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Distinct synovial tissue macrophage subsets regulate inflammation and remission in rheumatoid arthritis

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Title: *Distinct tissue macrophage subsets regulate inflammation and provide a novel cellular and molecular mechanism for disease remission in rheumatoid arthritis*

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Abstract

Treatment-refractory rheumatoid arthritis (RA) is a major clinical challenge. Drug-free remission is uncommon but provides proof-of-concept that articular immune-homeostasis can be reinstated. Here we identify active cellular and molecular mechanisms of sustained remission in RA. Single-cell transcriptomics (32,000 cells) identified phenotypic changes in synovial tissue macrophages (STM) spanning health, early/active RA, treatment-refractory/active RA, and RA in sustained remission. Each clinical state is characterised by different frequencies of 9 discrete phenotypes of 4 distinct STM subpopulations with diverse homeostatic, regulatory and inflammatory functions. This cellular atlas combined with deep-phenotypic, spatial and functional analyses of synovial biopsy FACS-sorted STMs revealed two STM subpopulations (MerTK/TREM2^{high} and MerTK/FOLR2/LYVE1^{pos}) with unique remission transcriptomic signatures enriched in negative regulators of inflammation. In response to damage signals these cells are potent producers of inflammation-resolving lipid mediators and are the only STMs that induce the repair response of synovial fibroblasts. A low proportion of MerTK^{pos} STMs in remission RA is a prognostic biomarker predictive of flare after treatment cessation. Therapeutic enhancement of MerTK^{pos} STM-subsets could encourage resolution of inflammation and reinstate synovial homeostasis in inflammatory arthritis.

Introduction

Rheumatoid Arthritis (RA), the most common autoimmune-inflammatory arthropathy, is characterised by breach of self-tolerance to post-translationally modified self-proteins and chronic synovitis. Current therapies mainly target inflammatory cytokine and receptor pathways, or cells of adaptive immunity. While these deliver therapeutic benefit, partial or non-response in up to ~50% of patients remain a significant challenge. Furthermore, approximately half of those who respond will relapse within months of treatment-reduction or cessation[1,2]. However, long-term drug-free remission does occur and provides proof-of-concept that articular immune-homeostasis can be reinstated in RA[3]. The cellular and molecular mechanisms mediating such remission are unknown yet could offer remarkable opportunity to enact cure, informing both RA and other immune-mediated disease therapeutics.

The healthy *synovial membrane* is a specialized, multifunctional structure consisting of a lining layer of synovial fibroblasts and macrophages, a supporting sublining layer of loose connective tissue with sublining fibroblasts, and a rich network of nerves and blood/lymphatic vasculature[4]. The origin of human synovial tissue resident macrophages (STMs) is unclear; recent mouse studies suggest they originate from embryonic precursors, distinct from adult bone-marrow monocyte precursors, that populate the synovium during early embryogenesis and proliferate *in situ*[5-7]. Their joint-specific functions in humans remain undefined but emerging murine evidence suggests that they help maintain joint immune-homeostasis[5,7]. Recent elegant work by Culemann *et al* found that murine Trem2^{pos} lining-layer macrophages are long-lived, locally-renewing and formed a protective epithelial-like barrier that was disrupted during experimental arthritis, and this disrupted synovial distribution was seen in RA patients[7].

In active RA, the synovial membrane becomes populated by leukocytes including macrophages[8-10]. Human[11] and mouse[5,12] studies suggest that the latter may differentiate locally from blood-derived monocytes recruited by synovial chemokines[13], and comprise the main producers of pathogenic TNF[3,9,14,15].

Recently, single-cell transcriptome sequencing (scRNAseq) revealed complex heterogeneity of synovial fibroblasts[16-19] and synovial tissue macrophages[17,20] from patients with active RA and osteoarthritis. A distinct biology associated with different synovial fibroblast clusters has since been experimentally validated and will have important implications for therapies aimed at modulation of inflammation or tissue repair[16]. Identifying the phenotypic and functional heterogeneity of human synovial myeloid populations has been hampered by the relatively few cells sequenced, restricted functional validation *ex vivo*, and the lack of comparative phenotype profiles of macrophages in healthy and remission human synovium[17,20]. Our earlier studies[2,3,21,22] demonstrated that although most synovial inflammation resolves in RA in sustained clinical and ultrasound remission [2,3], clusters of synovial tissue macrophages persist, predominantly in the lining layer[3]. The phenotype and function of these cells are unknown. In this study we explored the phenotypic and functional changes

in synovial tissue macrophage subpopulations spanning health, inflammation and disease remission and uncovered MerTK-dependent mechanisms that actively maintain remission, mediated by tissue resident STMs.

Results

Synovial tissue of RA patients in sustained remission is enriched in MerTK/CD206^{pos} STMs; with a decline predictive of flare.

To determine the phenotypic spectrum of human synovial tissue macrophages (STMs) spanning health, immune-mediated synovitis and resolution, we characterised expression of candidate immune-receptors. These included CD163; previously found on healthy STMs[9,23], and MerTK and CD206; key markers of *murine* mature resident macrophages with immune-homeostatic function[24,25,26]. Using these markers, we investigated the relative composition of STMs in dispersed synovial tissue biopsies from RA patients (45 treatment-naive active RA, 31 treatment-resistant active RA, 36 RA in sustained remission) and 10 healthy donors **(Suppl Table 1)**. STMs were identified firstly by their expression of CD64, CD11b, MHCII and the absence of other cell-lineage markers, including DCs, T/B/NK-cells, mast cells and neutrophils. (**Suppl Fig 1a-b**). Two predominant STM populations were then identified by co-expression of MerTK and CD206: MerTK/CD206^{pos} and MerTK/CD206^{neg} **(Fig.1a** and **extended data Suppl Fig.2a)**. The relative proportions of these populations (**Fig.1b**) indicated that STMs from healthy donors were almost exclusively MerTK/CD206^{pos}. A predominance of MerTK/CD206^{pos} also distinguished RA patients in sustained remission from those with active RA. These STMs were fewer in RA patients with active disease, with commensurately increased MerTK/CD206^{neg} STMs **(Fig.1a-b)**. CD163 was co-expressed as a subpopulation of MerTK/CD206^{pos} STMs (**Fig.1c and Suppl Fig.1c-d)** and this MerTK/CD163/CD206^{pos} STM population was increased in RA patients in sustained remission compared to active RA **(Fig.1d-e and Suppl Fig.3a)**. The relative proportion of the MerTK/CD206^{pos}, CD163/CD206^{pos} and MerTK/CD163^{pos} STMs phenotypes that dominated in RA patients in disease remission correlated negatively with clinical parameters of RA disease activity including DAS28 (CRP) **(Fig.1f),** SDAI, synovial hypertrophy and changes in US-measured synovial blood flow whereas these clinical parameters correlated positively with MerTK/CD206^{neg} and CD163/CD206^{neg} STMs **(extended data Suppl Fig.2b and 3b)**.

We validated the relationship between STM populations and tissue resolution using the highly stringent Boolean criteria for disease remission in RA[27]. Among the 36 RA patients in clinical DAS28-defined remission, 11 also met the Boolean remission criteria at the time of biopsy. Their STMs were characterized by an increased density of MerTK expression on the MerTK/CD206^{pos} population (**Fig.1g**), and an increased proportion of CD163-expressing STMs as compared to equivalent populations in sustained DAS28-defined remission **(Fig.1h-j** and **extended data Suppl Fig.4a-d)**. This indicates that the difference in proportion between STM populations is enhanced in more established remission.

Next, we investigated the clinical significance of the relative proportion of the MerTK^{pos} and MerTK^{neg} STM populations associated with subsequent occurrence of flare after treatment withdrawal. All 36 RA patients that achieved sustained clinical and ultrasound remission had

received identical prior treatment (MTX plus TNF-inhibitor; **Suppl. Tab.1**). Among them, 22 RA patients consented to taper then discontinue treatment. Remission was maintained in 11, whereas 11 patients flared. Those patients who maintained remission had, at the time of synovial tissue biopsy, a different STM population composition compared to those who subsequently flared **(Fig.1k)**. The most prominent difference was a higher proportion of MerTK/CD206^{pos} STMs and correspondingly lower MerTK/CD206^{neg} **(Fig. 1l** and **extended data Suppl Fig.4e-i)**.

We hypothesised that this STM phenotypic profile may provide a prognostic biomarker predictive of disease flare after treatment change. To test this, logistic regression analysis was performed on the relative proportions of MerTK/CD206^{pos} and MerTK/CD206^{neg} STM populations **(Suppl Fig.4j-k)**. The proportion of MerTK^{neg} STMs alone was insufficient to predict flare. However, the ratio of MerTK/CD206^{pos} to MerTK/CD206^{neg} ≤2.5 or a proportion of MerTK/CD206^{pos} STMs ≤47.5% emerged as independent factors predicting disease flare after treatment tapering and discontinuation [Odds ratios: 16.2 (95%CI: 2.61–100.45) and 13.5 (95%CI: 2.26–80.79)], respectively) **(Fig.1m)**. These data suggest that reinstatement of MerTK/CD206^{pos} STMs in synovium and their potential local governance of other STM phenotypes may be key for maintaining synovial tissue homeostasis in RA.

Next, we used IHC/IF to explore the synovial tissue localization of MerTK^{pos} STMs comparing RA in remission with active RA. MerTK^{pos} STMs localized mainly in the lining layer both in remission and in active RA **(Fig.1n-p)**. In accordance with flow-cytometry (Fig1a), the majority of STMs (CD68^{pos}) in remission RA are MerTK^{pos} and form a tight layer **(Fig.1n)**. In contrast, in active RA the many lining-layer CD68 cells lack MerTK expression **(Fig.1p)** but if present, the MerTK^{pos}CD68^{pos} cells are dispersed in the sublining layer **(Fig.1o)**

Together, these data suggest that the distribution, localisation and relative proportions of MerTK/CD206 positive vs negative STM populations characterize distinct clinical phases of RA and are indicative of persistence of remission.

scRNAseq of STMs defines heterogeneity within MerTK/CD206^{pos} and MerTK/CD206^{neg} populations associated with synovial homeostasis, and progression and resolution of synovitis.

To better understand the heterogeneity and changing patterns of human MerTK/CD206^{pos} and MerTK/CD206^{neg} STM populations during development and resolution of arthritis, we performed unbiased molecular profiling (scRNAseq), followed by validation with flow cytometry and immunofluorescent IHC staining, of all CD11b/CD64^{pos} STMs from treatment-naive RA (n=5), treatment-resistant RA (n=6) and RA in sustained remission (n=6). As comparators for RA, we included STMs from healthy (n=4) and pathological control (autoantibody-negative, undifferentiated peripheral arthritis[28], n=4) **(Suppl Table 2-3)**. Our initial analysis of whole synovium by scRNAseq

provided unbiased confirmation that CD11b and CD64 markers captured the whole STM population. However, some CD11b/CD64^{pos} STMs also expressed CD1c therefore these STMs were included in subsequent scRNAseq analyses **(Suppl Fig.5).** The transcriptome of 32,141 STMs (>5,000/condition) revealed 9 STM clusters (phenotypes) **(Fig.2a, Suppl Table 4)**; each characterized by the expression of 63-432 unique genes **(Fig.2b-c, Suppl Table 5)**.

