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Arthritis flares mediated by tissue-resident memory T cells in the joint

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SUMMARY

Rheumatoid arthritis is a systemic autoimmune disease, but disease flares typically affect only a subset of joints, distributed in a distinctive pattern for each patient. Pursuing this intriguing pattern, we show that arthritis recurrence is mediated by long-lived synovial resident memory T cells (T_{RM}). In three murine models, CD8⁺ cells bearing T_{RM} markers remain in previously inflamed joints during remission. These cells are bona fide T_{RM} , exhibiting a failure to migrate between joints, preferential uptake of fatty acids, and long-term residency. Disease flares result from T_{RM} activation by antigen, leading to CCL5-mediated recruitment of circulating effector cells. Correspondingly, T_{RM} depletion ameliorates recurrence in a site-specific manner. Human rheumatoid arthritis joint tissues contain a comparable CD8⁺-predominant T_{RM} population, which

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AUTHOR CONTRIBUTIONS

M.H.C., A.L., R.C.F., and P.A.N. conceived and designed the study. K.W., L.A.H., R.A.C., and D.A.R. provided expertise and assisted with studies design. N.N.-M., R.B.B., K.D.W., A.M., S.B., A.W., and R.G.-B. performed the experiments. Y.I. provided essential reagents. M.H.C. and K.D.W. analyzed the data. M.H.C. and P.A.N. drafted the manuscript. All authors edited the manuscript.

SUPPLEMENTAL INFORMATION

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DECLARATION OF INTERESTS

R.A.C. has a pending patent application describing depletion of T_{RM} as a strategy to treat autoimmune and inflammatory diseases. The other authors declare no relevant competing interests.

is most evident in late-stage leukocyte-poor synovium, exhibiting limited T cell receptor diversity and a pro-inflammatory transcriptomic signature. Together, these findings establish synovial T_{RM} as a targetable mediator of disease chronicity in autoimmune arthritis.

Graphical abstract

In brief

Rheumatoid arthritis is characterized by recurrent inflammation of some, but not all, joints. Chang et al. show that CD8⁺ tissue-resident memory T cells accumulate in previously inflamed joints, nucleating local disease recurrence when triggered by antigens. T_{RM} depletion attenuates arthritis flares, defining a mechanistic basis for joint-specific memory.

INTRODUCTION

Rheumatoid arthritis (RA) affects millions of individuals world-wide (Cross et al., 2014). This chronic autoimmune disorder is characterized by immune-mediated inflammation within joint tissue (Chang and Nigrovic, 2019). Although arthritis can in many cases improve with immunomodulatory therapies, treatment is often lifelong, and episodic flares of joint inflammation continue to affect most patients (Prince et al., 2012).

Arthritis flares exhibit a strong tendency to recur in a restricted subset of joints. In RA, most joints that will ever be affected become inflamed within the first year of disease;

subsequent involvement of a previously spared joint is unusual (Roberts et al., 1988). This finding highlights the presence of a mechanism mediating local memory, but the nature of this mechanism remains unexplored.

Memory T cells are immune cells that develop after antigen exposure and persist for a lifetime. Resident memory T cells (T_{RM}) develop in tissues after antigen exposure, taking up long-term residence to provide local site-specific immune defense against pathogens previously encountered in these locations (Clark, 2015; Gebhardt et al., 2018; Mackay and Kallies, 2017; Szabo et al., 2019). Unlike other memory T cells, T_{RM} do not recirculate by the blood or lymphatics (Clark et al., 2012; Jiang et al., 2012). Belonging to either CD4 or CD8 T cell subsets, T_{RM} have been described in the lung, gut, brain, reproductive tract, and skin but not in synovial tissue (Gebhardt and Mackay, 2012; Gebhardt et al., 2018; Mackay and Kallies, 2017; Purwar et al., 2011; Seneschal et al., 2012; Szabo et al., 2019). They exhibit effector functions such as cytokine production and cytotoxicity, activation of local innate and adaptive immune cells, and recruitment of other effector cells from circulation (Di Rosa and Gebhardt, 2016; Gebhardt and Mackay, 2012; Schenkel et al., 2013, 2014). T_{RM} contribute to local immune defense and tissue homeostasis (Gebhardt and Mackay, 2012; Jiang et al., 2012), but they can also drive localized recurrent inflammation; for example, psoriasis and fixed drug eruptions are both mediated by T_{RM} (Boyman et al., 2004; Clark, 2015; Mizukawa et al., 2002; Park and Kupper, 2015). We sought to investigate a role for T_{RM} in recurrent site-specific joint inflammation.

Using three murine models of arthritis, we show the long-term persistence of CD8⁺ T_{RM} in arthritic joints during remission. Activation of these cells by antigens led to the recruitment of circulating lymphocytes and arthritis flare. Site-specific depletion during remission curtailed arthritis recurrence, demonstrating an essential role for T_{RM} within quiescent joints in instigating recurrent joint-specific flares. Correspondingly, we identified T_{RM} in human RA synovial tissue, most prominently in late-stage non-inflamed synovium. These T_{RM} are primarily CD8⁺ and exhibit limited T cell receptor (TCR) diversity, as well as transcription profiles consistent with immune activation and cell recruitment, suggesting a pro-inflammatory role. Together, these findings provide a mechanistic explanation for joint-restricted disease recurrence in autoimmune arthritis.

RESULTS

Generation of a murine model of recurrent synovitis

To study arthritis flare, we developed an animal model of recurrent synovitis by adapting a T-cell-driven, joint-specific model of antigen-induced arthritis (Figure 1A; Lawlor et al., 2001; Staite et al., 1990; van den Berg et al., 2007). Arthritis was established by intra-articular (I.A.) injection of methylated bovine serum albumin (meBSA) without adjuvant into one wrist, knee, and ankle of a B6 mouse, while contralateral joints were injected with vehicle (phosphate-buffered saline [PBS]) as an internal control. Mice also received interleukin-1 β (IL-1 β) injected into the footpad on either side on days 0–2. By 1 week, mice developed joint swelling restricted to the antigen-exposed joints (Figure 1B), quantified by measurement of the anterior-posterior diameter of the wrist, as the joint measured most reliably (Figure 1C). Clinical swelling subsided by 4 weeks without further intervention

but could be re-activated by intraperitoneal (i.p.) injection of meBSA, resulting in recurrent inflammation limited to previously inflamed joints, which we termed a “flare” in this model (Figures 1C and 1D). Characterization of the joint by histology revealed synovial thickening and infiltration of inflammatory cells in the antigen-exposed joint at week 1, corresponding to acute joint swelling assessed clinically (Figure 1E). Inflammatory infiltrates resolved without intervention by 4 weeks but recurred in previously inflamed joints during the flare response to a systemic antigen challenge (Figure 1E).

Arthritis mediated by meBSA depends on T cells, as RAG-1^{-/-} mice and CD4-depleted mice fail to develop disease (Lawlor et al., 2001). To investigate the immune cells involved in the arthritis flare, we assessed dissociated synovium by using flow cytometry 3 days after systemic antigen re-stimulation (day 31). We found CD19⁺ B cells, CD3⁺ T cells, CD11b⁺Ly6G⁻ monocyte/macrophage cells, and a large CD11b⁺Ly6G⁺ neutrophil population within the synovium of joints previously exposed to an antigen; these cells were rare in the contralateral joints not injected with the antigen (Figure S1). Although no clinical swelling or histological evidence of inflammation remained during remission, a population of T cells persisted within the synovium of previously inflamed joints (Figure 1F, remission). After systemic antigen challenge, the number of T cells increased markedly (Figure 1F, flare), supporting a potential role in disease reactivation.

Antigen-specific synovial CD8 T cells mediate recurrent synovitis

To determine a role for CD4 and CD8 T cells in arthritis flares, we used an adoptive transfer approach by using donor mice transgenic for ovalbumin (OVA)-specific TCR (OT-I, CD8 T cells; OT-II, CD4 T cells). We collected peripheral lymph nodes from 5- to 6-week-old CD45.2⁺ OT-I or OT-II mice, isolated CD3⁺ cells through negative selection, and transferred CD3⁺ cells intravenously (i.v.) into recipient wild-type B6 CD45.1 mice on day -1. Joints were then injected with OVA (instead of meBSA) on day 0, and during remission, the mice were challenged with i.p. OVA to trigger an arthritis flare. Of note, the inflammation generated by I.A. meBSA injection was similar to inflammation from I.A. OVA injection without prior adoptive transfer of antigen-specific cells. However, the presence of pre-existing antigen-specific T cells yielded a more robust response (Figures S2A–S2C). The adoptive transfer of antigen-specific OT-I yielded a flare response, as characterized by T cell infiltrates, whereas the transfer of antigen-specific OT-II did not, indicating dependence of the flare on CD8 T cells (Figure 1G). When OT-I and OT-II were transferred together, all donor (CD45.2⁺) T cells remaining in the synovium during remission were CD8⁺, whereas both CD4⁺ and CD8⁺ donor T cells were recruited back to the synovium during a flare (Figures 1H and 1I).

