to integrate data from different tissue samples and across different experiments uses the Seurat v.3 pipeline66. QC-filtered matrices from all patients were individually normalized using the “SCTransform” function before running the “SelectIntegrationFeatures” function to determine the top 3000 variable genes. TCR genes were then excluded from these variable features and the matrices integrated according to the standard Seurat version 3 SCT integration pipeline (“PrepSCTIntegration,” “IntegrateData,” “IntegrateBatch”)66. TCR genes were excluded from variable features to prevent downstream clustering based on clonality, which can differ between patient cohorts, and potentially distort clustering based on cell phenotype. A Treg cluster identified using the “CTRL” function in CellRanger (10x Genomics, v. 2.1 for PsA samples, and v. 3.0.2 for AS samples). All five samples had similar coverage in terms of unique mRNA molecules and genes represented, altogether surveying a total of 33,694 genes (sequencing metrics detailed in Supplementary Table F1). The generated consensus annotation file for each patient and sample type (blood or SF) were then used to construct clonality tables and input files for further downstream analysis using the jsoilite (v. 1.6.1) package.

Quality control. Downstream analysis of the count matrices was carried out using R (version 3.6.1) and the Seurat package (v. 3.1.4). After cell-containing droplets were identified, gene expression matrices were first filtered to remove cells having >10% mitochondrial gene transcripts, <250 or >4000 genes expressed or >25,000 UMI (unique molecular identifiers). The Seurat demultiplexing function (“HTODeindex,” with a threshold set at the 99th quantile of the negative binomial distribution for the oligo) was then used to demultiplex the hashing library in order to identify Tregs and to remove doublets. Cells were further filtered to exclude cells not expressing any transcripts from CD3 complex-associated genes (CD3E, CD3D, and CD3G) and TCR and multiplets (defined as cells with >1 TCR β-chain or >2 TCR α-chains).

To further remove any CD14+ cells or multiplets that may have escaped exclusion by cell sorting, a preliminary round of dataset integration, dimensionality reduction and cell clustering as described below was used to identify cells belonging to CD14+ clusters. These cells, along with any additional cells expressing CD14, were then excluded from the input used in generating a final integrated dataset.

Dataset integration. The analytical strategy used to integrate data from different tissue samples and across different experiments uses the Seurat v.3 pipeline66. QC-filtered matrices from all patients were individually normalized using the “SCTransform” function before running the “SelectIntegrationFeatures” function to determine the top 3000 variable genes. TCR genes were then excluded from these variable features and the matrices integrated according to the standard Seurat version 3 SCT integration pipeline (“PrepSCTIntegration,” “IntegrateData,” “IntegrateBatch”)66. TCR genes were excluded from variable features to prevent downstream clustering based on clonality, which can differ between patient cohorts, and potentially distort clustering based on cell phenotype. A Treg cluster identified using the “CTRL” function in CellRanger (10x Genomics, v. 2.1 for PsA samples, and v. 3.0.2 for AS samples). All five samples had similar coverage in terms of unique mRNA molecules and genes represented, altogether surveying a total of 33,694 genes (sequencing metrics detailed in Supplementary Table F1). The generated consensus annotation file for each patient and sample type (blood or SF) were then used to construct clonality tables and input files for further downstream analysis using the jsoilite (v. 1.6.1) package.

Dimensionality reduction and clustering. The function “RunRPA” was performed on the integrated assay to compute principal components (PCs), the first 30 of which were selected, based on the Seurat elbow plot, and specified as the dims argument to the “FindNeighbors” and “RunUMAP” functions. Clusters were then

ARTICLE COMMUNICATIONS BIOLOGY | https://doi.org/10.1038/s42003-021-02931-3 | www.nature.com/commsbio
Supplementary Data 9. Sequencing data from the PsA dataset15 have been deposited in
Reactome Pathway67 repositories. The gene module score was calculated with the
command “AddModuleScore” in Seurat66.