The developmental relationship between these 9 clusters revealed by diffusion map **(Fig.2d)**, hierarchical-clustering analyses **(Fig.2e)** and by gene expression of MerTK, CD163 and CD206 (**Fig.2f and 2c)** classified them into 4 subpopulations (TREM2^{pos}, FOLR2^{pos}, HLA^{pos} and CD48^{pos}) comprising two main MerTK^{pos} and MerTK^{neg} STM populations as previously defined by flow cytometry **(Fig 1)**. The TREM2^{pos} and FOLR2^{pos} are MerTK^{pos} STMs whereas the HLA^{pos} and CD52^{pos} comprise MerTK^{neg} STMs (*STM taxonomy is proposed in* **Fig.2g)**. Analysis of 9 clusters provided insight into the phenotypic heterogeneity within the MerTK^{pos} and MerTK^{neg} STM subpopulations. The TREM2^{pos} subpopulation contains two phenotypes (clusters); TREM2^{low} and TREM2^{high} and the latter is further distinguished by co-expression of TIMD4 and CD163. The FOLR2^{pos} subpopulation contains three distinct phenotypes (clusters) categorized by their top marker genes as ID2^{pos}, LYVE1^{pos} or ICAM1^{pos}. The MerTK^{neg}HLA^{pos} subpopulation contains two clusters distinguished by CLEC10a expression and either an interferon signature (ISG15^{pos} cluster) or an antigen presenting cell signature (CLEC10A^{high} cluster). The latter resembles dendritic cells[29] and likely represents synovial tissue antigen presenting cells. The CD48^{pos} subpopulation of MerTK^{neg} STMs is enriched in either alarmins (S100A12^{pos} cluster) or osteopontin (SPP1/CD9^{pos} cluster). The SPP1^{pos} and the ISG15^{pos} clusters (i.e. MerTK negative) were previously noted in the synovium of active RA[17] thus validating our analysis strategy.

To discover condition-specific STM profiles, and genes indicative of mechanisms of homeostasis, pathogenesis and resolution of arthritis, we compared differences in the relative proportions of the 9 clusters and their unique pathways between clinical states (**Fig.2h-i, Suppl Fig.6a)**. *The healthy synovium* contains predominantly MerTK^{pos} STMs (validating flow cytometry data from Fig.1) comprising TREM2^{pos} and FOLR2^{pos} subpopulations. Pathway analysis revealed that these subpopulations are enriched in complement and defensin pathways (contrasting with MerTK^{neg} STMs), suggesting efferocytosis and anti-microbial functions (**Suppl Fig.7a and Suppl Table 6)**. They also express genes of retinoic acid production (*ALDH1A1, RBP4*) which drive regulatory T-cell differentiation[30], and show high expression of the B7-related co-inhibitory molecule *VSIG4* which inhibits effector T-cells[31], suggesting a role in the local regulation of adaptive immunity. Healthy synovium had the highest proportion of the TREM2^{high} cluster compared with all RA synovia, consistent with recent murine studies suggesting that these TREM2^{pos} cells have a protective function[7]. In addition, human TREM2^{high} STMs have a distinct transcriptome indicative of phagocytosis *e.g.* high expression of scavenger receptors (*e.g. TIM4, MARCO*) and lipid (*e.g.*

cholesterol) binding proteins (*APOE, APOC1, FABP5*), and components of the phagosome, together suggesting a role in clearing microbes, apoptotic cells and oxysterols (**Suppl Fig.7a**). High expression of *MERTK* and *LILRB5*, which inhibit TLR/cytokine[32] and integrin/FcγR[33] driven activation respectively, suggests they may also restrain inflammation **(Suppl Fig.7a)**. Interestingly, *early undifferentiated arthritis (UPA)* showed increased proportions of the MerTK^{pos}-TREM2^{low} STM cluster **(Fig.2g-i**) which is closely related to the TREM2^{high} cluster in terms of transcriptomics and cell trajectory **(Fig.2b,e, Suppl Fig.7)** but has increased oxidative phosphorylation as evidence of a change in metabolism and cytoskeletal activation **(Suppl Fig.7a)**, and may represent an early activation phenotype of the protective TREM2^{high} STMs.

Treatment-naïve and treatment-resistant *active RA* had increased proportions of the MerTK^{neg} -CD48/SPP1^{pos} cluster, and treatment-resistant RA additionally had an increased MerTK^{neg} -CD48/S100A12^{pos} cluster (**Fig.2i**). Their transcriptomes indicate pro-inflammatory phenotypes *e.g.* increased expression of glycolytic enzymes (*LDHA, ALDOA, PKM, ENO1*; **Suppl Fig.7b**) indicating that their activation is fuelled by glycolysis. The top marker of the SPP1^{pos} cluster (osteopontin) has multiple pro-inflammatory and bone-resorbing properties[34], and high levels of cytoskeletal proteins and integrins suggesting a migratory phenotype (**Suppl Fig.7b**). This confirms and considerably extends the observation of SPP1^{pos} STMs in active RA[17]. The S100A12^{pos} STM cluster is a novel finding of importance in active RA because of its abundance and high expression of inflammation-triggering alarmins S100A8/9/12. These are chemoattractant for neutrophils and monocytes and can bind RAGE/TLR4 on fibroblasts and monocytes to induce pro-inflammatory cytokines IL-6 and TNF[35]

In *sustained remission* RA, the MerTK^{neg}-CD48/SPP1^{pos} cluster was absent, yet the MerTK^{neg}-CD48/S100A12^{pos} cluster persisted. This STM cluster may constitute the persisting MerTK^{neg} cells in RA patients in remission who flare **(Fig.1l)**. Moreover, in contrast to active RA, and in accordance with our flow cytometry **(Fig.1b)**, RA patients in remission have an increase in MerTK^{pos} STMs shown by scRNAseq to be the FOLR2/LYVE1^{pos} cluster. Specific expression of LYVE1 suggests they are perivascular tissue macrophages[38]. Their distinct transcriptome (*e.g. BLVRB, HMOX1*) suggests heme-degradation and iron homeostasis functions. Also, their transcriptome is selectively enriched in regulators of tissue collagen turnover *(e.g. STAB1[39], TGFBI[40]*), antiprotease enzymes (*e.g. A2M*), coagulation factors (*F13A1*) and regulators of VEGFR on endothelial cells (*SERPINF1*) **(Suppl Fig.7a)**, together suggesting a role of this STM cluster in the control of synovial tissue remodelling and homeostasis.

The proportions of MerTK^{neg}-CLEC10a^{pos}, MerTK^{pos}-ID2^{pos} and MerTK^{pos}-ICAM1^{pos} clusters were similar in healthy and RA synovia. The CLEC10a^{pos} cluster is enriched in antigen-presentation pathway genes and DC markers[29], and in DC transcription factors (*e.g.* NR4A3[41]), strongly suggesting that this population represents synovial tissue-resident antigen presenting cells **(Fig.2i).** This cluster has a recently described myeloid phenotype expressing HBEGF, EREG and PLAUR[20]

that potentially promotes synovial fibroblasts invasiveness in active RA. We confirmed that the expression of HBEGF in this cluster is increased only in early inflammation, consistent with a role in initiating synovitis **(Suppl Fig.6d).**

To validate the classification of STM clusters based on scRNAseq analysis, we investigated the expression of markers of the most sizeable subpopulations/clusters by multiparameter flow cytometry of additional RA (n=29) and healthy (n=9) synovial tissues, in conjunction with the initial STM MerTK/CD206 classification (Fig with your nice cluster definition). Regarding the MerTK^{pos}population, patients with active RA had a decrease in the proportion of TREM2^{pos} subpopulation (defined as MerTK/TREM2^{pos}) and LYVE1^{pos} clusters (defined as MerTK/LYVE1^{pos}) compared to healthy synovium, and their proportions were restored in patients in sustained disease remission to the levels similar (TREM2^{pos}) to, or significantly higher (LYVE1^{pos}) than of those in healthy tissue **(Fig.2j-k and Suppl Fig.8-9).** Regarding the MerTK^{neg} population, the scRNAseq data identified CD48 as a composite marker **(Fig.2l).** Antibodies against CD48 with CLEC10a and CD9 defined the different MerTK^{neg} clusters and demonstrated that SPP1^{pos} (identified as CD48^{pos}/CD9^{pos}/CLEC10a^{neg}) and S100A12^{pos} (identified as CD48^{pos}/CD9^{neg}/CLEC10a^{neg}) clusters were scarce in health, abundant in active RA and strongly reduced in sustained disease remission **(Fig.2m and Suppl Fig.8-9)**. The scRNAseq analysis suggested similar proportions of the MerTK^{neg}/CLEC10a^{pos} cluster between clinical conditions, however additional flow cytometry revealed that the CLEC10a^{pos}/CD48^{pos}/CD9^{pos} cluster was increased in active RA compared to health and RA in remission **(Fig.2m).** This suggest these markers includes related but distinct phenotypes, grouped by their expression of CLEC10a and antigen presenting pathways (*e.g.* DCs) not included in Umap (Fig.2a). We investigated the STM cluster signatures of active RA using the independent Pathobiology of Early Arthritis Cohort (PEAC; synovial biopsy RNA from 90 RA patients[36,37]). This analysis confirmed that SPP1 and S100A9 were the top two markers of the MerTK^{neg} clusters and that their expression correlated positively with disease activity **(Fig.2n)**. All together, these observations validate the scRNAseq-based classification of STMs within the main MerTK/CD206^{neg} and MerTK/CD206^{pos} populations **(Fig 1a)** and corroborate differences in their proportions in health, synovitis and sustained disease remission.

Next, to localise the scRNAseq-categorised STM clusters by fluorescence IHC within synovial tissue structures, we used cluster-specific markers in conjunction with the macrophage marker CD68. This showed that MerTK^{pos} STM clusters (TREM2^{pos} and LYVE1^{pos}) are predominantly localised in the lining layer **(Fig.1o and Suppl Fig.10-11** and **Suppl Table 8),** while MerTK^{neg} STM clusters (CLEC10a^{pos}, S100A12^{pos} or SPP1^{pos}) **(Suppl Fig.12-14)** are predominantly localised in the sublining layer. TREM2^{pos} STMs form a lining-layer in the healthy synovium and in the synovium of RA patients in disease remission. However, they were dispersed and distributed in both layers in active RA **(Suppl. Fig.10)**, consistent with recent findings of disruption of tight linining

layer in active RA[7]. LYVE1^{pos} (FOLR2^{high} cluster) STMs were predominantly found in the lining layer in health and in RA in remission, and to a lesser extent in active RA. In the latter, the LYVE1^{pos} (FOLR2^{high}) STM cluster was also located around specific structures *e.g.* blood vessels in the sublining layer **(Suppl Fig.11)**. The CLEC10a^{pos} STM cluster, which based on their transcriptome represent HLA^{high} antigen presenting cells, are exclusively distributed in the sublining layer in close proximity to lining layer TREM2^{pos} STMs in healthy, active RA and remission RA synovial tissue. Only a proportion of these cells are CD68^{pos}, which is consistent with the scRNAseq data suggesting that CLEC10a is a marker for both tissue resident DCs (CD68^{neg}) and the interferon signature ISF15^{pos} STM cluster with high HLA expression **(Suppl Fig.12)**. The S100A12^{pos} and SPP1^{pos} clusters were mostly restricted to the sublining layer and were abundant in active RA and scarce in health and in RA in remission, confirming the scRNAseq and FACS data **(Suppl Fig.13-14)**.

Together, these comprehensive data systematically map the transcriptomic heterogeneity in MerTK^{neg} and MerTK^{pos} STM populations spanning different clinical states, suggesting distinct inflammatory and regulatory functions that may actively contribute to RA synovitis or disease remission. Next, we investigated their functional complexity *ex vivo*.

MerTK/CD206^{neg} STMs produce pro-inflammatory cytokines and alarmins, and MerTK/CD206^{pos} STMs produce inflammation-resolving mediators

To evaluate the functions of MerTK^{pos} and MerTK^{neg} STM populations, we tested their *ex-vivo* responses to components of the inflammatory synovial microenvironment, and compared their functions in patients in remission and with active RA. We FACS-sorted MerTK/CD206^{pos} and MerTK/CD206^{neg} STMs from patients with active RA, and MerTK/CD206^{pos} STMs from RA patients in remission and compared their response to LPS (surrogate for danger-signal ligand binding TLR4) and/or GAS6 (endogenous ligand for suppressive MerTK). Culture supernatants were assayed for multiple cytokines, chemokines and resolving mediators **(Fig.3a)**.