To determine whether the arthritis flare is antigen specific, we generated arthritis with intra-articular meBSA and challenged with i.p. injection of a naive antigen (OVA) or non-specific inflammatory trigger such as inflammatory cytokines (IL-1 β) or TLR ligands (lipopolysaccharide [LPS]) (Figure S2D). Only re-stimulation with the original antigen (meBSA) was able to produce an arthritis flare in the previously inflamed joint (Figure S2E). Extending this line of investigation, we generated arthritis with intra-articular meBSA and introduced OT-I during remission to confer OVA reactivity (Figure S2F). We confirmed

that adoptively transferred OT-I are widely distributed within the spleen and peripheral lymph nodes of the recipient mouse (Figure S2G). We then challenged the mice with i.p. injection of meBSA or OVA. The availability of OVA-specific T cells within local lymph nodes was insufficient to trigger joint disease, as joint inflammation recurred only with i.p. meBSA re-stimulation and not to i.p. OVA re-challenge, further confirming the key role of antigen-specific T cells remaining within the specific joint after the initial arthritis episode (Figures S2H and S2I).

These data indicate that antigen-specific CD8 T cells, recruited into the synovium during the initial inflammatory response and retained during remission, participate in joint-specific arthritis recurrence.

A population of memory T cells with a T_{RM} phenotype is present in previously inflamed joints

To identify the T cells involved in joint-specific memory, we assessed memory cell markers on synovial T cells from previously inflamed joints. Arthritis was induced by I.A. OVA or PBS injection into mice that received adoptive transfer of OT-I. After 1 month of remission, flare was triggered by i.p. OVA, and synovium was collected 3 days later for flow cytometry analysis. A t-distributed stochastic neighbor embedding (tSNE) analysis with surface markers CD3, CD4, CD8, CD44, CD62L, CD103, CD69, and ST2 lymphocytes showed a population of T cells within OVA-treated joints that was distinctly absent from PBS-injected joints (Figure 2A). These cells expressed CD8⁺CD44⁺CD62L[−]CD69⁺ CD103^{+/−}ST2⁺ surface proteins, consistent with the cellular profile of T_{RM}. Strikingly, cells expressing the T_{RM} signature (see Figure S3 for gating strategy) remained in the synovium of previously inflamed joints during remission (Figure 2B, left; Figure 2C, remission), despite the absence of joint swelling or histological evidence of inflammation (Figures 1C–1E). CD44⁺CD62L[−]CD69⁺ cells represented approximately 20% of the T cells in remission, and these T_{RM}-like cells expanded during an arthritis flare, again consistent with an active role in joint inflammation (Figure 2B, right; Figure 2C, flare).

Synovial memory T cells persist in the synovium and do not migrate in response to tissue egress signals

To establish that these synovial memory T cells are in fact T_{RM}, we assessed their persistence in tissues by using two-color fluorescent Cre-reporter mice (mT/mG mice) that express tdTomato at baseline but switch to EGFP after Cre-mediated recombination (Muzumdar et al., 2007). We induced arthritis in both knees with OVA and then injected adeno-associated virus expressing Cre recombinase (AAV-Cre) into a single knee to label the cells remaining in that joint during remission (Figure 3A). Adeno-associated viruses are not replication competent, and any circulating viral particles are cleared from the animal within 72 h, restricting labeling of cells to the single point (Kaspar et al., 2002; Reuter et al., 2012). One month after AAV-Cre injection, we assessed the synovium for EGFP-positive T cells. We found a distinct population of EGFP⁺ T cells in joints injected with AAV-Cre that expressed CD8⁺CD44⁺CD62L[−]CD69⁺, which is consistent with results of CD8⁺ T_{RM} (Figures 3B and 3C). Importantly, no EGFP⁺ T cells were found in the contralateral knee, confirming that synovial T cells remained anchored within the previously inflamed joint.

We also tested the capacity of synovial T cells to migrate in response to the tissue egress signal CCL21, a chemokine that binds to CCR7 and attracts T cells to secondary lymphoid organs. Sorted CD3⁺ T cells isolated from the synovium of mice in an arthritis flare were placed in the top of a transwell chamber, and CCL21 chemoattractant was placed in the bottom. After 2 h, cells in the top and bottom chambers were analyzed by flow cytometry. Consistent with *in vivo* results from the AAV-Cre experiment, synovial T_{RM}-like cells (CD3⁺CD44⁺CD62L[−]CD69⁺) failed to migrate across the transwell membrane, whereas synovial naive T cells (CD3⁺CD44[−]CD62L⁺) showed the expected dose-dependent migration in response to CCL21 (Figure 3D). Thus, T_{RM}-like cells from inflamed joints remain sessile both *in vivo* and *in vitro*.

Synovial T_{RM}-like cells display increased fatty acid uptake

Skin T_{RM} preferentially use exogenous free fatty acids for metabolism, a process essential to their maintenance, longevity, and function (Pan et al., 2017). To determine whether synovial T_{RM}-like cells similarly exhibit enhanced uptake of free fatty acids, we collected synovium from mice in an arthritis flare and incubated disaggregated synovial cells with Bodipy FL C16, a green-fluorescent free fatty acid. After 30 min, we assessed free fatty acid uptake by each T cell subtype by using flow cytometry. As with T_{RM} from the skin, synovial T_{RM}-like cells (CD3⁺CD44⁺CD62L[−]CD69⁺) incorporated higher amounts of free fatty acids than naive T cells (CD3⁺CD44[−]CD62L⁺) and effector memory T cells (CD3⁺CD44⁺CD62L[−]CD69[−]) (Figures 3E and 3F).

Synovial T_{RM}-like cells retain a long-term capacity to initiate arthritis flares

T_{RM} provide long-term immunity in tissues, but aberrant activity can also lead to enduring autoimmunity (Clark, 2015; Park and Kupper, 2015). To determine the longevity of synovial T_{RM}-like cells, we used the OT-I adoptive transfer method to induce arthritis in mice and waited for over 8 months of remission before challenging mice with a systemic antigen (Figure 3G). After 243 days, mice still exhibited a flare response restricted to joints that had previously been exposed to antigen (Figure 3H). Evaluation of synovial T cells showed a continued presence of CD3⁺CD44⁺CD62L[−]CD69⁺ cells in the antigen-injected joint (Figures 3I and 3J), demonstrating long-term retention and function. Together, the surface signature, metabolic features, tissue retention, and long-term persistence allow us to conclude that T_{RM}-like T cells from inflamed joints in this murine model are bona fide T_{RM}.

T_{RM} persist in remission in a spontaneous murine model of RA

Both meBSA and OVA + OT-I models of arthritis are mediated by exogenous antigen in the B6 background. We sought to identify T_{RM} in a spontaneous model within a different genetic background. BALB/c mice lacking the endogenous IL-1 receptor antagonist (IL-1Ra) spontaneously develop an intense T-cell-dependent inflammatory arthritis with age (Akitsu et al., 2015). We noted that some *IL1rn*^{−/−} mice begin with unilateral joint inflammation before accruing additional inflamed joints over time (Figures 4A and 4B). Remission at this stage could be induced using recombinant IL-1Ra (anakinra); a subsequent withdrawal of treatment resulted in an arthritis flare that preferentially occurred in the previously inflamed joint and a pattern of recurrent joint-specific flare reminiscent of human RA (Figures 4C–4F). Evaluation of synovial T cells showed a population of cells with

a T_{RM} signature that persisted during remission (Figures 4G and 4H). These cells were unaffected by antibody-mediated depletion of circulating T lymphocytes with anti-Thy1.2 antibody, establishing these memory T cells to be tissue resident (Figures 4I–4L). These findings further support the generalizability of the T_{RM} mechanism in arthritis.

Arthritis flare is dependent on recruitment of circulating lymphocytes

To delineate whether the local proliferation of T_{RM} or recruitment of T cells is responsible for the expansion of synovial T cells during flare, we pre-treated arthritic mice in remission with FTY720, a S1P inhibitor that prevents lymphocyte circulation, prior to re-stimulation for flare (Figure 5A). S1P inhibition blocked recurrent joint inflammation, indicating that flare is dependent on the recruitment of lymphocytes (Figures 5B and 5C). By staining for Ki67 expression, a marker of cell proliferation, we showed that although there is some local synovial T_{RM} expansion upon antigen re-stimulation (Figure 5D), the proliferating cell numbers are small in comparison to the total T cell population in the tissue (Figure 5E).

Chemokines are secreted proteins that act to induce cellular migration during inflammation. CCL5 is prominent in human arthritic synovial fluid, and CD8+CD45RA⁺ T cells have been implicated as major producers of the chemokine (Pharoah et al., 2006). We assessed synovial T cells for CCL5, in the absence of exogenous stimulation, and found that some synovial memory T cells contained CCL5 during remission and that this proportion increased markedly during flare (Figures 5G and 5H). To investigate the causal contribution of CCL5, we treated mice with an anti-CCL5 neutralizing antibody prior to re-stimulation on days 27 and 29 (Figure 5F). CCL5 inhibition blocked arthritis flare and T cell influx into the joint (Figures 5I and 5J). These data suggest that CCL5 production is a mechanism by which synovial memory T cells recruit other cells to generate an arthritis flare.