**TCR reconstruction and analysis of clonality.** Chromium 10× V1(DJ) single-cell sequencing data were mapped and quantified using the software package Cell-
Ranger (v2.1 for the PsA samples and v3.1 for the AS samples) against the GRC38
reference provided by 10× Genomics with that release. The generated consensus
annotation files were then used to construct clonality tables and input files for
further downstream analysis. After TCR reconstruction, the proportion of cells
having the same clone was compared between sample types for each clone using a
two-sided Fisher’s exact test with Benjamini and Hochberg correction for multiple
comparisons (R Stats Package) considering all clones with three or more cells in
either SF or PB. Clonotypes were defined as cells having identical complementary
determining region 3 (CDR3) nucleotide sequences for the α- and β-chain
CDR3 sequences assigned to each cell. As it was not possible to deduce β- and α-
chain pairing for partitions with multiple β-chains, these partitions were treated as
a single clone. When analyzing both gene expression and clonality of the same cells,
partitions containing more than one β-chain or more than two α-chains were
considered multiplets and were excluded from analysis.

**Monocyte LPS stimulation.** Monocytes were isolated using a CD14+ magnetic
positive selection kit (CD14 Microbeads, Miltenyi Biotec) from patients PBMCs,
achieving a purity of 85–95%. Isolated CD14+ cells (or, in some experiments, whole
PBMCs) were plated at a concentration of 0.5 × 10^6 cells per well in 96-well round-bottom
plate. LPS (LPS-EB, InvivoGen) was added at a dose 10 ng/ml. After LPS stimulation,
cells were kept in culture overnight. When determination of intracellular cytokine production was desired, brefeldin A was added four hours
after LPS stimulation. For experiments that evaluated the effect of LAG-3 ligation on monocytes, a recombinant human LAG-3 IgG1 Fc chimera protein (R&D) at a concentration of 2.5 μg/ml was used. Recombinant human IgG1 Fc control and
anti-human HLA class II (anti-DQ-DR-DP) (clone Tu39, BD Biosciences) were also
used at 2.5 μg/ml and were added 2 h before the addition of LPS.

**Statistics and reproducibility.** Statistical analysis was performed using the soft-
ware GraphPad Prism 8.4. Data are presented in the form of box-and-whisker plots
(minimum, maximum, and interquartile range). Statistical analysis on the scRNA-
seq data was performed using the R “Stats” package or the built-in statistical tools
for each R package used. No sample size calculation was performed for RNA-
sequencing experiments. The number of cells sequenced was based on previous
published datasets. Gene expression validation of 10× 5’-data was done in a second
dataset of a related condition, obtained from a dataset generated previously by our
group15.

**Reporting summary.** Further information on research design is available in the Nature
Research Reporting Summary linked to this article.

**Data availability**
The scRNA-seq data and scTCR-seq datasets included in this study are deposited in
ArrayExpress with the accession code E-MTAB-10948. Source data used for the generation of Figs. 4a and 5a are provided in Supplementary Data 2, 5, and 7,
respectively. Data for the generation of Fig. 6b, i are provided in Supplementary
Data 8. Source data to generate Figs. 1c, d, 2c, h, 4b, 5d, g, and 6c, f are provided in
Supplementary Data 9. Sequencing data from the PsA dataset15 have been deposited in
ArrayExpress with the accession code E-MTAB-9492 and in the European Genome-
phenome Archive (EGA) with accession code EGA50000102014.

**Code availability**
All code used to analyze data and synthesize the figures is available upon reasonable
request from the corresponding authors.