MerTK^{neg} and MerTK^{pos} STMs differed significantly in their responses to TLR4-stimulation **(Fig.3b-d)**. MerTK^{neg} STMs produced significantly more IL-6, TNF, IL-1β, CCL2 and CCL3 than MerTK^{pos} STM from either active RA or RA in remission. After stimulation, both populations produced comparable concentrations of tissue remodelling cytokines (IL-13), and cytokines involved in the response against pathogens (*e.g.* IL-8, IL-12p70, IFNα2). In contrast to MerTK^{pos} STMs from active RA, the MerTK^{pos} STMs from RA in remission did not spontaneously produce any of the pro-inflammatory mediators tested, and the concentrations of cytokines produced per cell after stimulation were lower than from STMs from active RA. Interestingly, following LPS stimulation, IL-10 was produced in similar concentrations by all STM populations regardless of clinical state. In contrast to pro-inflammatory cytokines, the inflammation-resolving lipid mediator resolvin D1 was released only by MerTK^{pos} STMs, and the concentrations were strikingly higher in culture supernatants of STMs from disease remission (**Fig.3d**). This differential production of resolvins is

consistent with the transcriptomic profile of the MerTK/TREM2^{high} STM cluster which is enriched in pathways involved in the production of lipid mediators **(Suppl Fig.7a)**. This, together with the high ratio of IL-10 to TNF produced by MerTK^{pos} STMs, suggests that their functions include preventing excessive synovial inflammation and favouring resolution.

As expected, there was no immunomodulatory effect of GAS6 on the receptor-negative MerTK^{neg} STM population. Interestingly, there was no influence of GAS6 on the LPS-induced cytokine production by MerTK^{pos} STMs from patients with active RA. Rather, GAS6 reduced further the low production of LPS-induced pro-inflammatory cytokines, especially IL-6, by MerTK^{pos} STMs from RA patients in remission. This difference in response to GAS6 could be attributable to the lower surface density of MerTK that we identified by FACS on MerTK^{pos} STMs from active RA (**Fig.3e**).

Together, these data indicate that MerTK^{neg} STMs have a pro-inflammatory phenotype, whereas MerTK^{pos} STMs, in particular those from RA patients in remission, have a resolving phenotype, and utilize a GAS6/MerTK negative-feedback regulatory loop to attenuate the response to inflammatory stimuli.

Our scRNAseq profiling revealed that the MerTK^{neg} STM population persisting in RA patients in remission was MerTK^{neg}-CD52/S100A12^{pos}. This cluster expressed alarmins (**Fig.2c and Suppl Fig.7b**) and may contribute to flares of arthritis. To test whether these MerTK^{neg} STMs release alarmins, and to compare the production of alarmins by MerTK^{neg} STMs from RA patients in remission with those with active RA, we quantified S100A12 in culture supernatants from LPS-stimulated MerTK^{neg} and MerTK^{pos} STMs, FACS-sorted from biopsies of RA patients with active RA and RA in remission. As expected, MerTK^{pos} STMs from RA patients in remission produced negligible concentrations (2.1±1.4pg/ml) of S100A12. In contrast, MerTK^{neg} STMs produced high levels (155±43pg/ml); similar to levels produced by MerTK^{neg} STMs from active RA (176±39pg/ml). Consistent with this, the transcriptomic analysis of the MerTK^{neg} -CD52/S100A12^{pos} STM cluster confirmed high expression of S100A12, 8 and 9 in remission RA; equivalent to that of RA patients with treatment-naïve active RA (**Fig.3f-h**). Thus, the MerTK^{neg} -CD52/S100A12^{pos} cluster, when present in patients in remission, has the potential to produce alarmins and initiate inflammation and flare, with the same potency as STMs from patients with active RA.

In summary, MerTK^{neg} and MerTK^{pos} STM populations have distinct pro-inflammatory and resolving properties respectively. MerTK^{neg} STMs from RA patients in remission can produce pro-inflammatory alarmins upon stimulation and may contribute to flare of arthritis upon treatment modification if not counterbalanced by the governing functions of MerTK^{pos} STM.

MerTK^{pos} STM clusters from RA in disease remission have a unique regulatory signature

To investigate the molecular signature underlying the resolving phenotype of the MerTK^{pos} clusters in remission (*i.e.* TREM2^{low}, TREM2^{high}, FOLR2^{high}/LYVE^{pos}) we compared their

transcriptomes between health, joint inflammation and resolution. These evolving programs included pathways induced during RA inflammation that resolve in disease remission (*e.g.* glycolysis: *ALDOA, ENO1*), or pathways inhibited by inflammation that return to levels similar to those of healthy STMs (*e.g.* scavenger receptor *MARCO*, and the leukotriene and resolvin regulator *ALOX5AP* **(Suppl Fig.15 and Suppl Table 12**). However, some programs underwent transcriptomic changes during inflammation that did not return to normal levels in remission. These include sustained *upregulation* of the antigen-presentation pathway, and sustained *repression* of the regulatory signature typical of healthy STMs (*e.g.* retinoic acid pathway[30] and a B7-relate co-inhibitory molecule *VSIG4*[31]). These findings suggest long-term epigenetic imprinting triggered by inflammation that does not resolve **(Fig.4a-b; Suppl Fig.15.**

Interestingly, the MerTK^{pos} clusters: TREM2^{low}, TREM2^{high} (and to a lesser extent FOLR2/LYVE1^{pos}) in remission STMs had a regulatory transcriptomic signature different from the regulatory transcriptomic signature of healthy STMs **(Fig.4a,c, Suppl Table 13).** This signature is characterized by upregulation of transcription factors (*KLF2, KLF4, NR4A1, NR4A2* and *ATF3*), and upregulation of dual-specificity phosphatase 1 (*DUSP1*). These emerged in the top 30 upregulated genes in each of the MerTK^{pos} clusters in remission. Murine studies suggest that these transcription factors and DUSP1 are negative regulators of inflammation that can reinstate tissue homeostasis. Specifically, DUSP1 drives destabilization of pro-inflammatory mRNA transcripts[42] and lack of DUSP1 increases susceptibility to experimental arthritis[43]. KLF2 and KLF4 coordinate the expression of receptors that recognize and remove apoptotic cell (*e.g.* MARCO, TIM4) and inhibitors (*e.g.* SOCSs, A20) that limit the responses to intracellular TLR ligands[44], while ATF3 inhibits type I interferon production induced by those ligands[45]. NR4A1 and NR4A2 coordinate a metabolic switch from pathological glycolysis to homeostatic oxidative phosphorylation, and trans-repress NFκB to limit the pro-inflammatory response to extracellular danger signals, respectively[46-48]. We confirmed that this remission-specific transcriptomic signature is linked to upstream activation of MerTK by demonstrating *in vitro* that their expression was reduced by a MerTK inhibitor **(Fig 4d).** A subsequent analysis of this signature in RA synovium using the independent PEAC cohort[36] confirmed that KLF4 and NR4A2 expression in RA synovium correlates negatively with disease activity **(Fig.4e)**.

In summary, these data clearly indicate that MerTK^{pos} STM clusters in RA disease remission have regulatory functions characterised by a unique set of transcription factors.

MerTK/CD206^{neg} STMs and MerTK/CD206^{pos} STMs induce inflammatory and repair responses respectively in primary fibroblast-like synoviocytes (FLS)

We next tested the potential resolving versus inflammatory roles of CD206/MerTK^{pos} and CD206/MerTK^{neg} STMs in modulating the synovial tissue environment by *ex vivo* micro co-culture with primary FLS. We FACS-sorted synovial tissue fibroblasts from biopsies of RA patients as we described previously[16] **(Fig.5, Suppl Fig.16 a-c and Suppl Table 9)**. FLS were seeded at 3000/well

alone or in contact with either 3000/well FACS-sorted MerTK/CD206^{neg} or MerTK/CD206^{pos} STMs from patients with active or remission RA respectively, for 48h. The modulatory effect on the FLS was evaluated by comparing their expression of 446 immune/stromal genes (scRNAseq BD Rhapsody, **Fig.5a**). 23,629 FLS were evaluated. Based on this gene panel, FLS cells alone exhibited 4 distinct activation states: FLS cluster 1 (FLS1) expressed extracellular matrix proteins (*e.g. COL1A1, COL1A2*) and TGFβ (*TGFBI, TGFB3*); FLS2 expressed cell adhesion molecules (*e.g. ITGB2, SELPLG*); FLS3 expressed receptors for TGFβ and resolvin (*e.g.CMKLR1 and TGFBR1*); and FLS4 expressed high levels of glycolytic enzymes and proliferation markers (*e.g. LDHA, PGK1, ENO1* and *PCNA*) **(Fig.5b-c)**

Interestingly, following co-culture with MerTK/CD206^{neg} STMs an additional fifth cluster (FLS5) emerged characterised by high expression of cartilage and bone destructive mediators (*e.g. MMP1, MMP3 and RANKL*), proinflammatory cytokines (*e.g. IL6*) and chemokines that recruit neutrophils *(e.g. CXCL8, CXCL1, CXCL2, CXCL5*) monocytes (*e.g.CCL2* and *CXCL3*) and T-cells (*e.g.CCL20* and *CXCL10*) **(Fig.5d-f and Suppl Fig.16f)** together suggesting inducible pro-inflammatory properties. This FLS5 cluster was similar to a 'positive control' FLS cluster that emerged when the MerTK/CD206^{neg} STMs isolated from patients with active RA were additionally pre-treated with LPS before co-culture with primary FLS **(Fig.5d-f).** The increase in the expression of inflammatory mediators in FLS co-cultured with MerTK/CD206^{neg} but not MerTK/CD206^{pos} STMs was also evident at the pseudobulk (mean expression) level **(Fig.5g-h).** Importantly, this differential influence of MerTK/CD206^{neg} and MerTK/CD206^{pos} STMs on FLS was observed with the STM populations isolated from the same biopsy, supporting the concept that these populations may have intrinsic distinct functional states. In contrast to the pro-inflammatory effect of the MerTK/CD206^{neg} STMs modulating FLS, the MerTK/CD206^{pos} STMs, especially those isolated from biopsies of RA patients in sustained disease remission, induced FLS repair responses *e.g.* increased expression of collagens (*e.g. COL1A*) and TGFβ response genes *(e.g. TGFBI*) **(Fig.5i)**. These data further support the opposing roles of MerTK/CD206^{neg} and MerTK/CD206^{pos} STMs in modulating the synovial pro-inflammatory versus protective environment.

To test mechanistically the specific role of the MerTK receptor characteristic of MerTK/CD206^{pos} STMs in the interactions between MerTK^{pos} macrophages and FLS, we used a surrogate system of human monocyte-derived macrophages (MoM), which have M-CSF-driven expression of MerTK **(Suppl Fig.15a)**. These MoMs were pre-incubated with or without a MerTK-selective inhibitor, and with or without TLR4 stimulation (LPS) before co-culturing with FLS[49] in direct-contact or in trans-well indirect-contact systems. Macrophages pre-treated with LPS increased the FLS expression of MMP1, MMP3 and IL-6 that was contact dependent. Pre-treatment of these macrophages with a MerTK inhibitor further increased FLS expression of MMP1, MMP3, IL-6 and MMP14 **(Suppl Fig.17a-f and Suppl Fig.18)**. Comprehensive comparison of the FLS transcriptome

in these two co-culture conditions **(Suppl Fig17g**, **Suppl Table 10 and Suppl Table 11)** revealed eighty-two differentially expressed genes under macrophage MerTK regulation **(Suppl Table 11)**. String pathway analysis highlighted the pro-inflammatory cytokine pathway (15 of 216 pathway genes, $p=4.10^{-15}$) and the pro-inflammatory chemokine pathway (9 of 48 pathway genes, $p=1.79^{-12}$). In contrast, repair mediators (*e.g. FGF14*) and extracellular matrix (e.g. *COL21a*) were downregulated **(Suppl Fig.17g).** These data suggest that macrophage membrane MerTK limits the proinflammatory (cytokine) and tissue-destructive properties (MMP) of synovial fibroblasts.