Synovial resident T cells are required for arthritis flare

To test whether synovial T_{RM} are necessary for arthritis flare, we used mice with conditional expression of the diphtheria toxin receptor (DTR). Mice expressing Cre recombinase driven by the *Lck* (lymphocyte protein tyrosine kinase) promoter were crossed with inducible DTR (iDTR) mice that express DTR in the presence of Cre recombinase. *Lck* is expressed primarily by T cells, associates with CD4 and CD8, and is involved in TCR signaling (Barber et al., 1989). The resulting *Lck*-iDTR mice express DTR on all T cells, rendering them susceptible to diphtheria toxin (DT)-mediated depletion. Arthritis was induced in both knees of *Lck*-iDTR mice (Figure 6A). During remission, DT was injected into one joint while the contralateral knee received I.A. saline. I.A. DT partially depleted synovial T cells (Figures 6B and 6C). Importantly, I.A. DT did not deplete circulating T cells, as the percentage of T cells in the peripheral blood remained unchanged during remission, after DT injection, and at re-stimulation (Figure S4). Arthritis flare was then triggered with systemic antigen challenge 2 weeks after local T cell depletion, and joint inflammation was assessed after 72 h. Localized T cell depletion during remission attenuated arthritis flare as measured by joint histology (Figures 6D and 6E), T_{RM} expansion, and myeloid cell recruitment (Figures 6F–6I; myeloid cells were selected as a marker of inflammation to avoid confounding findings resulting from DT-mediated lymphocyte depletion), demonstrating an

essential role of resident synovial T cells within quiescent joints in instigating recurrent joint-specific flares.

A subset of T cells within human RA synovial tissue displays T_{RM} markers

To determine whether T_{RM} contribute to human RA, we sought T cells with a resident memory signature in human RA synovium. Mantra multispectral immunofluorescence imaging, a tyramide-based immunostaining technology, was performed on formalin-fixed paraffin-embedded synovium obtained from four RA patients. Tissues were stained with antibodies against CD3, CD8, CD4, CD45RO, CD69, and CD103. CD8+ or CD4+ memory (CD3+CD45RO+) T cells were evaluated for co-expression of CD69 and CD103, which are the most commonly used T_{RM} markers (Gebhardt et al., 2018; Mackay and Kallies, 2017; Szabo et al., 2019). We found cells bearing a CD3+CD45RO+ CD69+CD103+ signature, consistent with T_{RM}, within areas of aggregated lymphoid cells in RA synovium (Figure 7A).

To quantify these cells, we performed unbiased automated identification and analysis of staining within synovial sections. A total of 29.1% ($\pm 2.9\%$ SEM) of T cells within human RA synovium were CD8+ memory T cells, while 43.6% ($\pm 5.9\%$) were CD4+ memory T cells (Figure 7B). A total of 8.1% ($\pm 2.1\%$) and 14.5% ($\pm 4.8\%$) were CD69+CD8+ and CD4+ memory T cells. Despite the numeric predominance of CD4+ cells overall, CD69+ CD103+ cells were largely CD8+ rather than CD4+ ($1.13\% \pm 0.27\%$ versus $0.16\% \pm 0.04\%$ of all memory T cells, respectively) (Figure 7B). Lymphocytes with the CD3+CD45RO+CD69+ CD103+ T_{RM} signature were found in the synovium of all RA donors.

Memory T cells within RA synovium exhibit gene expression consistent with T_{RM}

To test whether these RA synovial T cells are T_{RM}, we performed single-cell RNA sequencing (scRNA-seq). Leukocytes were harvested from disaggregated synovial tissue from five human RA donors (Figure S5A). Memory T cells (CD3+ CD45RO+) were enriched by fluorescence-activated cell sorting (FACS). Cells were clustered into a tSNE plot based on gene expression profiles (Figure 7C). Cells from all five donors were represented in each cell cluster, suggesting that clustering was robust to donor-to-donor variability (Figures S5B and S5C). Memory CD8 T cells formed clusters 3 and 8, and memory CD4 T cells were found in clusters 1, 4, 5, 6, and 8 (Figure 7D).

Expression of published T_{RM}-associated genes was evaluated within each cluster (Figure 7D; Gebhardt et al., 2018; Kumar et al., 2017; Mackay and Kallies, 2017; Szabo et al., 2019). Transcription factors such as RUNX3, Hobit (*ZNF683*), or Blimp (*PRDM1*) are essential to T_{RM} differentiation (Mackay et al., 2016; Milner et al., 2017). CD49a (*ITGA1*), CD103 (*ITGAE*), and E-cadherin (*CDH1*) are cell adhesion molecules upregulated to anchor T_{RM} in tissue (Cheuk et al., 2017; Hofmann and Pircher, 2011; Jiang et al., 2012). T_{RM} downregulate receptors S1PR1 and CCR7 that facilitate the emigration of lymphoid cells from tissue while expressing tissue retention mediators regulator of G-protein signaling-1 (*RGS1*) and *RGS2* (Gibbons et al., 2011; Jennrich et al., 2012; Mackay et al., 2013, 2015). Only clusters 3 and 4 displayed upregulation of at least one T_{RM}-associated transcription

factor; downregulation of *SIPR1* and *CCR7*; upregulation of either *ITGA1*, *ITGAE*, or *CDH1*; and upregulation of *RGS1* and *RGS2* (Figure 7E). We assessed the hypergeometric probability of overlap between the differentially expressed (DE) genes of each cluster and the established T_{RM} transcriptional signature identified by Kumar et al. (2017) based on the CD4 and CD8 cell type. Of CD8 cells, cluster 3 overlapped significantly with the CD8 lung T_{RM} transcriptome, with a negative log₁₀ p value of 30 (Figure 7F). CD4 cells in cluster 4 and cluster 5, to a smaller degree (negative log₁₀ p value of 5 and 2, respectively), also showed overlap with the CD4 lung T_{RM} transcriptome.

RA synovial T_{RM} express genes involved in inflammation and cellular recruitment

Gene ontology analysis of DE genes within cluster 3 compared to all other clusters showed enrichment for several functional categories, including immune cell activation, cytokine and chemokine signaling, and other immune responses (Figure S6A). Furthermore, Ingenuity Pathway Analysis revealed an enrichment of cellular recruitment pathways among genes upregulated within cluster 3 and identified *CCL5* to be a hub gene within the gene network (Figure S6B). This finding suggests that T_{RM} serve a pro-inflammatory role within the human arthritic joint, particularly through recruitment of infiltrating immune cells, operating by pathways similar to those used by murine synovial T_{RM}.

Synovial T_{RM} signature widely identified in inflammatory arthritis with a lymphocytic pathotype

To establish the general prevalence of T_{RM} in inflammatory arthritis, we assessed whole-synovium microarray expression data from two published studies (n = 30 and n = 15 samples) that compared RA tissues with healthy synovium and osteoarthritis (OA) synovium (Ungethuem et al., 2010; Woetzel et al., 2014). Using our cluster 3 T_{RM} gene signature, we found that this signature was present in each of the RA samples but not the healthy or OA synovial tissues (Figures 7G and 7H).

We further interrogated whole-synovium RNA-seq from 66 well-characterized early-RA samples with various RA pathotypes (fibroid, lymphoid, and myeloid) (Lewis et al., 2019). Using either our cluster 3 gene signature or the Kumar et al. (2017) signature, we found evidence for T_{RM}-pattern gene expression in many samples (Figures S7A and S7B). Intriguingly, but perhaps not surprisingly, the signature was most evident in the lymphoid subset, although a few donors within the myeloid and fibroid subset also exhibited a T_{RM} signal. No difference in T_{RM} signal was noted as a function of time between the first symptom and sampling (all less than 12 months), which is consistent with the observation that disease duration in these early-arthritis samples was also not associated with pathotype (Figures S7C and S7D). Using available clinical data, we found that the presence of T_{RM} correlated with systemic inflammation, reflecting the association of inflammation with the lymphoid pathotype (Figures S7E and S7F). These data confirm the wide-spread presence of T_{RM} in RA, particularly in inflamed synovium with a lymphocytic pathotype.

T_{RM} gene signature is enriched in late-stage leukocyte-poor RA synovium

We assessed bulk RNA-seq data from T cells sorted from 34 RA synovial tissue biopsy samples across a spectrum of synovial inflammation. Zhang et al. (2019) classified these

synovial tissues as leukocyte rich, with T and B lymphocyte infiltration, or leukocyte poor, showing low inflammation comparable to OA tissues. Using our cluster 3 gene signature, we found the T_{RM} -pattern gene expression to be relatively enriched in synovial tissues that were leukocyte poor compared to those that were leukocyte rich (Figure 7I). By further comparing cluster 3 gene signature in RA synovial tissues obtained early in disease (disease duration less than 12 months) to late disease without synovitis on histopathology (disease duration over 5 years with Krenn inflammation score of 1) (Figure 7J), we found the T_{RM} signature to be enriched in late-stage tissues with low inflammation scores, suggesting that T_{RM} remain in the synovium after the dense leukocytic infiltrates characterizing inflamed joints resolve.