**References**
1. Sakaguchi, S. et al. Regulatory T cells and human disease. Annu. Rev. Immunol. https://doi.org/10.1146/annurev-immunol-042718-041717 (2020).
2. Panduro, M., Benoist, C. & Mathis, D. Tissue Tregs. Annu. Rev. Immunol. 34, 609–633 (2016).
3. DiSpirito, J. R. et al. Molecular diversification of regulatory T cells in nonlymphoid tissues. Sci. Immunol. 3, eaat5861 (2018).
4. Simone, D., Al Mossawi, M. H. & Bowness, P. Progress in our understanding of the pathogenesis of ankylosing spondylitis. Rheumatology (Oxf) 57, vi4–vii (2018).
5. Smith, J. A. & Colbert, R. A. Review: the interleukin-23/interleukin-17 axis in spondyloarthropathies pathogenesis: Th17 and beyond: IL-23/IL-17 axis in SpA. Arthritis Rheum. 66, 231–241 (2014).
6. Lathrop, S. K. et al. Peripheral education of the immune system by colonic commensal microbiota. Nature 478, 250–254 (2011).
7. Cebula, A. et al. Thymus-derived regulatory T cells contribute to tolerance to commensal microbiota. Nature 497, 258–262 (2013).
8. Romagnoli, P., Hudrisier, D. & van Meerwijk, J. P. M. Preferential recognition of self antigens despite normal thymic deletion of CD4+CD25+ regulatory T cells. J. Immunol. 168, 1644–1648 (2002).
9. Zemmour, D. et al. Single-cell gene expression reveals a landscape of regulatory T cell phenotypes shaped by the TCR. Nat. Immunol. 19, 291–301 (2018).
10. Yu, Y. et al. Recent advances in CD8+ regulatory T cell research. Oncol. Lett. 15, 8187–8194 (2018).
11. Woo, S.-R. et al. Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T cell function to promote tumoral immune escape. Cancer Res. 72, 917–927 (2012).
12. Fergusson, J. R. et al. CD161 defines a transcriptional and functional phenotype across distinct human T cell lineages. Cell Rep. 9, 1075–1088 (2014).
13. International Genetics of Ankylosing Spondylitis Consortium (IGAS) et al. Identification of multiple risk variants for ankylosing spondylitis through high-density genotyping of immune-related loci. Nat. Genet. 45, 730–738 (2013).
14. Ohmacht, C. et al. The microbiota regulates type 2 immunity through RORγt+ T cells. Science 349, 989–993 (2015).
15. Ponzava, F. et al. Single-cell sequencing reveals clonal expansions of pro-
inflammatory synovial CD8 T cells expressing tissue-homing receptors in psoriatic arthritis. Nat. Commun. 11, 4767 (2020).
16. Bromley, S. K., Thomas, S. Y. & Luster, A. D. Chemokine receptor CCR7 guides T cell exit from peripheral tissues and entry into arteriolar lymphatics. Nat. Immunol. 6, 895–900 (2005).
17. Yamazaki, T. et al. CCR6 regulates the migration of inflammatory and regulatory T cells. J. Immunol. 181, 8391–8401 (2008).
18. Selif, E. et al. Mucosal immunology. Individual intestinal symbionts induce a distinct population of RORγt+ regulatory T cells. Science 349, 993–997 (2015).
19. James, K. R. et al. Distinct microbial and immune niches of the human colon. Nat. Immunol. 21, 343–353 (2020).
20. Gracey, E. et al. Revisiting the gut–joint axis: links between gut inflammation and spondyloarthritis. Nat. Rev. Rheumatol. 16, 415–433 (2020).
21. Chaudhry, A. et al. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. Science 326, 986–991 (2009).
22. Thaker, Y. R., Andrews, L. P., Workman, C. J., Vignali, D. A. A. & Sharpe, A. H. Treating specific LAG-3 deletion reveals a key role for LAG3 in regulatory T cells to inhibit CNS autoimmunity. J. Immunol. 200, 101.7–101.7 (2018).
23. Xu, M. et al. c-MAF-dependent regulatory T cells mediate immunological tolerance to a gut pathobiont. Nature https://doi.org/10.1038/nature25500 (2018).
24. Al-Mossawi, M. H. et al. Unique transcriptome signatures and GM-CSF expression in lymphocytes from patients with spondyloarthritis. Nat. Commun. 8, 1510 (2017).
25. Pesenacker, A. M. et al. CD161 defines the subset of FoxP3+ T cells capable of producing proinflammatory cytokines. Blood 121, 2647–2658 (2013).
26. Duurland, C. L., Brown, C. C., O’Shaughnessy, R. F. L. & Wedderburn, L. R. CD161+’ Activated T cells share a transcriptional and functional phenotype despite limited overlap in TCR repertoire. Front. Immunol. 8, 103 (2017).
27. Powolny, G. A. M. et al. Human retinoic acid–regulated CD161 + regulatory T cells support wound repair in intestinal mucosa. Nat. Immunol. 1, https://doi.org/10.1038/s41590-018-0230-z (2018).
28. Billerbeck, E. & Thimme, R. CD8+ regulatory T cells in persistent human viral infections. *Hum. Immunol.* **69**, 771–775 (2008).