Together these data indicate that MerTK/CD206^{neg} STMs trigger synovial inflammation while MerTK/CD206^{pos} STMs may re-instate joint immune homeostasis by enhancing the tissue remodelling functions of FLS.

The scRNAseq profiles of synovial fibroblasts from patients with active RA and RA in remission reveals that they have distinct transcriptomes that infer local interactions with inflammatory MerTK/CD206^{neg} or resolving MerTK/CD206^{pos} STMs, respectively.

To compare the phenotypes of active and remission FLS and investigate whether FLS of patients in remission have a transcriptome indicative of local interaction with CD206/MerTK^{pos} STMs, we performed scRNA sequencing (10xGenomics) of synovial fibroblasts rapidly FACS-sorted from RA patients in sustained remission and from patients with active RA. Unsupervised clustering of 13,949 FLS confirmed the existing classification of FLS[16-18] that distinguished one lining-layer FLS cluster expressing MMPs, and four sublining-layer FLS clusters (HLA^{high}, THY1^{high}, THY1/CXCL14^{pos}, THY1/CD34^{pos}) expressing collagens and immune-mediators **(Fig.6a-e and Suppl Table 14)**.

There were no differences in the relative proportion of these clusters between active RA and RA in sustained remission**.** However, crucially, their transcriptome differed **(Suppl Table 15**). In particular, we observed decreased expression of mediators induced by MerTK/CD206^{neg} STMs and identified as negatively regulated by MerTK. These include metalloproteinases *(MMP1, MMP3)* and chemokines (*CXCL1, CXCL8)*. In contrast, mediators of tissue repair and resolution (*e.g.* LTBP4, IGFBP5/6 and AXL) were increased. Thus, lining-layer FLS of RA patients in disease remission have a transcriptomic signature of a 'resolved/repair' phenotype potentially induced by interaction with MerTK/CD206^{pos} STMs.

We showed that GAS6 regulate the functions of remission MerTK/CD206^{pos} STMs (Fig.3). thus, we investigated potential sources of the MerTK ligand GAS6 in the synovium. Our single-cell transcriptome analysis of FLS revealed that sublining FLS clusters express GAS6 mRNA; abundantly in the small THY1/CXCL14^{pos} cluster **(Fig.6e).** GAS6 expression increased in the THY1^{high} cluster from RA in remission as compared to active RA (**Fig.6i)** suggesting a potential increase in GAS6 in specific tissue niches of resolving synovium. Experimentally, *in vitro* production of abundant GAS6 has been shown by cultures of primary FLS lines derived from biopsies of RA patients **(Suppl Table 16** and **Fig.6j)** and experimental gene-deletion of GAS6 in FLS with siRNA,

increased FLS pro-inflammatory phenotype following contact with macrophages **(Suppl Fig.19).** Thus, GAS6 derived from sublining FLS may contribute to the homeostatic regulatory functions of lining layer STMs expressing MerTK.

Discussion

This comprehensive study provides the first comparative description of the functional biology of human synovial tissue macrophages in health, active RA synovitis, and RA in sustained disease remission. Our data define the presence and function of different human macrophage subpopulations. This knowledge will inform future biomarker and therapeutic target opportunities in RA and potentially other chronic immune-mediated inflammatory diseases. Notably, we provide a novel cellular and molecular explanation suggesting that RA remission as an actively restrained state.

We combine integrated analysis of scRNAseq of >32,000 STMs from 25 synovial biopsies, FACS-phenotyping of STMs from 132 biopsies, and functional analysis of STMs from 42 biopsies. This spanned healthy homeostasis, early undifferentiated joint inflammation (UPA), treatment-naïve early RA, treatment-resistant RA and RA in sustained remission. Multiparameter flow cytometry showed that STMs consisted of two main populations; positive and negative for MerTK/CD206, and scRNAseq uncovered deep phenotypic and functional heterogeneity in both, revealing RA stage-specific mechanisms of pathogenesis and of remission. The MerTK/CD206^{pos} STMs are dominant in healthy tissue and in RA in disease remission, whereas MerTK/CD206^{neg} STMs are enriched in active RA. Of prognostic importance, their relative proportion is predictive of persistent remission or flare upon drug withdrawal that is commensurate with their functional roles. Patients in disease remission whose STMs are composed of less than 47.5% MerTK/CD206^{pos} or alternatively their MerTK/CD206^{pos} to MerTK/CD206^{neg} ratio is less than 2.5 have a higher likelihood of flare after treatment cessation. This can be explained by different functions of these two populations. MerTK/CD206^{neg} STMs produce proinflammatory cytokines and alarmins and induce inflammatory responses in synovial fibroblasts. In contrast, the MerTK/CD206^{pos} STMs from the synovium of RA patients in sustained disease remission produce lipid mediators that resolve inflammation and induce a repair response of FLS, consistent with our previous findings of increase repair in RA synovium in disease remission compared with active RA[3]. Thus, an inter-cellular crosstalk between MerTK/CD206^{pos} and synovial fibroblasts during remission may govern and maintain joint immune homeostasis.

We show that regulatory MerTK^{pos} STMs consist of distinct subsets including *MerTK/TREM2*^{pos} and *MerTK/FOLR2/LYVE1*^{pos} STMs; both increased in remission, while *MerTK/TREM2*^{pos} is the dominant subpopulation of MerTK^{pos} in the healthy synovium. Their transcriptomes suggest distinct but complementary functions controlling the local immune-response and tissue homeostasis, respectively. Hierarchical clustering of orthologous human and mouse cluster-specific transcripts **(Suppl Fig.20-21)** indicates that human TREM2^{high} STMs are homologs of mouse lining-layer *Trem2/Cx3cr1*[7] STMs, and human FOLR2/LYVE1^{pos} STMs closely resemble interstitial *Relma*^{pos} STMs. These murine counterparts differentiate from locally proliferating precursors and are key for maintaining immune homeostasis. Furthermore, in remission RA these

clusters gain a unique phenotypic and transcriptomic signature that is different from similar cells in active RA and in the healthy synovium. We show that this is driven by MerTK activation, potentially by GAS6 produced locally by Thy1^{pos} synovial fibroblasts, and includes low production of pro-inflammatory cytokines, high production of resolvins and an increased expression of the set of transcription factors KLF2/4, NR4A1/2 and ATF3 that inhibit inflammation.

Of the four pro-inflammatory MertK^{neg} STM clusters, we identified that the CD52/S100A12^{pos} cluster can persist in RA patients in disease remission, and that these cells produce high levels of the S100A12 alarmin upon stimulation thereby providing a potential mechanism for flare during remission. Thus, sustained remission of arthritis appears to be an active process maintained by tissue-resident subsets of MerTK^{pos} STM subpopulations (e.g. *TREM2*^{pos} and *FOLR2/LYVE1*^{pos}) governing pro-inflammatory CD52/S100A12^{pos} STMs and synovial fibroblasts to reinstate and maintain homeostasis. Of interest, recent mouse and human studies have suggested a protective function for TREM2^{+} resident-macrophages in adipose tissues that counteracts inflammation and metabolic deregulation. Our study adds to and highlights a broad regulatory role of TREM2^{pos} tissue resident macrophages[50].

Of the three MerTK/FOLR2^{pos} STM subpopulations, the relative proportions of the minor FOLR2/ID2^{pos} and FOLR2/ICAM1^{pos} clusters remained unchanged between health and the different RA disease states. We speculate that FOLR2/ID2^{pos} may be the human equivalent of mouse M-CSF driven *in situ* precursors that give rise to mouse RELMa^{pos} [7] which is the homolog of human FOLR2. This is supported by their high expression of M-CSF-R, and ID2 which is a key driver of self-renewing haemopoietic stem cells[51] **(Suppl Fig.7a)**. We also identified a small population of cycling STMN1^{pos} STMs that also expressed M-CSF-R, and cluster with the TREM2^{low} phenotype, suggesting that they might be precursors of the resolving MerTK/TREM2^{pos} STMs subset **(Suppl Fig.20e)**. Synovial macrophages proliferating *in situ* was recently reported in patients with inflammatory osteoarthritis[52] supporting the concept of self-renewing STMs in the human synovium. This introduces the possibility for therapy-directed differentiation of STM phenotypes to reinstate and maintain synovial homeostasis.

Of interest, FOLR2/ICAM1^{pos} STMs (~0.025% of STMs) are present in the healthy synovium and their frequency did not change in inflammation and disease remission. They constitutively express high levels of mRNA for pro-inflammatory cytokines (*e.g.*TNF and IL-1β) and chemokines. Their MerTK expression and position on the ontogeny dendrogram suggest that they are members of the MerTK^{pos} population. Little is known about these intriguing cells that may form the first line of defence against pathogens in the joint.

In summary, we identified dynamic phenotypic changes in synovial tissue macrophage subpopulations spanning health, RA inflammation and disease resolution, and describe for the first time in a human autoimmune disease, active mechanisms mediating sustained disease remission facilitated via tissue resident STMs **(summary in Suppl Fig.22)**. Our findings may help to generate

novel therapeutic strategies to exploit STM-based endogenous mechanisms of resolution of synovitis and restoration of synovial homeostasis. Moreover, our scRNAseq analysis revealed, for the first time, that specific clusters of STMs (MerTK^{pos}/CD206^{pos}) with anti-inflammatory properties are predictive biomarkers of maintained remission and protective factors against disease recurrence after treatment discontinuation. The current direct anti-macrophage therapy which shows beneficial effect in RA targets GM-CSFR (mavrilimumab) *is a case in point*[53]. Our scRNAseq dataset indicates that mRNA for this receptor is preferentially expressed in inflammatory sublining MerTK^{neg} clusters: CLEC10a^{pos}, SPP1^{pos} and ISFG15^{pos}, suggesting that protective MerTK^{pos} STM located in the lining layer may persist and contribute to resolution of inflammation in patients treated with anti-GM-CSF/GM-CSFR therapies. However, a previous study showed that GM-CSFR can co-localise with CD163[54] which is expressed on protective MerTK/TREM2^{pos} subpopulation, thus a comprehensive study quantifying STM heterogeneity in RA patients before and after treatment with anti-GM-CSF/GM-CSFR therapies may be of value to predict their short and long-term responses and the balance between the two MerTK populations.

Our discovery of the clinical significance of the relative proportions of STM subpopulations in RA patients in sustained disease remission suggest this may represent a novel synovial tissue biomarker predictive of persistent remission versus flare. This cellular basis for remission could provide a molecular and cellular definition of sustained and persistent remission in RA that could be incorporated within personalized protocols for the management of RA patients in sustained “cellular” remission that would be transformative in terms of management of biologics discontinuation.