RA synovial T_{RM} exhibit a restricted TCR repertoire

We found murine T_{RM} to be activated by a specific antigen. To assess comparable antigen restriction in human T_{RM} , we assessed TCR repertoire. The third complementarity determining region (CDR3) is the hypervariable region on each alpha and beta TCR chain responsible for recognizing processed antigen peptides. Using the 10x Genomics Single Cell Immune Profiling platform, we were able to integrate targeted amplification of full-length V(D)J sequences with primers for the TCR CDR3 region of both alpha and beta chains into the single-cell sequencing workflow. The CDR3 region of paired alpha and beta TCR chains for each cell was sequenced and plotted by frequency (Figure 7K). The vast majority of memory T cells showed unique TCR sequences, but 0.5% of clonotypes included at least 5 cells bearing the same CDR3 TCR, representing expanded clones. Notably, clonal T cells largely mapped to cluster 3 (Figure 7L), which was the cluster exhibiting T_{RM} gene expression, across multiple donors, although the precise TCR sequences were not shared between donors (Figure S5D). T cells within cluster 3 showed the lowest TCR diversity normalized to the number of T cells (clonality index) (Figure 7M), confirming repertoire restriction among these synovial human T_{RM} and supporting a role for antigen specificity.

DISCUSSION

RA is an autoimmune disease characterized by recurrent joint inflammation (Chang and Nigrovic, 2019). Despite the systemic nature of this disease, arthritis flares commonly affect only a subset of joints, typically in a recurrent manner, implying the existence of a mechanism of local memory (Roberts et al., 1988). Here, we show that T_{RM} mediate joint-specific memory and arthritis flares and are targetable to ameliorate disease recurrence.

T cell studies in RA have focused primarily on CD4⁺ T cells because anti-citrullinated protein-antibody-positive (ACPA⁺) RA is strongly associated with the major histocompatibility complex class II (MHC class II) HLA-DRB1 alleles; however, RA also exhibits genetic associations with alleles in the MHC class I HLA-B locus, highlighting the likely importance of CD8⁺ T cells (Han et al., 2014; Raychaudhuri et al., 2012). Although CD8⁺ T cells are described in the synovium and have been implicated in germinal center formation (Kang et al., 2002), their role in RA disease has not been further defined.

We found that CD8⁺ rather than CD4⁺ T_{RM} play a predominant role in arthritis recurrence. These cells were triggered by an antigen because neither non-specific activating signals

nor circulating antigen-specific T cells could replicate their role in initiating disease flare in mice. Together with our observation that human synovial T_{RM} use a restricted TCR repertoire, with most of the 0.5% most abundant TCR clones residing within cluster 3, these findings identify antigen exposure as the likely mechanism by which T_{RM} are activated in the context of joint flare, a finding consistent with a recent study using Nur77 reporter mice to establish TCR-driven activation of T_{RM} in the lung (Low et al., 2020).

T_{RM} are defined by their capacity for long-term persistence in tissues, which is mediated through resistance to tissue egress and therefore to recirculation by the blood and lymphatics (Jiang et al., 2012; Mackay and Kallies, 2017). Parabiosis studies have been used to demonstrate tissue residency in skin. When the vascular systems of two animals are joined, circulating leukocytes establish equilibrium between animals, whereas tissue-resident cells, including T_{RM}, do not (Jiang et al., 2012). In our experimental system, we achieved a comparable demonstration by studying contralateral joints within a single animal. To show that T_{RM} do not traverse to other sites, we labeled synovial cells in one joint during remission and found that labeled T cells did not appear within other joints. Synovial T_{RM} also failed to migrate to tissue egress signals *in vitro* and exhibited a preferential uptake of fatty acids consistent with the known metabolic skew of T_{RM} toward oxidative phosphorylation. T_{RM} persisted in joints for more than 8 months, triggering an arthritis flare in response to systemic antigen stimulation even after extended remission. Thus, the synovial T_{RM} we identify here are bona fide tissue-resident memory T cells by virtue of surface signature, tissue retention, and metabolic features, providing the immunologic substrate for long-term autoimmunity in the joint (Boyman et al., 2004; Clark, 2015; Mackay and Kallies, 2017).

In human RA synovium, we identified T_{RM} by cell surface markers and the gene expression profile. By immunofluorescence microscopy, we found that CD8+CD69+CD103+ T_{RM} represent 1% of memory T cells in RA synovium, outnumbering CD4+CD69+CD103+ cells 10-fold. These figures likely underestimate the T_{RM} population, as T_{RM} do not always express CD103 and display considerable heterogeneity between tissues (Gebhardt et al., 2018; Szabo et al., 2019; Watanabe et al., 2015; Wong et al., 2016). Using scRNA-seq, we found that cells with a T_{RM} gene expression signature represent up to 15% of synovial memory T cells (cluster 3). Consistent with our immunofluorescence results, more than 80% of these cells are CD8+, establishing CD8+ T_{RM} as the most common resident memory population within RA synovium. The discrepancy in T_{RM} abundance between immunofluorescence and RNA-seq likely reflects the overly conservative nature of the T_{RM} surface signature we used for imaging (Mackay and Kallies, 2017). These findings in human RA synovium complement our murine observations in underscoring the importance of CD8+ over CD4+ T_{RM}.

We found that the recruitment of circulating lymphocytes, particularly through the production of CCL5, is necessary for arthritis flare to occur in mice. In human RA, gene ontology analysis of differentially expressed genes showed that cells in cluster 3 exhibit transcriptional evidence for immune effector activity, further supporting an active role for T_{RM} in synovial inflammation. In particular, the Ingenuity Pathway Analysis of cluster 3 identified enrichment for cellular recruitment pathways, with CCL5 as a key hub within the

network, consistent with our murine experimental evidence that T_{RM} use CCL5 to recruit immune effector cells during an arthritis flare.

By depleting resident T cells from the joint during remission, we demonstrated a role for T_{RM} in the initiation of joint-specific flares. We highlight that attenuation of arthritis could be achieved even though T cell depletion was only partial, limited in our system by the maximum dose of I.A. DT that did not also deplete circulating T cells. This result suggests that a more complete elimination of synovial T_{RM} could further attenuate or even abort recurrent disease. Targeting synovial T_{RM} therefore represents a novel therapeutic approach to treat disease chronicity in arthritis.

No animal model exactly mimics human disease. Thus, it is important to consider when murine data inform the understanding of human pathology. Perhaps most compelling is when orthogonal model systems reach the same conclusion. Here, we characterized T_{RM} in models of arthritis driven by antigen injection into the joint followed by systemic antigen challenge (meBSA and OVA + OT-I transfer models), both in the C57BL/6 background. We find the same biology in a third model, in which BALB/c mice lacking endogenous IL-1Ra spontaneously develop chronic joint inflammation driven by T cells, IL-1, and IL-17 (Akitsu et al., 2015). Thus, T_{RM} are not limited to one animal model or to arthritis induced by an exogenous antigen, confirming the generalizability of T_{RM} in arthritis pathogenesis. Disease flares in our models were joint restricted, and T_{RM} were predominantly CD8+ and antigen specific, which are findings echoing those in human RA.

Importantly, we could identify the T_{RM} gene signature in more than 100 RA synovial tissue samples. Using transcriptome data from 45 synovial tissue biopsies published by Ungethuen et al. (2010) and Woetzel et al. (2014), we found that the T_{RM} gene signature is present in RA synovium but not OA and healthy control tissue. In 66 early RA synovial tissue samples from diverse synovial pathotypes (Lewis et al., 2019), the T_{RM} gene signature was preferentially found in RA synovium with lymphoid-type histology. In 34 RA synovial tissue biopsies across a spectrum of synovial inflammation (Zhang et al., 2019), the T_{RM} gene signature was enriched in late-stage non-inflamed synovium compared to early and inflamed synovium, suggesting a relative retention of T_{RM} in RA synovium during remission. These data establish the generalizability of our murine T_{RM} findings, human scRNA-seq data, and immunofluorescence observations across human RA.

Our findings establish that T_{RM} occur in arthritic synovial tissue and play a direct role in disease pathogenesis. Cells with a related phenotype have been identified in arthritic synovial fluid. Petrelli et al. (2018) described a clonally expanded subset of PD-1-expressing CD8+ T cells within inflamed synovial fluid that express a higher level of the T_{RM} markers CD69 and CD103 than their PD-1- counterparts. Qiayum et al. (2019) found a population of CD103+β7+CD29+CD49a+ “integrin-expressing” memory CD8+ T cells reminiscent of T_{RM} (CD62L–S1PR1–) in the synovial fluid of patients with ankylosing spondylitis, as well as in the gut, suggesting a possible gut-joint connection (Wong et al., 2016). It remains unclear whether the cells identified by these groups persist when synovial effusions resolve between flares.

Our results establish T_{RM} as a mechanism of joint-specific memory; however, other mechanisms may also exist. Synovial fibroblasts represent a key effector lineage in arthritis, acquiring an invasive and pro-inflammatory character through epigenetic reprogramming (Ai et al., 2018; Croft et al., 2019; Lee et al., 2007). Synovial macrophages could potentially acquire a pathogenic character that persists even after a primary immune stimulus resolves (Misharin et al., 2014). In arthritis mediated by autoantibodies, joint inflammation could recur through deposition of circulating antibodies or by autoantibodies generated locally by B cells with the assistance of tissue-resident T helper populations (Chang and Nigrovic, 2019; Rao et al., 2017). Depletion of synovial T_{RM} may therefore block recurrent inflammation in some contexts but not others. Therapeutic implications across the range of human arthritides remain to be established (Nigrovic et al., 2021; Nigrovic et al., 2018).