29. Joosten, S. A. et al. Identification of a human CD8+ regulatory T cell subset that mediates suppression through the chemokine CC chemokine ligand 4. *PNAS* **104**, 8029–8034 (2007).

30. Patterson, S. J. et al. Regulatory T cell chemokine production mediates pathogenic T cell attraction and suppression. *J. Clin. Invest.* **126**, 1039–1051 (2016).

31. Grossman, W. J. et al. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* **21**, 589–601 (2004).

32. Gracey, E. et al. Altered cytotoxicity profile of CD8+ T cells in ankylosing spondylitis. *Arthritis Rheumatol.* **72**, 428–434 (2020).

33. Hewitson, J. P. et al. Malat1 suppresses immunity to infection through the resolution of inflammation. *Nature* **572**, 481–487 (2019).

34. Andreae, S., Piras, F., Burdin, N. & Triebel, F. Maturation and activation of lymphocytes in (CD223) as a cancer immunotherapy target. *PNAS* **116**, 6625–6633 (2002).

35. Do, J.-S. et al. An IL-27/Lag3 axis enhances Foxp3+ regulatory T cell-suppressive function and therapeutic efficacy. *Mucosal Immunol.* **9**, 137–145 (2016).

36. Perretti, M. & D’angelica, F. Macrophages are essential for the resolution of inflammation. *Nat. Rev. Immunol.* **6**, 17–27 (2006).

37. Liang, B. et al. Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. *Arthritis Rheumatol.* **72**, 1405–1413 (1990).

38. Magnuson, A. M. et al. Identification and validation of a tumor-infiltrating Treg transcriptional signature conserved across species and tumor types. *PNAS* **115**, E10672–E10681 (2018).

39. Bacher, P. et al. Regulatory T cell specification directly correlates with age in humans. *Cell* **170**, 1029–1041.e10 (2017).

40. Barchet, W. et al. LAG-3, a novel lymphocyte activation gene closely related to CD223, as a cancer immunotherapy target. *PNAS* **116**, 3874–3880 (2002).

41. Plitas, G. et al. Regulatory T cells exhibit distinct features in human breast cancer. *Immunity* **45**, 1122–1134 (2016).

42. Piroir, L. et al. Expansion of human T cells to antigen-presenting cells using SIRPβ2–CD47 interaction costimulates T-cell proliferation. *Blood* **105**, 2421–2427 (2005).

43. Barchet, W. et al. LAG-3, a novel lymphocyte activation gene closely related to CD223, as a cancer immunotherapy target. *PNAS* **116**, 3874–3880 (2002).

44. Piroir, L. et al. SIRPβ2–CD47 interaction costimulates T-cell proliferation. *Blood* **105**, 2421–2427 (2005).

45. Barchet, W. et al. LAG-3, a novel lymphocyte activation gene closely related to CD223, as a cancer immunotherapy target. *PNAS* **116**, 3874–3880 (2002).

46. Zhang, B. et al. Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. *J. Immunol.* **180**, 5916–5926 (2008).

47. Bauchet, D. et al. LAG3+ regulatory T cells restrain interleukin-23–producing CXCR1+ gut-resident macrophages during group 3 innate lymphoid cell-driven colitis. *Immunity* **49**, 342–352.e5 (2018).