Material and Methods

Patients recruitment and management. One-hundred and twelve patients fulfilling the American College of Rheumatology 2010 revised criteria for RA[54] were enrolled and underwent ultrasound-guided synovial tissue biopsy of the knee at the Division of Rheumatology of Fondazione Policlinico Universitario A. Gemelli IRCCS – Università Cattolica del Sacro Cuore, Rome, Italy. These samples comprised the SYNGem cohort. RA patients were stratified into naïve to treatment (n=45), inadequate responder to Methotrexate (Treatment resistant RA) (n=31) and patients in sustained (for minimum 6 months) clinical and US remission under a combination of MTX+TNF-inhibitor (n=36). Ten healthy donors attending arthroscopy for meniscal tear or cruciate ligament damage, with normal synovium (MRI and macroscopically) were included as a control group (University of Glasgow). The study protocol was approved by the Ethics Committee of the Università Cattolica del Sacro Cuore (6334/15) and by the West of Scotland Research Ethics Committee (19/WS/0111). All subjects provided signed informed consent. Demographic, clinical and immunological features of the study RA and healthy cohorts are summarized in **Suppl Table 1- 3**. All treatment-resistant RA were taking stable doses of MTX (mean dose: 15.3±3.3 mg/week). All RA in sustained clinical (DAS28<2.6 for 3 sequential determinations each 3 months apart) and ultrasound remission (Power Doppler negativity at US assessment for 3 sequential determinations each 3 months apart) were selected based on published protocols[2,3]. For each RA patient enrolled, clinical and laboratory evaluations included the number of tender and swollen joints of 28 examined, Erythrocyte Sedimentation Rate (ESR), C-Reactive Protein (CRP) and Disease Activity Score (DAS28). Peripheral blood samples were tested for IgA and IgM-RF (Orgentec Diagnostika, Bouty-UK) and ACPA (Menarini Diagnostics-Italy) using commercial Enzyme-Linked Immunosorbent Assay (ELISA) and ChemiLuminescence Immunoassay (CLIA) methods respectively. After study enrolment, RA patients in sustained clinical and US remission (n=22) were first tapered on TNF-inhibitor (adalimumab 40mg/4weeks or etanercept 50mg/2 weeks) for 3 months. After 3 months of TNF-inhibitor tapering, patients who were still in US remission (Power-Doppler negative) discontinued TNF-inhibitor and were followed every 3 months while maintained on stable doses of Methotrexate (15.2±2.9 mg/week), with follow-up after treatment modification of 13.2±7.6 months during which there were no treatment modifications[2]. The relapse rate was recorded for each RA patient in sustained clinical and US remission after treatment modification[55].

Patient selection for single-cell RNA sequencing. Seventeen patients fulfilling the American College of Rheumatology 2010 revised criteria for RA[54] (5 treatment-naive RA, 6 treatment-resistant RA and 6 RA patients in sustained clinical and US remission, respectively) and 4 patients with Undifferentiated Peripheral Arthritis (UPA)[56] with at least one active knee joint, seronegative for IgA/IgM-Rheumatoid Factor (RF) and Anti-Citrullinated Peptide Antibody (ACPA) and naive to any pharmacological treatment were enrolled in the study at the Division of Rheumatology of Fondazione

Policlinico Universitario A. Gemelli IRCCS – Università Cattolica del Sacro Cuore, Rome, Italy. For each RA and UPA patient enrolled, clinical and laboratory evaluations included the number of tender and swollen joints on 28 sites, Erythrocyte Sedimentation Rate (ESR), C-Reactive Protein (CRP) and Disease Activity Score-28 (DAS). Peripheral blood samples were tested for IgA and IgM-Rheumatoid Factor (RF) (Orgentec Diagnostika, Bouty-UK) and ACPA (Menarini Diagnostics-Italy) using commercial Enzyme-Linked Immunosorbent Assay (ELISA) and ChemiLuminescence Immunoassay (CLIA) methods respectively. Each enrolled patient underwent US-guided synovial tissue biopsy and synovial tissue samples were processed following the protocol described below. Four healthy donor synovial tissues were included as control. Demographic, clinical and immunological features of patient and healthy donor samples used for scRNAseq are summarized in **Suppl Tables 2-3.**

Synovial tissue biopsies. All RA and UPA arthritis patients enrolled underwent ultrasound-guided synovial tissue biopsy of the knee following the published protocol[57] at the Division of Rheumatology of Fondazione Policlinico Universitario A. Gemelli IRCCS – Università Cattolica del Sacro Cuore (SYNGem cohort), Rome, Italy. Ultrasound (US) evaluation of the knee was performed using an ultrasound machine with a multi-frequency linear transducer (MyLab Twice, Esaote). Using the ultrasound view, the best point of entrance for the biopsy needle was identified on the lateral margin of the suprapatellar recess. Each patient was provided with a face-mask and cap, and the procedure was performed under sterile conditions. Skin was disinfected with iodine solution, performed twice, starting from the point of needle entrance up to 25cm proximally and distally. If joint effusion was present, arthrocentesis of the knee joint was performed using the lateral suprapatellar access. The skin, subcutaneous tissue and joint capsule was anaesthetized with 10ml 2% lidocaine. Next, a 14G needle (Precisa 1410-HS Hospital Service Spa, Italy) was inserted into the joint. Regions of synovial hypertrophy were identified under grey-scale guidance to ensure sampling of representative synovial tissue. All synovial tissue specimens obtained (at least eight pieces for histology and twelve pieces for singe-cell RNA-sequencing and functional experiments) were placed on a nonwoven wet sterile gauze for collection. For ***Histology***, tissue specimens were fixed in 10% neutral-buffered formalin and embedded in paraffin. Briefly, paraffin-embedded synovial tissue specimens were sectioned at 3μm. Sections were stained for Haematoxylin and Eosin as follows: sections were deparaffinized in xylene and rehydrated in a series of graded ethanol, stained in haematoxylin and counterstained in Eosin/Phloxine. Finally, sections were dehydrated, cleared in xylene and mounted with Bio Mount (Bio-Optica). Slides were examined using a light microscope (Leica DM 2000). The severity of synovitis was graded according to the three synovial membrane features (synovial lining cell layer, stromal cell density and inflammatory infiltrate), each ranked on a scale from none (0), slight (1), moderate (2), and strong (3). The values of the parameters were summed and interpreted as follows: 0–1 no synovitis, 2–4 low-grade synovitis, and 5–9 high-grade synovitis[58].

For ***MerTK Immunohistochemistry***, sections were stained with IgG2a mouse anti-human monoclonal antibody for CD68 (clone 514H12; antibody at 6.7mg/ml) (Leica Biosystem, Newcastle, UK) or IgG rabbit anti-human monoclonal antibody for MerTK (clone Y323, Abcam ab205718, dilution 1/1000) by immunostainer BOND MAX III (Leica, Newcastle, UK). Single immunohistochemical staining for CD68 or MerTK was performed as follows: 3µm sections from formalin-fixed paraffin-embedded tissue blocks and dried in a 60°C oven for 30min. Sections were placed in a Bond Max Automated Immunohistochemistry Vision Biosystem (Leica Microsystems GmbH, Wetzlar, Germany) according to the following protocol: Tissues were deparaffinized and pre-treated with the Epitope Retrieval Solution 1 (CITRATE buffer) or Solution 2 (EDTA-buffer) at 98°C for 10min according to the manufacturer's instructions. After washing, peroxidase blocking was carried out for 10min using the Bond Polymer Refine Detection Kit DC9800 (Leica Microsystems GmbH). Tissues were washed and incubated with the primary antibody for 30min then incubated with polymer for 10min, developed with DAB-Chromogen and finally counterstained with hematoxylin. Slides were examined using a light microscope (Leica DM 2000).

For ***MerTK Immunofluorescence****,* 3µm formalin-fixed RA synovial tissue sections were microwaved in a citric acid-pH 9.2 and pre-incubated with phosphate-buffered saline containing 10% bovine serum albumin (BSA) for 30min. Sections were then stained with a primary antibody against CD68 (clone L26 mouse anti-human monoclonal antibody, at 1.2 mg/ml, Leica Biosystem, Newcastle, UK), and anti-MerTK (rabbit IgG polyclonal Cy3-coniugated anti-human MerTK, clone 5770, BIOSS bs-0548R-Cy3, dilution 1/100) at 37°C for 1h. Sections were rinsed and incubated with secondary conjugated antibody fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG H&L, #ab6785, (Abcam, dilution 1/1000) at RT for 1h. Slides were mounted and scanned on a fluorescent microscope (Nikon).

Synovial tissue processing for synovial tissue macrophage phenotyping, subset FACS-sorting and scRNA-sequencing. Fresh synovial tissues were diced to 1-2 mm^3 fragments with sterile disposable no.22 scalpels and transferred into a sterile universal container containing 10ml sterile RPMI with Penicillin/Streptomycin 100/U/ml and L-Glutamine 2mM (RPMI medium) in 1/33 dilution of Liberase at 0.15mg/ml, 0.78 Wunsch units/ml [TM Research Grade (Thermolysin, Medium, Roche Diagnostics (000000005401127001, Sigma)]. Tissue pieces were incubated at 37ºC, 5% CO_2 in a humidified atmosphere 30-45min rotating on a Miltenyi MACSmix tube-rotator and shaken vigorously by hand twice during this incubation. After incubation, the digested mixture was filtered using an Easy Strain 100µM cell-strainer into a 50ml falcon tube. Residual cell clumps retained on the filter were gently massaged using the rubber end of a 1 ml syringe plunger to optimise cell retrieval. Complete medium (RPMI above plus 10%FCS) was poured through the filter into the falcon tube up to 40ml then centrifuged 1800rpm for 10min at 4ºC and the supernatant was carefully removed. One ml of complete medium was added to gently resuspend the cell pellet using a wide

opening 1ml pipette tip to minimize cell damage, then the resuspended cells were transferred to a sterile Eppendorf tube. A 20µl aliquot was used to count the cells. Cells were centrifuged at 1500rpm for 5min at 4ºC. The supernatant was removed, and cells were either aliquoted for STM phenotyping and/or STM FACS-sorting as described below, or for subsequent scRNA-sequencing (cells from 25 patients/healthy donors described above (Tables 1-3). Cells were added to 1ml of ice-cold freezing mix [Bambanker (302-14681; Wako)], immediately frozen at -80ºC then stored in liquid nitrogen.

Phenotyping and FACS-sorting of STM populations. Digested biopsies were centrifuged at 1800rpm for 10min, resuspended and washed with FACS buffer, and transferred to FACS tubes (BD Biosciences) in a final volume of 3ml FACS buffer (PBS/2%FSC/2mMEDTA). An 80µl aliquot was set aside for live-dead gating (unstained cells). To the rest of the cells, Fixable Viability Dye eFluor™ 780 (eBioscience) was added at 1:1000 in PBS and incubated for 20min at 4ºC. Cells were then washed with FACS buffer. Four tubes were labelled: a) unstained b) live-dead marker only c) Fluorescence Minus One Control (FMO) tube, FMO minus FITC, where cells were stained with antibodies specific for STM but not FITC-antibodies against all other lineage-positive cells d) cells stained with antibodies against STMs and FITC-antibodies against any unwanted lineage (Suppl Fig1a). Staining was performed in a final volume of 500µl with antibody dilution 1/100 for 30min on ice. All antibodies are listed in **Suppl Fig.1a**. Cells were washed twice with FACS buffer and resuspended in a finial volume of 500µl, filtered through an Easy Strain 100µm cell-strainer and analyzed or sorted with the use of FACS ARIAIII sorter (BD Bioscience). Synovial tissue macrophages were gated based on their membrane expression of CD45, CD64, CD11b, and HLA-DR after all other cell lineages (FMO-FITC gating) and cell-doublets were excluded (dump channel-1). FMO-FITC cells were used to set up a gate to exclude unwanted lineage-positive cells (dump channel). The expression of MerTK, CD163, CD206, TREM2, FOLR2 and TIM4 were evaluated on gated CD64^{pos}CD11b^{pos}HLA-DR^{pos} STMs **(Suppl Fig.1)**. In addition, MerTK/CD206^{pos} and MerTK/CD206^{neg} STM populations were FACS-sorted from 47 synovial biopsies. The cells were sorted into FACS tubes containing 2ml of complete RPMI1640. Post-sorting purity of macrophages was performed, and all data generated were analyzed using FlowJo software (TreeStar Inc., OR, USA).

Ex-vivo stimulation of sorted STMs. MerTK/CD206^{pos} and MerTK/CD206^{neg} STMs were FACS-sorted into complete medium and plated into a 96-well flat-bottom cell-culture plate, pre-coated with collagen (Sigma; bovine collagen at 1:300 dilution). The precoating protocol was as follow: wells were incubated with collagen at 37ºC, 5% CO_2 for 2h and then washed twice with PBS. STMs were seeded at 1000 cells/well and stimulated with LPS (10ng/ml, Sigma, L6529) or human recombinant Gas6 (100 ng/ml, R&D Systems, 885-GSB-050), or both in combination, or left unstimulated for 24h in a total volume of 100µl. The supernatants were then harvested and assayed

using an ultra-sensitive 19-plex assay (Meso Scale Discovery, Maryland, USA), Resolvin D1 (Cayman Chemical, 500380) and S10012A (DY 1052-05 R&D Systems).