Taken together, our data show that T_{RM} form in previously inflamed joints and persist in the synovium during remission. Activation of T_{RM} leads to arthritis flares with joint swelling and infiltration of circulating leukocytes, ultimately contributing to joint destruction. Although patients with RA currently require life-long treatment, our findings imply that targeting local T_{RM} could provide a new option to achieve durable disease control without systemic immunosuppression.

STAR★METHODS

RESOURCE AVAILABILITY

Lead contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Peter A. Nigrovic (peter.nigrovic@childrens.harvard.edu).

Materials availability—This study did not generate new unique reagents.

Data and code availability—Single-cell RNA-seq data have been deposited at GEO and are publicly available as of the date of the publication. These accession numbers are also listed in the key resources table. Additionally, this paper analyzes existing, publicly available data. These accession numbers for the datasets are listed in the key resources table. Graphs and original microscopy images have been deposited at Mendeley and are publicly available as of the date of this publication. The DOI is listed in the key resources table.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human subjects—Synovial tissues were obtained from joint replacement surgeries, synovectomy surgeries or synovial biopsies of RA patients (See Figure S5). The subjects were enrolled at Brigham and Women's Hospital (BWH) with appropriate informed consent as required. The study protocols were approved by the Institutional Review Board of BWH.

Murine arthritis models—All animal studies were approved by and performed in accordance with Animal Research Committee guidelines and regulations at BWH.

Inducible arthritis model: Arthritis was engendered in 8–12 week old C57BL/6J (B6) mice (B6, JAX strain 664) or B6.SJL-Ptprc^aPepc^b/BoyJ mice (B6 CD45.1, JAX strain 2014) by intraarticular injection of 400µg/10µL meBSA (Sigma-Aldrich) antigen into wrist, knee and ankle joints of a B6 mouse while the contralateral joints were injected with 10µL of PBS as an internal control. Both male and female mice were used and randomly assigned to experimental groups. The mice also received 250ng/20µL of recombinant human IL-1β (Peprotech) into either footpad on Days 0–2 (Lawlor et al., 2001; Staite et al., 1990). Arthritis flare was triggered by intraperitoneal injection of 400µg of meBSA. For adoptive transfer experiments, peripheral lymph nodes (cervical, brachial, inguinal, popliteal) were collected from 6 week old C57BL/6-Tg(TcrαTcrβ)110Mjb/J mice (OT-I, JAX strain 3831) or B6.Cg-Tg(TcrαTcrβ)425Cbn/J mice (OT-II, JAX strain 4194). Lymph nodes were disaggregated by mechanical dissociation over a 70µm cell strainer. T cells were purified by negative selection with the EasySep Mouse T cell isolation kit (STEMCELL Tech) as per manufacturer's instructions. $2.5\text{--}5 \times 10^6$ T cells were then transferred by retroorbital injection into B6 CD45.1 mice 24 hours before intraarticular injection of antigen. In adoptive transfer experiments, 500µg/10µL of OVA (Sigma-Aldrich) antigen was injected instead of meBSA. Intraperitoneal re-stimulation was conducted with 100µg of OVA.

Spontaneous arthritis model: *III^{rm}/–* mice were obtained from the Iwakura lab (Akitsu et al., 2015). These mice spontaneously develop arthritis with age. Male and female mice 4–10 weeks old with arthritis score less than 3 were utilized and randomly assigned to experimental groups. Remission was induced by daily injection of i.p. anakinra 20mg/kg (Sobi) for 15 days until there was no clinical signs of joint swelling. Arthritis flare was achieved by discontinuation of anakinra injections.

In vivo cell tracking: Arthritis was engendered in 8–12 week old B6.129(Cg)-Gt(ROSA)26Sor^{tm4}(ACTB-tdTomato,-EGFP)^{Luo}/J mice (mT/mG, JAX strain 7676) with intraarticular antigen injection as described above. During remission, 5µL of adeno-associated virus serotype 2/6 expressing Cre recombinase (3.88×10^{12} genome copies/mL) was intraarticularly injected into one knee while 5µL of PBS control was injected into the contralateral knee. 4 weeks later, knee synovium was collected and processed for analysis.

Localized T cell depletion in vivo: B6.Cg-Tg(Lck-cre)548Jxm/J mice (Lck-Cre, JAX strain 3802) were crossed with C57BL/6-Gt(ROSA)26Sortm(HBGEF)^{Awai}/J mice (B6-iDTR, JAX strain 7900) to generate mice with *Lck*-driven diphtheria toxin receptor expression (Lck-iDTR). These mice express the diphtheria toxin receptor on all T cells, rendering them susceptible to diphtheria toxin (DT)-mediated depletion. Arthritis was engendered in both knees of 8–12 week old Lck-iDTR mice with intraarticular antigen injection as described above. During remission, 25ng of DT (Sigma-Aldrich) was injected into one knee while the contralateral knee received intraarticular PBS. Arthritis flare was triggered 2 weeks later with i.p. antigen challenge. Synovium was collected 72 hours later and processed for analysis.

METHOD DETAILS

Human synovium processing—Formalin-fixed paraffin embedded (FFPE) synovial tissues used for microscopy were obtained from the pathology archives at BWH as unstained tissue sections mounted on slides. Fresh synovial tissues were obtained immediately after surgeries. Bone and adipose tissues were removed with scissors and the remaining synovium was cut into small pieces then subjected to enzymatic digestion and mechanical dissociation. Tissue fragments were homogenized with gentleMACS (Miltenyi) and digested in RPMI with Liberase TL 0.1mg/mL (Roche) and DNase I 0.1mg/mL (Sigma-Aldrich) for 30 minutes at 37°C before filtering through a 70µm cell strainer.

Mantra microscopy—Opal Multiplex IHC Detection Kit was used to stain synovial tissues per the manufacturer's directions (PerkinElmer). Briefly, unstained FFPE slides of synovial tissues were deparaffinized by heating in a dry oven at 58°C for 45 minutes, washed with xylene three times for a total of 40 minutes, then hydrated through an ethanol gradient ending with a distilled water wash. The slides were then fixed with 10% neutral buffered formalin for 10 minutes. Antigen retrieval was achieved by microwaving the slides in Tris-EDTA Buffer (10mM Tris, 1mM EDTA, pH 9.0) for 15 minutes. Slides were cooled then washed with TBS-Tween20 for 20 minutes, then blocked with BLOXALL (Vector Laboratories). Multiplex antibody staining was conducted with serial cycles of antigen retrieval, 1 hour incubation with primary antibodies against each target antigen, 10 minute incubation with Opal polymer HRP-labeled secondary antibodies, and 10 minute Opal fluorophore incubation for each fluorescent label. The slides were then mounted with media containing DAPI counter staining. Synovial sections were imaged with the Mantra Quantitative Pathology Workstation microscope platform by PerkinElmer. Images were captured using the Mantra Snap program version 1.0.4 at 20x magnification. 5–7 image fields containing CD3+ cells were chosen for each sample. Images were analyzed using the InForm software program version 2.4.8. Individual cells were segmented by identifying the nucleus of each cell through positive DAPI staining, cell membrane size was set to standard distance from the nucleus. Threshold for positivity for each fluorescent marker was generated automatically per image and confirmed manually. Positive expression of each cellular marker was defined by mean fluorescence intensity above the threshold for positivity for each individual cell. Co-expression was defined by positive expression of multiple cellular markers within the same cell.

Single-cell RNA sequencing—Viable cells were enriched by flow cytometric sorting using BD FACSAria Fusion and resuspended in 0.4% BSA in PBS at a concentration of 1,000 cells/µl. 12,000–15,000 cells were loaded onto a single lane (Chromium chip, 10X Genomics) followed by encapsulation in lipid droplets. cDNA and library generation per manufacturer protocol (Chromium Next GEM Single Cell 5' Library & Gel Bead Kit v1.1, 10X Genomics). Additional target amplification for immune cell profiling were all prepared as per manufacturer protocol (Chromium Single Cell V(D)J Human T Cell Enrichment Kit, 10X Genomics). cDNA libraries were sequenced to an average of 50,000 reads per cell using Illumina Nextseq 500. scRNA-seq reads were processed with Cell Ranger v3.1, which demultiplexed cells from different samples and quantified transcript counts per putative cell. Quantification was performed using the STAR aligner against the GRCh38 version of the

genome. scRNA-seq data was normalized using Cell Ranger. tSNE plots and clusters were calculated with Cell Ranger. Data were visualized using the Loupe Browser.

Differentially expressed genes between clusters were identified using Cell Ranger with thresholds $FDR < 0.1$ and $\log_2$ fold change > 0.5 . We then compared these differentially expressed genes to genes identified as signatures of CD8 and CD4 T_{RM} from the lung as described Kumar et al. (2017). The significance of the overlap between each comparison was calculated using a hypergeometric probability.

Gene ontology was performed using DAVID (Huang et al., 2009a, 2009b). Differentially expressed genes were input into DAVID, and GO_FAT categories Biological Process, Cellular Compartment, and Molecular Function were selected. Significantly enriched, non-redundant terms were selected. Ingenuity Pathway Analysis (IPA) (version 57662101) was used for pathway enrichment analysis in Ingenuity knowledge base (<https://www.qiagen.com/ingenuity>). Differentially expressed genes within cluster 3 ($p < 0.01$) were included in the analysis.