48. Ciocca, F. et al. Proinflammatory CXCR1+CD49+ tumor necrosis factor-like molecule 1A+ interleukin-23+ monocytes are expanded in patients with ankylosing spondylitis and modulate innate lymphoid cell 3 immune functions. *Arthritis Rheumatol.* **70**, 2003–2013 (2018).

49. Do, J.-S. et al. An IL-27/Lag3 axis enhances Foxp3+ regulatory T cell-suppressive function and therapeutic efficacy. *Mucosal Immunol.* **9**, 137–145 (2016).

50. Pot, C. et al. Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells. *J. Immunol.* **183**, 797–801 (2009).

51. Firestein, G. S. & Zöller, N. J. Peripheral blood and synovial fluid monocyte activation in inflammatory arthritis. I. A cytofluorographic study of monocyte differentiation antigens and class II antigens and their regulation by gamma-interferon. *Arthritis Rheum.* **30**, 857–863 (1987).

52. Knights, S. et al. Class II antigens on dendritic cells from the synovial fluids of patients with inflammatory arthritis. *Clin. Exp. Immunol.* **78**, 19–25 (1989).

53. Tsark, E. C. et al. Differential MHC class II-mediated presentation of rheumatoid arthritis autoantigens by human dendritic cells and macrophages. *J. Immunol.* **169**, 6625–6633 (2002).

54. Saligrama, N. et al. Opposing T-cell responses in experimental autoimmunity. *J. Immunol.* **180**, 5916–5926 (2008).

55. Joosten, S. A. et al. Identiﬁcation of a human CD8+ regulatory T cell subset that mediates suppression through the chemokine CC chemokine ligand 4. *PNAS* **104**, 8029–8034 (2007).

56. Grossman, W. J. et al. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* **21**, 589–601 (2004).

57. Gracey, E. et al. Altered cytotoxicity profile of CD8+ T cells in ankylosing spondylitis. *Arthritis Rheumatol.* **72**, 428–434 (2020).

58. Hewitson, J. P. et al. Malat1 suppresses immunity to infection through the resolution of inflammation. *Nature* **572**, 481–487 (2019).

59. Joeris, T. et al. Intestinal cDC1 drive cross-tolerance to epithelial-derived antigen via induction of Foxp3+CD25+ Tregs. *Sci. Immunol.* **6**, eabd3774 (2021).

60. Taylor, W. et al. Classification criteria for psoriatic arthritis: Development of new criteria from a large international study. *Arthritis Rheum.* **54**, 2665–2673 (2006).

61. Rudwaleit, M. et al. The assessment of SpondyloArthritis International Society classiﬁcation criteria for peripheral spondyloarthritides and for spondyloarthritis in general. *Ann. Rheum. Dis.* **70**, 25–31 (2011).

62. Stuart, T. et al. Comprehensive integration of single-cell data. *Cell* **177**, 1888–1900.e21 (2019).

63. Fabregat, A. et al. The reactome pathway knowledgebase. *Nucleic Acids Res.* **46**, D649–D655 (2018).

Acknowledgements

We thank the patients for their participation. D.S. was funded by the Henni Mester Stединship and by the Oxfordshire Health Services Research Committee (OHSHRC, project number 1284). F.P., S.S., and P.B. were funded by Versus Arthritis (grant number 22252). H.A.M. was funded by National Institute for Health Research (NIHR), the Academy of Medical Sciences (grant number S6G18100106), and had unrestricted research grants from UCB. The study received support from the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre (BRC) (A.R. and P.B.). The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health.

Author contributions

D.S., F.P., H.A.M., and P.B. conceived and designed the experiments. D.S., F.P., and H.A.M. performed the 10x experiments, designed, and performed the computational analysis aided by S.S. and P.B. co-directed this study. All authors read and approved the paper.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s42003-021-02931-3.

Correspondence and requests for materials should be addressed to Davide Simone or Paul Bowness.

Peer review information Communications Biology thanks Lars Rogge and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Primary Handling Editors: Damon Tunes and George Inglis.

Reprints and permission information is available at http://www.nature.com/reprints