Co-culture of distinct STM populations with primary synovial fibroblasts. Synovial tissue biopsies from RA patients (n=6) were digested as described above. Synovial fibroblasts were identified by surface membrane Thy and PDPN[17], and PDPN^{pos} (lining) plus PDPN/Thy1^{pos} (sublining) FLS were FACS-sorted into Eppendorf tubes containing 0.5ml of complete RPMI1640. FLS were seeded into T25 culture flasks (Merck) with complete RPMI1640. After attaching to the plastic surface (~1 week), they were harvested and seeded into 96-well plates at 3000 cells/well and freshly FACS-sorted MerTK/CD206^{pos} or MerTK/CD206^{neg} STMs from RA patients (n=5 patients with active RA and n=5 RA patients in sustained clinical and US imaging remission) were added to the co-cultures. One patient from each group was biopsied together on the same day so that paired cultures of STM populations from active and remission RA could be compared **(Suppl Table 9 and Suppl Fig.16)**. STMs were added to FLS at 3000 cells/well in 3 technical replicates.

FLS cultured without STMs were used as comparator, and as a positive inflammatory control, FLS co-cultured with MerTK/CD206^{neg} sorted from RA patients with active RA and pre-treated with LPS (10ng/ml) for 1h were used. LPS pre-treated MerTK/CD206^{neg} were extensively washed before being added to FLS (3 times). After 48h, changes in the phenotypes of the FLS during the co-cultures were investigated using scRNAseq (BD Rhapsody system), and their associated function investigated by soluble mediators in culture supernatants quantified by luminex (PPX-06/PROCARTAPLEX, Life Technologies) **(Fig.4a)**.

Co-culture of monocyte-derived macrophages (MoM) with synovial fibroblasts. Direct co-culture system. CD14^{pos} cells were isolated from PBMC using CD14^{pos} micro-beads and AutoMACSPro (Miltenyi BioTec) according to the manufacturer's protocol. These were differentiated to monocyte-derived macrophages in complete medium containing M-CSF. Briefly, cells were plated at a density of $1x10^6$ per well in a 6-well cell-culture plate in 3ml of RPMI 1640 compete medium containing M-CSF (PeproTech, UK) at 50ng/ml. On day 3, the medium was replaced with fresh medium containing M-CSF. On day 6, cells were pre-treated with LPS (1ng/ml) in the presence of absence of MerTK inhibitor UNC1062[43] (Aobious; 250μM). After 24h, macrophages were de-attached and labelled with CellTrace Far Red (5μM, Life Technologies) according to the manufacturer's protocol. These cells were added at $2x10^3$ per well of a 96-well plate containing $2x10^3$ primary fibroblast-like synoviocyte (FLS). The fibroblasts were obtained from US-guided synovial tissue biopsies (**Suppl Table 10**) and had been labelled with CellTracer Violet (5μM, Life Technologies) 24h before the co-culture with macrophages. In experiments to demonstrate the role of FLS-derived GAS6 in FLS-MoM interactions, prior to co-culture with STMs, FLS were transfected with 5pmol GAS6 siRNA (4390824) or Silencer™ Select Negative Control (4390843) using

Lipofectamine RNAiMAX Reagent (Life Technologies) on two consecutive days. 24h after the last transfection, FLS were washed and labelled with CellTracer Violet. After 24 or 48h co-culture, supernatant was collected for assay of mediators, and macrophages and synovial fibroblasts were de-attached and stained with antibodies against the synovial fibroblast marker podoplanin (PDPN), and the macrophage marker CD64 (both at 1/100 dilution, details in Supp Fig.1a). Fibroblasts and macrophages were FACS-sorted into RLT buffer (Qiagen) containing 1% β-mercaptoethanol, based on their specific CellTracer staining and cell type-specific markers, and stored at -80ºC for RNA isolation. ***Trans-well culture system.*** CD14^{pos} monocytes were plated in a 24-well culture plate in 3ml complete medium contained M-CSF (PeproTech) at 50ng/ml. On day 3, some cells were pre-treated with LPS (1ng/ml) for 4h, and to some for the last 2h, MerTK specific inhibitor, UNC1062[43] (Aobious) was added at 100 or 250nM. Cells were then washed with PBS and Transwell inserts (0.4µm pore size) containing 3x10^5 RA synovial fibroblasts (Suppl Table 10) were added to the wells to generate a co-culture system to test the effect of soluble mediators without direct cell-contact. After 48h, supernatants were collected, and the co-cultured macrophage and fibroblast were separately lysed in RLT buffer with 1% β-mercaptoethanol (Qiagen) and stored at -80ºC for RNA isolation. The MMP Luminex panel (PPX-05/ PROCARTAPLEX MMP1, MMP2, MMP3, MMP9 and MMP13 plex) and IL-6 Elisa (both from Life Technologies) was performed on supernatants from direct and trans-well co-cultures.

Evaluation of GAS6 production by synovial fibroblasts. FLS were FACS-sorted derived from biopsies of RA patients: treatment-naïve, treatment-resistant and in sustained disease remission **(Suppl Table 17)**. FLS were expanded in complete RPMI1640 medium supplemented with 2mM Glutamax, 1mM sodium pyruvate and 1% non-essential amino acid (Life Technologies). FLS at passage 2-3 were seeded in 48-wells cell culture plates at a density of 30×10^3 cells/well in complete medium containing 1% FCS. Cells were stimulated with dexamethasone 1µM or TNF or IL-1β or IL-10 or TGFβ, or LPS10 or 100ng/ml for 24h and 48h. GAS6 was quantified in culture supernatants using the Human GAS6 DuoSet ELISA kit (R&D Systems, Catalog # DY885B).

qPCR for MMPs, IL-6, GAS6 and transcription factors. RNA from macrophages and synovial fibroblasts was isolated using RNEasy micro-kit (Qiagen), and cDNA was prepared using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). TaqMan mRNA primers/probe assays and TaQman Gene Expression master mixes (both from Life Technologies) were used for semi-quantitative determination of the genes of interest. Data is presented as relative value (*i*) $2^{-\Delta CT}$ where ΔCt=Cycle threshold for 18S (housekeeping) minus Ct for gene of interests or (*ii*) fold change, where ΔCt for selected control condition =1 or 100%.
We used the following primers/ probe TaqMan assays:
Hs00231069_m1/ATF3,

Hs00374226_m1/NR4A,
Hs01117527_g1/NR4A2,
Hs01031979_m1/MERTK,
Hs00360439_g1/KLF2,
Hs00358836_m1/KLF4,
Hs01090305_m1/Gas6,
Hs00174131_m1/ IL-6,
Hs00899658_m1/MMP1,
Hs00899658_m1/MMP1,
Hs00968305_m1/MMP3,
Hs00957562_m1/MMP9,
Hs01037006_g1/MMP14.

Single Cell Sequencing of STM and whole synovial tissues. Our experiments were performed in two academic centres. The first set of samples ('Discovery Cohort') was sequenced at the Oxford Genomics Centre (Oxford University) Synovial tissue myeloid cells were sorted before sequencing, isolating CD11b and CD64 positive cells, and lineage negative for CD3, CD19, CD20, CD56, CD49, CD117 and CD15, as described in section "*Phenotyping and FACS-sorting of STM subsets"*. Typically, 2,000-10,000 synovial tissue macrophages per sample were sorted into qPCR 0.2ml tubes pre-coated with FSC and containing 10µl PBS/0.02%BSA according to 10xGenomics protocol available online. We compared the transcriptomic profile of synovial myeloid cells from 5 subject groups: healthy, patients with undifferentiated peripheral arthritis (UPA), treatment-naïve active RA, treatment-resistant active RA and RA in sustained remission.

We sequenced a second set of samples at Glasgow Polyomics, University of Glasgow ('Validation Cohort'). These included Undifferentiated Peripheral Arthritis (UPA), treatment-naive active RA, treatment-resistant active RA and RA in sustained remission. These synovial tissue samples were analyzed for both STMs and FLS. We used this 'validation' scRNAseq transcriptomic profile of synovial myeloid cells to validate the 'discovery' transcriptomic profile of FACS-sorted STMs that had scRNAseq profiled at Oxford. Detailed demographic, clinical, serological and histological characteristics of patients in both cohorts are provided in **Suppl Table 2-3 and Suppl Fig.5a-c**.

Data from both these cohorts were integrated using the following methods: ***Processing Raw Reads.*** All steps in primary data analysis, including read alignment and generation of count matrices, were performed using the Cell Ranger (2.1) pipeline. Raw base call files (BCL) generated by sequencing were previously demultiplexed into FASTQ files per sample. The "cellranger count" tool mapped the reads against the Human genome (hg19) and performed UMI counting. ***QC & Filtering.*** The Seurat package (3.0.1)[59] in R was used to create an object (CreateSeuratObject, min.cells=5).

Cell filtering involved removal of cells with less than 500 expressed genes (subset, subset=nFeatures_RNA > 500). We also set thresholds for level of gene expression, including expression of mitochondrial genes (percent.mt). This allows for exclusion of doublets and dying cells (see **Suppl Table 4** for exact values). The data was normalized using Seurat's NormalizeData function. For the analysis of synovial macrophages only, these cells were computationally isolated with the subset function from other cell types in Validation Cohort samples based on expression of CD14, MARCO and LYZ. These STM specific markers were selected based on Discovery cohort data. The top 2000 variable genes were then identified for all samples, using the FindVariableFeatures function. ***Integration.*** Sample integration was performed following the Seurat vignette, integrating all genes that are common between samples, using the functions: FindIntegrationAnchors, and IntegrateData (features.to.intergrate to find all common genes). These "integrated" batch-corrected values were then set as the default assay and the gene expression values are scaled before running principle component analysis. ***Clustering and Dimensional Reduction.*** UMAP based on PCA cell embeddings was generated from integrated counts batch-normalized by Seurat and the first 12 principle components (PCs) are visualized (RunUMAP). The same PCs were used in determination of the k-nearest neighbours for each cell during SNN graph construction before clustering at a chosen resolution of 0.5 (FindNeighbors, FindClusters). Rhe Destiny (2.14.0) R package *(https://academic.oup.com/bioinformatics/article/32/8/1241/1744143)* was used to plot a diffusion. A count matrix with the average expression of each cluster was generated before using Seurat's PlotClusterTree function to generate a dendrogram. ***Sample Filtering.*** In order to assess the quality of each sample, we determined the pseudo-bulk expression of each cluster per sample and performed PCA analysis on the result. Sample SA139 was removed due to low sequencing depth in the macrophages. SA225 was removed due to separation from all other samples in the PCA reduced dimensional space, **(Suppl Table 4** and **Suppl Fig.23d**). ***Differential Expression Analysis.*** In order to identify cluster markers and variable genes between conditions of RA, the Seurat function FindAllMarkers was used with the "test.use" function MAST[60]. As recommended in the best practice of Seurat, for DE comparison the non-batch normalized counts were used. For identification of cluster markers, we specify that any markers identified must be expressed by at least 40% of cells in the cluster ('min.pct' parameter 0.4). List of genes characterizing each of the cluster is provided in Supplementary Table 5. For differential expression analysis between conditions, we increase this value to 0.6 to reduce the risk of sample bias. We use the default values for all other parameters. Genes are considered significantly DE if the adjusted p-value (< 0.05) by Bonferroni Correction and multiple test correction (multiple by number of tests) **(Suppl Tables 12 and 13)**. To visualise heatmaps the pheatmap package was adapted. ***Pathway Analysis.*** To investigate the function of each of our identified synovial macrophage phenotypes, we performed pathway analysis using StringDB (https://string-db.org/) and IPA. Pathways associated with positive DE marker genes were investigated for each cluster. For each cluster, the Reactome

pathways were exported and compared between all clusters in a custom R script. The script a gene ratio (number of observed genes in the pathway divided by total number of genes in the pathways as provided by String-db) as well as the associated FDR value. Only pathways with p-values less than 0.05 are listed (**Suppl Table 6**, **Suppl Fig.7)**. Similar approaches were used to analyse FLS scRNAseq data from "Validation cohort". *Raw data is accessible at EMBL-EBI with the accession number E-MTAB-8322.*

***Validation of STM clusters identified by scRNASeq by flow cytometry*.** To validate the clusters identified by scRNAseq, we developed the antibody panel 1 and the antibody panel 2 (**Suppl Fig.7a**), which were used in conjunction with anti- MerTK, anti-CD206 and dump panel antibodies **(Suppl Fig.1a).** The tissue processing and total STM gating was performed as described above. The clusters were defined in each individual patient by panel 1 and /or panel 2 using FlowJo software (Tree Star Inc, OR, USA) and quantitative data are presented in Figure 1. In addition, the changes in clusters' distribution between different conditions were visualised by Cytobank software. Briefly, data were in the .fcs file format. All .fcs files were uploaded to Cytobank (www.cytobank.org) using the automated dimensionality reduction algorithm viSNE.