To compare the various clusters of memory T cells in synovium to known T_{RM} cell types, we measured the overlap of the differentially expressed genes within each cluster with the differentially expressed genes in the corresponding T_{RM} cell type identified previously in the lung (Kumar et al., 2017). Significance of the overlap between these sets of genes was calculated using the hypergeometric probability in R, and these p values were log-transformed for display on the barplot.

Gene expression data from additional synovial tissue specimens were obtained from publicly available datasets (ArrayExpress E-MTAB-6141 [Lewis et al., 2019]; ImmPort SDY998 [Zhang et al., 2019]; GEO DataSets GSE55235 [Woetzel et al., 2014]; GEO DataSets GSE1919 [Ungethuem et al., 2010]). Bulk RNA-seq data from different RA pathotypes (Lewis et al., 2019) were aligned using STAR (2.7.0f) and quantified using the HTSeq-count package (0.11.1) in python using all protein coding Ensembl genes as a reference. Processed RNA-seq data was obtained from sorted RA synovial T cells (Zhang et al., 2019) and microarray data from healthy, OA and RA synovial tissue (Woetzel et al., 2014) and (Ungethuem et al., 2010). Differentially expressed genes from cluster 3 were identified in each dataset, and the principal components analysis was performed using the singular value decomposition algorithm on scaled, centered expression data.

For donor differentiation using SNPs, scRNA-seq data was examined for the top 100 highest-expressed genes and reads mapping to these genes were extracted from mapped SAM files using samtools. SNPs present in the coding regions of these genes were extracted from the UCSC Genome Browser, and SNPs were limited to single nucleotide variants. We then examined each read to determine whether the SNP was present in the read. Genotyped SNPs were collected, and a determination of the genotype was attempted based on the information. If only the major allele was present in a given cell, then the SNP for that cell was given a value of $g(x) = 1 - (2 * [MaAF] * [MiAF]^N)$, where MaAF is the major allele frequency, MiAF is the minor allele frequency, and N is the number of reads containing the SNP. If both major and minor alleles were present, then the SNP was given a value of 0.5. If

only minor alleles were present, then the SNP was given a value of $g(x) = 0.5 - (MiAF)^N$. A matrix of all genotyped SNPs by all cells was created, and SNPs were limited by those with the greatest variance. Hierarchical clustering was performed, and the clusters were selected based on the number of donors.

Immune cell profiling—The 10x Genomics Single Cell Immune Profiling platform was used for immune cell profiling. This technology integrates a step into the 10x single cell RNA sequencing workflow for targeted amplification of full-length VDJ sequences with primers for the T cell receptor CDR3 of both alpha and beta chains (Chromium Single Cell V(D)J Human T Cell Enrichment Kit, 10X Genomics). Cell Ranger was used to associated cell barcodes with specific TCR clonotypes. Shannon entropy was calculated $H(X) = - \sum_{i=1}^n p(x_i) * \log_2(p(x_i))$ where $p(x_i)$ is the fraction of a single clonotype compared to all clonotypes present, and it was corrected for cluster size by $H(X)/\log_2(\text{cells in cluster})$.

Murine synovium processing—Synovium was dissected from the knees and ankles of mice and digested for 30 minutes with DNase I 0.1mg/mL (Sigma-Aldrich) and Collagenase type IV 1mg/mL (Worthington) in RPMI at 37°C. The tissue was then mechanically dissociated over a 70µm cell strainer into a cell suspension for subsequent assays.

Murine joint histopathology—The knees were dissected en bloc by amputation at mid-femur and mid-tibia. The skin, fat and muscles were trimmed off with scissors. The knee was fixed in 4% paraformaldehyde for 24 hours, protected from light at 4°C, then washed with PBS and placed into Osteosoft (Sigma-Aldrich) decalcification solution for 3–5 days. The tissue was embedded in paraffin, sectioned and stained with hematoxylin and eosin for light microscopy. Inflammatory score calculated as previously described (Ierna et al., 2010).

T cell migration—T cells were isolated from disaggregated synovium with the EasySep Mouse T cell isolation kit (STEMCELL Tech) as per manufacturer's instructions. T cells were placed in the top chamber of a transwell polycarbonate membrane insert with a 5.0µm pore (Corning) in serum-free RPMI 1640 media. RPMI 1640 medium containing 10% FBS and varying concentrations of murine CCL21 (Peprotech) was placed in the bottom of a 24-well plate. Cells were allowed to migrate for 2 hours at 37°C, after which cells were collected separately from the top and bottom chambers and stained for flow cytometry analysis.

Fatty acid uptake—Cell isolated from synovium were incubated with 1µM Bodipy FL C16 (ThermoFisher) in PBS with 20µM fatty acid-free BSA (Sigma-Aldrich) for 30 minutes at 37°C. Bodipy uptake was quenched by adding 4x volume of ice-cold PBS with 2% FBS, then cells were washed twice before antibody staining for flow cytometry analysis. All antibody staining and washing was performed on ice.

Antibodies—Anti-Thy1.2 antibody (Clone 30H12, Bio X Cell Cat# BE0066, RRID:AB_1107682) was used for the depletion of circulating lymphocytes. Rabbit polyclonal anti-CCL5 antibody (Origene Cat# PP1081P1, RRID:AB_1006884) was used for the neutralization of CCL5 chemokine.

The following antibodies were used for the analysis of human synovial cells with cell sorting: APC anti-CD3 (Clone OKT3, BioLegend Cat# 317317, RRID:AB_1937213), PE anti-CD45RO (Clone UCHL1, BioLegend Cat# 304206, RRID:AB_314422).

The following antibodies were used for the analysis of human synovium with microscopy: rabbit polyclonal anti-CD3 (Sigma-Aldrich Cat# C7930, RRID:AB_259074), anti-CD103 (Clone EP206, Sigma-Aldrich Cat# 437R, RRID:AB_2884943), rabbit polyclonal anti-CD69 (Sigma-Aldrich Cat# HPA050525, RRID:AB_2681157), anti-CD4 (Clone EPR6855, Abcam Cat# ab133616, RRID:AB_2750883), anti-CD8 (Clone C8/144B, BioLegend Cat# 372902, RRID:AB_2650657), anti-CD45RO (Clone UCHL1, BioLegend Cat# 304202, RRID:AB_314418).

The following antibodies were used for the analysis of murine synovium with flow cytometry or cell sorting: Alexa Fluor 700 anti-CD3 (Clone 17A2, BioLegend Cat# 100216, RRID:AB_493697), APC anti-CD3 (Clone 17A2, BioLegend Cat# 100236, RRID:AB_2561456), FITC anti-CD45 (Clone 30-F11, BD Biosciences Cat# 561088, RRID:AB_10562038), FITC anti-CD45.1 (Clone A20, BioLegend Cat# 110706, RRID:AB_313495), PE anti-CD45.2 (Clone 104, BioLegend Cat# 109808, RRID:AB_313445), FITC anti-CD4 (Clone GK15, BioLegend Cat# 100406, RRID:AB_312691), APC/Cyanine7 anti-CD4 (Clone GK1.5, BioLegend Cat# 100414, RRID:AB_312699), Brilliant Violet 650 anti-CD8a (Clone 53-6.7, BioLegend Cat# 100742, RRID:AB_2563056), PerCP/Cyanine5.5 anti-CD8a (Clone 53-6.7, BioLegend Cat# 100734, RRID:AB_2075238), Brilliant Violet 605 anti-CD44 (Clone IM7, BioLegend Cat# 103047, RRID:AB_2562451), APC anti-CD62L (Clone MEL-14, BioLegend Cat# 104412, RRID:AB_313099), Pacific Blue anti-CD69 (Clone H1.2F3, BioLegend Cat# 104524, RRID:AB_2074979), PE/Cyanine7 anti-CD103 (Clone 2E7, BioLegend Cat# 121426, RRID:AB_2563691), PE anti-CD103 (Clone 2E7, BioLegend Cat# 121406, RRID:AB_1133989), PE anti-ST2 (Clone DIH4, BioLegend Cat# 146608, RRID:AB_2728177), PE anti-CD19 (Clone eBio1D3, Thermo Fisher Scientific Cat# 12-0193-82, RRID:AB_657659), APC anti-CD11b (Clone M1/70, BioLegend Cat# 101212, RRID:AB_312795), Brilliant Violet 650 anti-Ly6G (Clone 1A8, BioLegend Cat# 127641, RRID:AB_2565881), Brilliant Violet 421 anti-NK1.1 (Clone PK136, BioLegend Cat# 108731, RRID:AB_10895916). 123count eBeads (Invitrogen) were used to calculate absolute cell counts.

QUANTIFICATION AND STATISTICAL ANALYSIS

Graphs and statistical analysis were generated using Prism v9 software. Statistical details for each experiment can be found in the figure legends and Results section, including the statistical tests used (i.e., Student's t test or Mann-Whitney U test depending on normality for single comparisons, ANOVA for multiple comparisons), number of cells, exact value of n, and what n represents. Statistical significance is defined as p value < 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Arthritic human and murine joints acquire CD8⁺ resident memory T cells (T_{RM})
- T_{RM} from rheumatoid joints display a restricted T cell receptor repertoire
- Antigen-activated T_{RM} recruit other immune cells to cause an arthritis flare
- Depletion of synovial T_{RM} blocks local disease recurrence

Figure 1. Arthritis flare model identifies CD8 T cells as mediators of recurrent synovitis

(A) Diagram of mouse model of arthritis flare. I.A., intraarticular; i.p., intraperitoneal.