Immunofluorescence staining for distinct STM markers. Synovial tissue biopsies from healthy donors (n=5) and RA patients (n=6 each; active RA and remission RA) **(Suppl Table 8 and Suppl Table 18)** were preserved in 10% formalin and embedded in paraffin following standard protocols. For antigen retrieval, tissue-fixed slides where immersed in 0.01M-citrate buffer, pH 6.0 (TCS HDS05-100) and boiled in a microwave at full power for 5mins followed by power reduced to 30% for a further 8mins. Slides were left to cool for 15mins before being washed in distilled water for 5mins and then washed twice in TBS/0.025% Triton X-100 (Invitrogen) buffer for 5mins. Sections were then incubated with TBS/1%BSA plus 10% normal human serum and 10% serum of the species in which the secondary antibodies ware raised (*e.g.* goat serum) at room temperature for 2h to minimise non-specific binding. Sections were then incubated with primary antibodies against TREM2 or LYVE1 or CLEC10A or S100A12 or Osteopontin (SPP1) in combination with antibody against the macrophage marker CD68, or appropriate isotope controls (dilutions provided in **Suppl Table 18)** overnight at 4ºC. The next day, sections were washed twice for 5mins in TBS/0.025% Triton X-100 and then incubated with secondary antibodies (Invitrogen) diluted in TBS/1% BSA buffer **(Suppl Table 18)** at room temperature for 1h. After incubation, the stained sections were washed 3 times in TBS and counterstained with mounting media containing DAPI (H-1800-2/VECTASHIELD® Vibrance™). The sections were visualised with confocal microscope Zeiss LSM 880 confocal microscope, using either a water immersion 40x (NA 1.3) or an oil immersion 63x (NA 1.4) objective.

Single-cell RNA sequencing and analysis of STM-FLS co-cultures. After 48h co-culture, cells were de-attached using Accutase solution (A6964, Merck) according to the manufacturer's protocol. Cells were transferred into U-bottom 96-well plates and harvested by centrifugation at 200g for 4 min at 4ºC. Supernatant was preserved at -80ºC for cytokine/chemokine/MMP analysis. The cells from each co-culture variant were labelled with unique Tags using Single-Cell Multiplexing Kit (Cat. No. 633781) for 15min at room temperature.

The following sample Tags were used:

Sample Tag 1— ATTCAAGGGCAGCCGCGTCACGATTGGATACGACTGTTGGACCGG Sample Tag 2—TGGATGGGATAAGTGCGTGATGGACCGAAGGGACCTCGTGGCCGG Sample Tag 3—CGGCTCGTGCTGCGTCGTCTCAAGTCCAGAAACTCCGTGTATCCT Sample Tag 4—ATTGGGAGGCTTTCGTACCGCTGCCGCCACCAGGTGATACCCGCT Sample Tag 5—CTCCCTGGTGTTCAATACCCGATGTGGTGGGCAGAATGTGGCTGG Sample Tag 6—TTACCCGCAGGAAGACGTATACCCCTCGTGCCAGGCGACCAATGC Sample Tag 7—TGTCTACGTCGGACCGCAAGAAGTGAGTCAGAGGCTGCACGCTGT Sample Tag 8—CCCCACCAGGTTGCTTTGTCGGACGAGCCCGCACAGCGCTAGGAT Sample Tag 9—GTGATCCGCGCAGGCACACATACCGACTCAGATGGGTTGTCCAGG Sample Tag 10—GCAGCCGGCGTCGTACGAGGCACAGCGGAGACTAGATGAGGCCCC Sample Tag 11—CGCGTCCAATTTCCGAAGCCCCGCCCTAGGAGTTCCCCTGCGTGC Sample Tag 12—GCCCATTCATTGCACCCGCCAGTGATCGACCCTAGTGGAGCTAAG. Cells were then washed 3x with PBS with centrifugation steps (200g for 4 min in 4ºC), after which the Tagged co-culture variants were pooled and stained with Fixable Viability Dye eFluor™ 780 (eBioscience) as described above. 50x10^4 live cells were sorted and immediately loaded onto the scRNAseq BD Rhapsody™ Cartridge using BD Rhapsody™ Cartridge Reagent Kit (Cat. No. 633731) according to the manufacturer's protocol. Single cell cDNA was prepared using BD Rhapsody™ cDNA Kit (Cat. No. 633773). This was followed by single cell mRNA and Tag library preparation using BD Rhapsody Targeted mRNA and Tag Amplification Kit (Cat. No. 633774), and primers for BD Rhapsody™ Immune Response Panel (399 genes; 633750) and custom-made panel that included additional genes expressed by FLS that were not represented in the Human Immune Panel (46 genes; **Suppl Table 19)**. Libraries were sequenced at the depth of 956374 ± 249958 (mean ± sem) reads per Tag using Illumina NextSeq 500 (Glasgow Polyomics). 1228 ± 370 cells (mean ± sem) per Tag were successfully sequenced **(Suppl Table 20).** *Analysis.* The Seurat package (3.1.2)[60] in R was used to create an object from the RSEC_MolsPerCell.csv file for each sample tag(CreateSeuratObject, min.cells=3). Fibroblasts were computationally isolated by selecting for cells with lack of expression of PTPRC (CD45) gene as illustrated in **Suppl Fig.16e**. The Seurat implementation of the sctransform package (0.2.0) was used for normalization and data scaling (SCTransform). Data from each run was integrated using functions compatible with SCTransform normalization (SelectIntegrationFeatures, PrepSCTIntegration, FindIntegrationAnchors, IntegrateData). Principle component analysis of

integrated counts, batch-normalized by Seurat was performed (RunPCA) before generating a UMAP (RunUMAP) from the first 10 priniciple components (PCs). The same PCs were used in determination of the k-nearest neighbours for each cell during SNN graph construction before clustering at a chosen resolution of 0.2 (FindNeighbors, FindClusters). Differential expression was performed using SCT normalized assay (FindAllMarkers, test.use=MAST) to identify cluster markers and variable genes between co-culture conditions. Genes are considered significantly DE if the adjusted p-value (< 0.05) by Bonferroni Correction and multiple test correction (multiple by number of tests). To visualise heatmaps the pheatmap package was adapted. The SCT normalized expression values were also used to perform pseudobulk expression analysis of each sample (AverageExpression) for investigation of previously identified genes of interest. *Raw data is accessible at EMBL-EBI with the accession number E-MTAB-8873.*

Bulk RNA seq of synovial fibroblasts. High-quality total RNAs (RIN >8) were used to construct Illumina mRNA sequencing libraries. cDNA synthesis and amplification were performed by using SMART-seq v4 Ultra Low Input RNA Kit for Sequencing (cat. no. 634890, Takara) starting with 10 ng of total RNA, following the manufacturers protocol. 10 ng of amplified cDNAs were sheared prior to preparing the final libraries using the Bioruptor® Pico system (Diagenode, 24 cycles of 30 sec on and 30 sec off). Dual indexed Illumina sequencing libraries were prepared by SMARTer® ThruPLEX® DNA-seq 48D Kit (cat. no. R400406, Takara) following the kit protocol. The pooled libraries were sequenced at Edinburgh Genomics (Edinburgh, UK) on a NovaSeq 6000 system using a read length of 100 bases in paired-end mode. The reads were mapped with STAR (version 020201) with default parameter against the Human genome version GRCh38, release 91. The read count matrix was constructed with featureCounts (Version 1.6.4) using default parameters. All differential expression analysis was performed in R using the DESeq2 package. All genes with an adjusted p value < 0.05 and a log fold change of > ± 1.5 were considered significantly differentially expressed. *Raw data is accessible at EMBL-EBI with the accession number E-MTAB-8316.*

Comparison of Human and Mouse scRNAseq Data. A recent publication by Culemann et al.[7] performed single cell transcriptional profiling on murine synovial tissue macrophages from the K/BxN serum transfer induced arthritis model (STIA). We downloaded the data (GSE134691) and integrated this mouse data with our human samples from healthy tissue, undifferentiated arthritis (UPA), naive active RA and treatment resistant active RA. This was performed in a stepwise-manner - firstly by disease group, by species and finally integrating across species - using Seurat's current integration methods (FindIntegrationAnchors, Integrate Data). The combined dataset was then scaled, before performing dimensional reduction and clustering using top 15PCs and a resolution of 0.3. Cluster marker genes were identified, and clusters were re-named accordingly. In addition, the datasets were sub-setted to create separate Seurat objects containing an assay of gene expression

normalized across species from the final integration step. The datasets were then clustered and analysed separately. Orthologs (genes present in both datasets, n=7954) were also identified using the intersect function in R and the average expression of such genes was calculated for each dataset, using the gene expression values from cross-species normalization. The outputs for each dataset were merged and a distance matrix (dist function) was generated before performing hierarchical clustering (hclust function). A dendrogram was plotted from the result to demonstrate the relationship between synovial macrophage clusters from different species. **(Suppl Fig.20-21).**

Analysis of candidate genes in the PEAC cohort. Detailed methodology and analytical pipeline of synovial tissue bulk RNA-Seq from 90 individuals with early treatment-naïve rheumatoid arthritis from the Pathobiology of Early Arthritis Cohort (PEAC) are described previously[37]. The study was approved by the UK Health Research Authority (REC 05/Q0703/198, National Research Ethics Service Committee London – Dulwich) and all patients gave written informed consent. Total RNA 1 µg/sample was extracted from whole synovial tissue retrieved from an inflamed peripheral joint using Trizol/Chloroform method. Bulk RNA-seq (50 million paired-end 75 bp reads/sample) was performed on Illumina HiSeq2500 platform. RNA-Seq data are uploaded to ArrayExpress (accession E-MTAB-6141). Data are expressed as regularised-log2 transformed reads.

Statistical evaluation of STM phenotyping and culture experiments. ROC analysis identified the optimal predictive values for each of $MerTK^{pos}/CD206^{pos}$, $MerTK^{neg}/CD206^{neg}$, $CD163^{pos}/CD206^{pos}$ and $CD163^{neg}/CD206^{neg}$ ST-derived macrophages associated with flare in the flowing groups. RA patients in sustained clinical and US remission who experienced disease flare and RA patients in sustained clinical and US remission who did not experience disease flare after treatment modification (n=11 in each group). Logistic regression was performed to determine the influence of the dependent variable “Disease flare occurrence” by the independent variables “fulfilling the cut-off values for $MerTK^{pos}/CD206^{pos}$, $MerTK^{neg}/CD206^{neg}$, $CD163^{pos}/CD206^{pos}$ and $CD163^{neg}/CD206^{neg}$ synovial macrophage subpopulations” in RA patients in clinical and US-remission. The values were expressed as Odds Ratio (OR) and 95% Confidential Interval (95% CIs), respectively. The Hosmer-Lemeshow test was used to assess the fitting of the model.

The difference in individual STM populations or cytokines between more than 2 joint conditions were evaluated using one-way ANOVA with Tukey’s correction for multiple comparison or Kruskal-Wallis test with Dunn’s correction for multiple comparison. Two-tailed nonparametric unpaired Mann-Whitney test was used if 2 groups were compared. Two-way ANOVA with Tukey’s correction for multiple comparison was used to evaluate (*i*) the differences between multiple cell clusters in multiple conditions and (*ii*) multiple conditions and different time points.

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Figure Legends

Figure 1. Synovial tissue of RA patients in sustained clinical and ultrasound remission is enriched in MerTK/CD206^{pos} macrophages, with decline predictive of flare. (a) Representative expression of MerTK and CD206 on STMs distinguishes two mainpopulations of STMs. **(b)** Distribution of MerTK/CD206^{pos} and MerTK/CD206^{neg} STMs in healthy controls (n=10), RA treatment naïve (n=43), RA treatment resistant (n=30), and RA in remission (n=36). **(c)** CD163 expression is exclusively on CD206/MerTK^{pos} STMs. **(d-e)** Distribution of CD163^{pos} cells in healthy donors and RA patients as described in **b**. **(f)** Spearman correlation between DAS28 and the frequencies of MerTK/CD206^{pos}, CD163/CD206^{pos} and MerTK/CD206/CD163^{pos} STMs in RA patients. **(g)** Comparison of the levels of MerTK expression, **(h)** distribution of CD163/CD206^{pos}, **(i)** MerTK/CD163/CD206^{pos} and **(j)** total CD163^{pos} STMs in RA patients in sustained DAS-based remission (n=24) compared to RA patients in Boolean remission (n=11). **(k)** Comparison of the distribution of STM phenotypes in RA patients who maintained in remission (n=11) compared to those who flared (n=11) after treatment cessation (n=22, total number of patients who consented to treatment modification). **(l)** Relative proportions of MerTK/CD206 and CD163/CD206 positive and negative STMs in patients as in **k** showing that remission is associated with a higher proportion of MerTK/CD206^{pos} compared to flare. **(m)** Odds Ratio (OR, 95% CI) showing the proportion of MerTK/CD206^{pos} STMs ≤47.5%, or the MerTK/CD206^{pos} to MerTK/CD206^{neg} ratio ≤2.5 as independent factors predicting flare. **(n-p)** Representative IHC image of CD68 (brown) and IF staining of CD68 (green), MerTK (red) and nuclei (blue) in synovial tissue sections of RA patient in remission (n) and with active disease (o-p). MerTK^{pos} STMs are mainly localized in the lining layer in remission **(n)**. MerTK^{pos}CD68^{pos} STMs are dispersed **(o)** or not present in tissues from RA patients with active disease **(p)**, the dotted lines are reference edges of lining/sublining areas (20X), white arrows indicate CD68^{pos}/MerTK^{pos} STMs and white dotted arrows indicate CD68^{pos}/MerTK^{neg} STMs (40X). Data are mean ± sem; p values are provided on graphs or marked * (<0.05). Differences (**a-l**) in individual STM populations between different RA conditions evaluated by one-way ANOVA with Tukey correction, or two-tailed Mann-Whitney test.

Figure 2. Single-cell transcriptomics defines STM subpopulations and phenotypes in health and arthritis. (a) UMAP visualization of 9 clusters of synovial tissue macrophages identified by scRNAseq analysis; each cell represented by an individual point colored by cluster identity. **(b)** Heatmap illustrating scaled expression of the top 20 differentially expressed genes per cluster (rows are genes, columns are cells). Marker genes of interest are annotated, and the total number of genes differentially expressed by each cluster is written next to the cluster. All genes expressed in at least 40% cells in each cluster. Average log-fold change ≥0.25. **(c)** Expression of cluster marker genes on log-normalized violin plots; black dot (median). **(d)** Transcriptional relationship between clusters demonstrated by the top 50 PCs were embedded in the top 3 diffusion-map components. **(e)**

Hierarchical clustering of STMs. **(f)** MerTK expression in the 9 STM clusters. **(g)** Proposed classification of human STMs based on scRNAseq and flow cytometry. **(h)** Split UMAP visualization demonstrating relative changes in the STM phenotypes between disease groups; cell numbers normalized to ~5,000/group. **(i)** Bar and dot plots illustrating changes in cluster distribution across conditions. Significant differences between the given condition and at least one other condition highlighted * (two-way ANOVA, with Tukey correction). Each dot represents individual patient. Flow cytometry validation of FOLR2/MerTK^{pos} **(j),** TREM2/MerTK^{pos} and LYVE1/MerTK^{pos} **(k)** in healthy controls (n=10), active RA (n=17), and RA in remission (n=313) depicted by multi-panel flow cytometry. **(l)** An opposite expression of *MerTK* and *CD48* mRNA in STMs clusters, suggesting that CD48 is a marker of MerTK negative STM clusters. An increased proportion of CD48^{pos} cells in patients with active RA compared to healthy and RA in disease remission. **(m)** Flow cytometry validation of MerTK^{neg}-S100A12^{pos} cluster (defined by CD48^{pos}/CD9^{neg}/CLEC10a^{neg}) **(m),** MerTK^{neg}-SPP1^{pos} cluster (defined by CD48^{pos}/CD9^{pos}/CLEC10a^{neg}) and MerTK^{neg}-CLEC10a cluster (defined by CD48^{pos}/CD9^{pos}/CLEC10a^{pos}) in healthy controls (n=8), active RA (n=13), and RA in remission (n=7) depicted by multi-panel flow cytometry. Dot plots show an increase in proportion of these clusters in active RA as compared to healthy and disease remission. (j-m) * $p<0.05$, one-way Anova adjusted for multiple comparison; &<0.05 Mann-Whitney test between two groups. **(n)** Correlation between synovial expression of S100A9 and SPP1 with disease activity in the validation PEAC cohort.

Figure 3. MerTK/CD206^{neg} and MerTK/CD206^{pos} STM populations have distinct pro- and anti-inflammatory phenotypes respectively. (a) Overview of sorting strategy for MerTK positive and negative STMs (n=47 patients), and functional studies. *In vitro* production by MerTK positive and negative STMs of **(b-c)** pro- and anti-inflammatory mediators, and (**d)** resolvin D1. **(e)** Expression of MerTK on MerTK/CD206^{pos} STMs are reduced in active RA. **(f)** UMAP of S100A12 mRNA expression on STM **(g)** Production of S100A12 by LPS-stimulated MerTK/CD206^{neg} and MerTK/CD206^{pos} STMs FACS-sorted from active RA or remission RA. **(h)** Soluble mediators differentially expressed in the MerTK^{neg}-CD52^{pos}/S100A12^{pos} cluster between conditions. * $p<0.05$, adjusted by Bonferroni.

Figure 4. TREM2^{pos} and FOLR2^{pos} clusters of MerTK^{pos} STMs from RA patients in remission share a unique transcriptomic signature. (a) Heatmaps illustrating scaled expression of the top 30 marker genes of each condition within the TREM2^{low}, TREM2^{high} and FOLR2/LYVE1^{pos} clusters. Rows are genes and columns show equal pseudo-bulk expression per condition within each cluster. All genes are expressed in at least 60% of cells in that condition, with an average log fold-change ≥0.25, (p<0.05 corrected for multiple comparisons. **(b)** The numbers of genes differentially

expressed between conditions for each cluster. **(c)** Violin plots illustrating the expression of top marker genes of TREM2^{pos} and FOLR2^{pos} clusters unique for healthy or remission STMs. **(d)** Transcription factors identified in TREM2^{pos} and FOLR2^{pos} clusters in remission are inhibited in macrophages incubated with MerTK inhibitor, *p<0.05 paired t-test. **(e)** Synovial tissue expression of KLF4 and NRA42 in (PEAC cohort) negatively correlates with DAS28.

Figure 5. Single-cell transcriptomics of STM reveals distinct influence of MerTK/CD206^{neg} and MerTK/CD206^{pos} STMs on FLS activation in co-culture. (a) Schematic of STM-FLS co-culture experiments. **(b)** UMAP visualization of 5 clusters of FLS from all co-cultures; FLS represented by an individual point; colored by cluster identity. **(c)** Heatmap illustrating scaled expression of the top 20 differentially expressed genes per cluster (rows are genes, columns are cells). All genes are expressed in at least 40% of cells in each cluster. Average log-fold change ≥0.25. **(d)** Split UMAP visualization demonstrating relative changes in the FLS phenotypes between distinct co-culture conditions. **(e)** Bar and **(f)** dot plots illustrating the change in FLS cluster distribution across conditions, *p<0.05 Mann-Whitney. Each symbol in f represents FLS from an individual patient (n=5) co-cultured with sorted MerTK^{neg} or MerTK^{pos} STMs from active RA (n=3) or remission RA (n=3). **(g)** Heatmap of scaled expression of the top differentially genes in total FLS induced by MerTK^{neg} STMs from active RA as compared to MerTK^{pos} STMs from the same patients and MerTK^{pos} from RA in disease remission. Rows are genes and columns show pseudo-bulk expression per condition. All genes are expressed in at least 60% of cells in that condition with an average log fold-change ≥0.25, and p<0.05 corrected for multiple comparisons. **(h)** Representative genes differentially expressed in FLS upon co-culture with MerTK^{neg} from RA patients with active RA as compared to MerTK^{pos} STM from remission RA (pseudobulk RNA analysis; *p<0.05 paired t-test). **(i)** Heatmap of scaled expression of the top differentially expressed genes in total FLS, induced by MerTK^{pos} STMs from RA in disease remission compared to MerTK^{neg} STMs from active RA. Rows are genes and columns show pseudo-bulk expression per condition. All genes are expressed in at least 60% of cells in that condition with an average log fold-change ≥0.25, and p<0.05 corrected for multiple comparisons. Violin plots represent an expression of *Col1a* in collagen producing FLS cluster 1 and 2 induced by MerTK^{pos} STMs from RA in disease remission.

Figure 6. Synovial lining-layer FLS of RA patients in remission express reduced levels of MMPs and chemokines compared to active RA, while sublining FLS are a source of GAS6. (a) UMAP visualization of heterogeneity of synovial fibroblasts in active RA (n=4) and RA in remission (n=3). Each cell (n=13,949) is visualized by an individual point and colored by cluster identity. **(b)** Stacked bar plots demonstrate the relative proportion of cells within each cluster per clinical group.

(c) Bar plots illustrate the change in cluster distribution between clinical groups; each point represents an individual sample. **(d)** Heatmap of scaled, batch-normalized expression of the top 10 differentially expressed genes per cluster, showing that THY1^{pos} sublining clusters express GAS6; predominantly in cluster 5 (CXCL14^{pos}). Rows are genes and columns represent cells. All genes are expressed in at least 40% of cells per cluster. Average log-fold change ≥0.25. **(e)** Violin plots of log-normalized expression of genes of interest, median value represented by a black dot. **(f)** Differentially expressed genes in the lining-layer FLS of remission RA compared to active RA. Scatterplot illustrates pseudobulk expression of each differentially expressed gene, and the top 20 genes for each condition are annotated. Heatmap shows the scaled, sample-specific pseudo-bulk expression of the top 20 differentially expressed genes, rows are genes and columns represent pseudobulk expression. All genes are expressed in at least 60% of cells per group, average log-fold change ≥0.25, $p<0.05$ corrected for multiple comparison**. (g-h)** Violin plots showing log-normalized expression of genes for MMPs, chemokines and anti-inflammatory mediators in lining-layer FLS, median value represented by white dot. **(i)** mRNA expression of GAS6 in distinct sublining FLS clusters comparing active and remission RA. The THY1^{high} cluster shows greater expression of GAS6 in remission RA compared to active RA, median values represented by white dots. **(j)** GAS6 is produced by cultured primary FLS from biopsies of RA patients (treatment naïve, treatment resistant, and RA in remission). Each dot represents a separate FLS line (n=5 per condition) *$p<0.05$, two-way Anova corrected for multiple comparisons.