(B) Representative images of wrists, knees, and ankles of arthritic mouse at day 7. Red circle denotes antigen-injected joint.

(C) Measured wrist thickness at day 7, day 28, and day 31. Each dot represents one animal (n = 15, day 7 and 28; n = 9, day 31). p values from two-tailed paired Student's t test.

(D) Difference in wrist thickness of meBSA- versus PBS-injected joints over time (n = 15 mice).

(E) Representative H&E of contralateral PBS- versus meBSA-injected knees from the same mouse at indicated time points. P, patella; F, femur; T, tibia; arrows mark inflammatory infiltrates. Scale bar, 50 μ m.

(F) Number of synovial T cells during remission and flare. Each dot represents one animal (n = 11, remission; n = 16, flare). p values from two-tailed Wilcoxon matched-pairs signed rank test.

(G) Synovial T cell count after adoptive transfer of OT-I and OT-II. Each dot represents one animal (n = 6, OT-I; n = 6, OT-II). p values from two-tailed paired Student's t test.

(H) Representative contour plots of CD45.2+ donor-derived versus CD45.1+ host-derived CD4 and CD8 T cells in the synovium during remission and flare.

(I) Graph quantifying CD45.2+ donor-derived CD4 and CD8 T cells in remission and flare. Each dot represents one animal (n = 6, remission; n = 7, flare). p value from two-way ANOVA with Sidak's multiple comparisons test.

Figure 2. Distinct population of TRM-like memory T cells identified in previously inflamed synovium

(A) tSNE analysis comparing cellular protein expression in synovial T cells from PBS-injected (top row) versus OVA-injected (bottom row) joints pooled from the knees and ankles. Plot reflects 99 CD3⁺ T cells. Red circle highlights distinct cell population found in OVA-injected joints.

(B) Representative contour plots comparing CD69 and CD103 expression in CD3⁺CD44⁺CD62L⁻ T cells in the synovium of paired PBS-injected (top row) versus meBSA-injected (bottom row) joints from the same mouse. The left column reflects a mouse in remission, and the mouse in the right column was challenged with a systemic antigen to induce a flare.

(C) Quantification of CD69⁺ and CD69⁺CD103⁺ CD44⁺CD62L⁻ T cells during remission and flare. Each dot represents one animal (n = 12, remission; n = 7, flare). p values from two-tailed Wilcoxon rank-sum test.

Figure 3. Synovial memory T cells are true T_{RM}

(A) Experimental design for tracking cells from a specific joint.

(B) Representative dot plots of T cells pooled from the synovium of 4 mice at day 54.

AAV-Cre-injected and contralateral PBS-injected knees are depicted in paired dot plots with a subanalysis of CD8 and CD4 expression in AAV-Cre-labeled T cells (EGFP⁺).

(C) Number of AAV-Cre-labeled EGFP⁺ T cells (left) and percentage of CD44⁺CD62L⁻CD69⁺ T_{RM} within EGFP⁺ T cells (right) at day 54. Each dot represents an independent experiment pooling synovium from 4 mice (n = 3).

(D) Proportion of synovial naive T cells (T_N , CD44–CD62L+) and resident memory T cells (T_{RM} , CD44+CD62L–CD69+) migrating to an increasing concentration of CCL21 chemoattractant. Values displayed as fold change of cells migrated to the bottom compared to cells on top of the transwell. Each dot represents an independent experiment pooling synovium from 5 animals ($n = 4$). p value from one-way ANOVA.

(E) Representative histogram of Bodipy FL C_{16} fatty acid uptake by T_N , effector memory T cells (T_{EM} , CD3+C44+CD62L–CD69–), and T_{RM} pooled from synovium of 5 mice. Histogram reflects 225 T_{RM} .

(F) Bodipy FL C_{16} fatty acid uptake, depicted as mean fluorescence intensity. Each dot represents pooled synovium from one animal ($n = 10$). p value calculated from one-way ANOVA.

(G) Experimental design for assessing T_{RM} longevity.

(H) Difference in wrist size between OVA- and PBS-injected wrists within the same animal. Each dot represents one animal ($n = 5$). p value from two-tailed Student's t test.

(I) Representative dot plot of CD69 and CD103 expression in CD44+CD62L– T cells from synovium on day 246.

(J) Percentage of CD44+CD62L– T cells that are CD69+ or CD69+CD103+ joints at day 246. Each dot represents one animal ($n = 6$). p values from two-tailed Wilcoxon matched-pairs signed rank test.

Figure 4. T_{RM} persist in drug-induced remission in spontaneous murine model of RA

(A) Representative photo of unilateral arthritis in *IL-1* receptor antagonist deficient (*IL-1ra*^{-/-}) BALB/c mice.

(B) Prevalence of unilateral arthritis by age.

(C) Experimental design for treating *IL-1ra*^{-/-} mice with unilateral joint swelling with anakinra and subsequent flare after withdrawal of treatment. (D and E) Arthritis score (D) and difference in joint measurements

(E) of the inflamed joint (red) and contralateral uninflamed joint (green) (n = 10).

(F) Laterality of flare in relation to initial arthritic joint after withdrawal of anakinra. p value from chi-square test.

(G) Representative dot plots of CD3⁺ and CD44⁺CD62L[−] T cells in the synovium of joints without clinical swelling, with inflammation, and during anakinra-induced remission.

(H) Graphs quantifying CD44⁺CD62L[−] memory T cells and T_{RM} (CD44⁺CD62L[−]CD69⁺) within synovium of the described disease states. Each dot represents one animal. p values from one-way ANOVA.

(I and J) Circulating T cells were depleted in *IL-1 α* ^{−/−} mice with i.p. injection of anti-Thy1.2 antibody (250 μ g) 7 days prior to collection. Representative dot plots showing anti-Thy1.2 antibody-mediated depletion of CD3⁺ T cells in the spleen (I) and T cell subsets in synovium (J).

(K and L) Graphs quantifying naive and CD44⁺CD62L[−] memory T cells (n = 6, PBS; n = 5, anti-Thy1.2) (K) and CD69⁺ and CD69⁺CD103⁺ memory T cells (n = 9, PBS; n = 5, anti-Thy1.2) (L) within synovium. Each dot represents one animal. p values from two-tailed Student's t test.

Figure 5. Arthritis flare is dependent on recruitment of circulating lymphocytes

(A) Experimental design for inhibiting circulating lymphocytes with FTY720 (20 μ g) in meBSA-induced arthritis (black) or with OT-I adoptive transfer and OVA-induced arthritis (red).

(B and C) Graphs depicting wrist thickness (B) and prevalence of T_{RM} (C) in PBS- versus meBSA-injected joints at day 31 flare with FTY720 inhibition. Each dot represents one mouse; line connects contralateral joints within the same animal (n = 10, PBS; n = 14, meBSA without FTY720; n = 16, meBSA with FTY720). p values from two-tailed paired Student's t test.

- (D) Number of proliferating Ki67+ T_{RM}.
- (E) Ki67+ T_{RM} as a percentage of total CD3+ T cells. Each dot represents one mouse (n = 10, PBS; n = 5, OVA). p values from two-tailed Mann-Whitney test.
- (F) Experimental design for inhibiting lymphocyte recruitment with isotype or anti-CCL5 antibody (1 µg/dose).
- (G) Representative dot plot of CCL5 expression in CD3+CD44+CD62L- T cells with and without re-stimulation with OVA.
- (H) CCL5 expression in synovial CD3+ cells and CD44+CD62L- T cells during flare assessed by intracellular cytokine staining. Each dot represents one mouse (n = 3, IA PBS; n = 8, IA OVA). p values from two-tailed Student's t test.
- (I and J) Graphs depicting wrist thickness (I) and CD3 T cells (J) in PBS- versus OVA-injected joints at day 31 flare with isotype or anti-CCL5 neutralizing antibody. Each dot represents one mouse; line connects contralateral joints within the same animal (n = 11, PBS; n = 13, OVA without inhibitor; n = 8, OVA with isotype or anti-CCL5 antibody). p values from two-tailed paired Student's t test.

Figure 6. Depletion of synovial-resident T cells in remission abrogates arthritis flare

(A) Experimental design for localized synovial T cell depletion.

(B) Representative dot plot of synovial lymphocytes from DT-injected and PBS-injected knees collected 72 h after I.A. injection.

(C) Graph quantifying CD3+ T cells as a percentage of total CD45+ lymphocytes in synovium of DT-treated versus non-treated knees. p value calculated from two-tailed paired Student's t test. Each dot represents one animal (n = 6 mice).

(D and E) Representative H&E images (D) and inflammatory score (E) of contralateral knees from the same mouse with or without DT injection after i.p. re-stimulation for flare at day 45. Each dot represents one animal (n = 15). p value from two-tailed Wilcoxon rank-sum test. S, synovium. Scale bar, 50 μ m.

(F–I) Graphs showing percentage of T_{RM} or CD45+ myeloid cells (see Figure S1) in the synovium in meBSA-injected joints with or without I.A. DT treatment. (F and H) Data compare the flare response with and without DT treatment. (G and I) Data evaluate the effect of DT injection compared to the contralateral control joint. p values calculated from two-tailed paired Student's t test. Each dot represents one animal (n = 17 mice/condition). Line connects contralateral joints within the same mouse.

Figure 7. Oligoclonal CD8 TRM enriched in late-stage, non-inflamed RA synovial tissue

(A) Representative immunofluorescence image of human RA synovium co-stained for CD3, CD8, CD45RO, CD69, and CD103 proteins and DAPI nuclear stain. Scale bar, 50 μ m. Red circle indicates cells positive for all five biomarkers. Insert magnifies a circled cell; scale bar, 5 μ m.

(B) Prevalence of CD8 and CD4 T cells expressing CD45RO memory and CD69 and CD103 markers. Each dot represents the average cell count from five fields from each donor ($n = 4$). p values from two-tailed paired Student's t test.

(C) tSNE plot of CD3+CD45RO+ cells from disaggregated RA synovium pooled from five donors. Each dot represents one cell. Color-coded clusters designate cells with similar gene expression profiles.

(D) Violin plot of CD4 and CD8 expression in each cluster.

(E) Gene expression heatmap of T_{RM}-associated genes. Red, increased expression; blue, decreased expression.

(F) Hypergeometric probability of overlap between differentially expressed (DE) genes of each cluster and T_{RM} gene expression signature from the lung based on CD4 and CD8 cell type (Kumar et al., 2017).

(G and H) Principal component analysis (PCA) of cluster 3 DE genes in microarray expression data from healthy controls (HC), osteoarthritis (OA), and rheumatoid arthritis (RA) synovium published in (G) Woetzel et al. (2014) (n = 30) and (H) Ungethuem et al. (2010) (n = 15). Each bar represents one donor.

(I and J) PCA of cluster 3 DE genes in T lymphocytes from RA synovial tissue (Zhang et al., 2019). Bar plots showing cluster 3 T_{RM} gene signature in (I) leukocyte-rich versus leukocyte-poor RA synovium and (J) early-stage RA versus late-stage non-inflamed RA synovium. Each bar represents one donor (n = 34).

(K) Histogram of cells with the same TCR-alpha and -beta CDR3 sequence.

(L) Top 0.5% of most frequent T cell clones overlaid on the tSNE plot.

(M) Clonality index for each cell cluster. Each dot represents one donor (n = 5). p values from one-way ANOVA.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Thy1.2 antibody (Clone 30H12)	Bio X Cell	Cat# BE0066; RRID: AB_1107682
Rabbit polyclonal anti-CCL5 antibody\	Origene	Cat# PP1081P1; RRID: AB_1006884
APC anti-CD3 (Clone OKT3)	BioLegend	Cat# 317317; RRID: AB_1937213
PE anti-CD45RO (Clone UCHL1)	BioLegend	Cat# 304206; RRID: AB_314422
rabbit polyclonal anti-CD3	Sigma-Aldrich	Cat# C7930; RRID: AB_259074
anti-CD103 (Clone EP206)	Sigma-Aldrich	Cat# 437R; RRID: AB_2884943
rabbit polyclonal anti-CD69	Sigma-Aldrich	Cat# HPA050525; RRID: AB_2681157
anti-CD4 (Clone EPR6855)	Abcam	Cat# ab133616; RRID: AB_2750883
anti-CD8 (Clone C8/144B)	BioLegend	Cat# 372902; RRID: AB_2650657
anti-CD45RO (Clone UCHL1)	BioLegend	Cat# 304202; RRID: AB_314418
Alexa Fluor 700® anti-CD3 (Clone 17A2)	BioLegend	Cat# 100216; RRID: AB_493697
APC anti-CD3 (Clone 17A2)	BioLegend	Cat# 100236; RRID: AB_2561456
FITC anti-CD45 (Clone 30-F11)	BD Biosciences	Cat# 561088; RRID: AB_10562038
FITC anti-CD45.1 (Clone A20)	BioLegend	Cat# 110706; RRID: AB_313495
PE anti-CD45.2 (Clone 104)	BioLegend	Cat# 109808; RRID: AB_313445
FITC anti-CD4 (Clone GK15)	BioLegend	Cat# 100406; RRID: AB_312691
APC/Cyanine7 anti-CD4 (Clone GK1.5)	BioLegend	Cat# 100414; RRID: AB_312699
Brilliant Violet 650 anti-CD8a (Clone 53–6.7)	BioLegend	Cat# 100742; RRID: AB_2563056
PerCP/Cyanine5.5 anti-CD8a (Clone 53–6.7)	BioLegend	Cat# 100734; RRID: AB_2075238
Brilliant Violet 605 anti-CD44 (Clone IM7)	BioLegend	Cat# 103047; RRID: AB_2562451
APC anti-CD62L (Clone MEL-14)	BioLegend	Cat# 104412; RRID: AB_313099
Pacific Blue anti-CD69 (Clone H1.2F3)	BioLegend	Cat# 104524; RRID: AB_2074979
PE/Cyanine7 anti-CD103 (Clone 2E7)	BioLegend	Cat# 121426; RRID: AB_2563691
PE anti-CD103 (Clone 2E7)	BioLegend	Cat# 121406; RRID: AB_1133989
PE anti-ST2 (Clone DIH4)	BioLegend	Cat# 146608; RRID: AB_2728177
PE anti-CD19 (Clone eBio1D3)	Thermo Fisher Scientific	Cat# 12-0193-82; RRID: AB_657659
APC anti-CD11b (Clone M1/70)	BioLegend	Cat# 101212; RRID: AB_312795
Brilliant Violet 650 anti-Ly6G (Clone 1A8)	BioLegend	Cat# 127641; RRID: AB_2565881
Brilliant Violet 421 anti-NK1.1 (Clone PK136)	BioLegend	Cat# 108731; RRID: AB_10895916
Bacterial and virus strains		
adeno-associated virus serotype 2/6 expressing	Boston Children's Hospital	N/A
Cre recombinase	Virology Core	
Biological samples		
Human rheumatoid arthritis synovial tissue	Brigham and Women's OR	N/A
Chemicals, peptides, and recombinant proteins		
methylated Bovine Serum Albumin	Sigma-Aldrich	A1009
ovalbumin	Sigma-Aldrich	A5503

REAGENT or RESOURCE	SOURCE	IDENTIFIER
human interleukin-1b	Peprtech	200-01B
anakinra	Sobi	N/A
diphtheria toxin	Sigma-Aldrich	D0564
liberase TL	Roche	5401020001
dnase I	Sigma-Aldrich	D4527
BLOXALL	Vector Laboratories	SP-6000-100
Collagenase type IV	Worthington	LS004188
Osteosoft	Sigma-Aldrich	1017289010
murine CCL21	peprtech	250-13
Bodipy FL C16	ThermoFisher	D3821
fatty acid-free BSA	Sigma-Aldrich	A8806
Critical commercial assays		
EasySep Mouse T cell isolation kit	STEMCELL Tech	19851
Opal Multiplex IHC Detection Kit	PerkinElmer	NEL811001KIT
Chromium chip	10X Genomics	N/A
Chromium Next GEM Single Cell 5' Library & Gel Bead Kit v1.1	10X Genomics	N/A
Chromium Single Cell V(D)J Human T Cell Enrichment Kit	10X Genomics	N/A
Deposited data		
human rheumatoid arthritis synovial memory T cells gene expression matrices	GEO DataSets	GEO: GSE184582
original data and images	Mendeley Data	https://dx.doi.org/10.17632/332n4pdxh9.1
Lewis et al., 2019. bulk RNA-seq data from different rheumatoid arthritis synovial tissue pathotypes	ArrayExpress	E-MTAB-6141
Zhang et al., 2019. bulk RNA-seq data from rheumatoid arthritis synovial T cells	ImmPort	SDY998
Woetzel et al., 2014. microarray data from rheumatoid arthritis, osteoarthritis and healthy synovial tissues	GEO DataSets	GEO: GSE55235
Ungethuem et al., 2010. microarray data from rheumatoid arthritis, osteoarthritis and healthy synovial tissues	GEO DataSets	GEO: GSE1919
Experimental models: Organisms/strains		
C57BL/6J (B6) mice (B6)	JAX	strain 664
B6.SJL-Ptprc ^a Pepc ^b /BoyJ mice (B6 CD45.1)	JAX	strain 2014
C57BL/6-Tg(TcraTcrb)110Mjb/J mice (OT-I)	JAX	strain 3831
B6.Cg-Tg(TcraTcrb)425Cbn/J mice (OT-II)	JAX	strain 4194
BALB/cA-III ^m -/-	Iwakura lab	N/A
B6.129(Cg)-Gt(ROSA)26Sor ^{tm4} (ACTB-tdTomato,-EGFP)Luo/J mice (mT/mG)	JAX	strain 7676
B6.Cg-Tg(Lck-cre)548Jxm/J mice (Lck-Cre)	JAX	strain 3802
C57BL/6-Gt(ROSA)26Sor tm (HBGEGF)Awai/J mice (B6-iDTR)	JAX	C57BL/6-strain 7900
Software and algorithms		
Cell Ranger v3.1	10X Genomics	N/A
Loupe Browser	10X Genomics	N/A
InForm software program version 2.4.8	Perkin Elmer	N/A