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

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Abstract **(~150 words; now 181)**

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 (31,000 cells) identified phenotypic changes in synovial tissue macrophage (STM) populations spanning health, early/active RA, treatment-refractory RA, and RA in sustained remission. Each clinical state is characterised by different frequencies of 9 discrete phenotypes of 4 distinct STM populations with diverse homeostatic, regulatory and inflammatory functions. This cellular atlas combined with phenotypic and functional analyses of synovial biopsy FACS-sorted STM clusters reveal two STM subpopulations (MerTK/TREM2/TIM4^{pos} and MerTK/FOLR2/LYVE1^{pos}) with unique remission transcriptomic signatures enriched in negative regulators of inflammation. In response to danger signals these cells are potent producers of inflammation-resolving lipid mediators, and engagement of their MerTK pathway restricts synovial fibroblasts activation. A low proportion of MerTK^{pos} STMs of RA patients in remission is predictive of flare. Therapeutic modulation targeting MerTK^{pos} STM-subsets could encourage resolution of inflammation and aberrant remodelling and reinstate synovial homeostasis in inflammatory arthritis.

Introduction

Rheumatoid Arthritis (RA) is the most common inflammatory arthropathy associated with the breach of self-tolerance to citrullinated peptides and production of pathogenic anti-citrullinated peptide antibodies (ACPA). 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 therapeutic opportunity to enact cure.

The healthy *synovial membrane* is a highly specialized, multifunctional structure consisting of a lining layer of synovial fibroblasts and macrophages, and 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; however recent mouse studies suggest they originate from embryonic precursors that are 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 evidence from mouse studies suggests that they help maintain joint immune-homeostasis[5,8]. Recent elegant work led by Culemann *et al* found in mice that lining layer macrophages are long-lived, locally-renewing and form a protective epithelial-like barrier[8]. In active RA, the synovial membrane becomes populated by leukocytes including macrophages[9-11]. Human[12] and mouse[5,13] studies suggest that these differentiate locally from blood-derived monocytes recruited by locally-produced chemokines[14], and might be the main producers of pathogenic TNF[3,10,15,16].

Recently, single-cell transcriptome sequencing (scRNAseq) has revealed complex heterogeneity of both synovial fibroblasts[17-20] and synovial tissue macrophages[18,21] 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 distinct modulation of inflammation or tissue repair[17]. In contrast, 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 resolved human synovium[18,21]. Our earlier studies[2,3,22,23] demonstrated that most synovial inflammation resolves in RA in sustained clinical and ultrasound remission (defined by attenuation of synovial hypertrophy, normalised blood flow and histology[2,3]). However, clusters of synovial tissue macrophages persist, predominantly in the lining, but also in the sublining 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 subsets spanning

health, inflammation and disease remission and uncovered MerTK-dependent active mechanisms of remission driven by tissue resident STMs.

Results

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

To determine the phenotypic spectrum of human synovial tissue macrophages (STMs) spanning health, immune mediated synovitis and resolution of disease, we surveyed their expression of candidate immune-receptors. These included CD163, expressed in healthy STMs[10,24], MerTK and CD206, key markers of murine tissue-resident macrophages with immune-homeostatic function[25,26,27]. Using these markers, we investigated the relative composition of STMs in digested synovial tissue biopsies from RA patients (45 active RA patients naive to treatment, 31 treatment resistant RA, 36 RA in sustained remission) and 10 healthy donors **(Suppl Table 1).** STMs were analyzed based on expression of CD64, CD11b, MHCII and absence of other cell lineage markers **(Fig.1a-b; Suppl Table 2**). Co-expression of MerTK and CD206 defined two distinct STM populations (MerTK/CD206^{pos} and MerTK/CD206^{neg}) **(Fig.1b).** Healthy STMs 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 were fewer in RA patients with active disease, with commensurately increased MerTK/CD206^{neg} STMs **(Fig.1c-d)**. Furthermore, CD163 was foremostly co-expressed with CD206 and MerTK as a subpopulation of MerTK/CD206^{pos} STMs (**Fig.1b,e)** and importantly the CD163/CD206^{pos} occurred in RA patients in sustained remission **(Fig.1e)**. The phenotypes that dominated in patients in disease remission MerTK/CD206^{pos} and CD163/CD206^{pos} STM inversely, whereas MerTK/CD206^{neg} and CD163/CD206^{neg} STM positively correlated with the clinical parameters of disease **(Fig.1f and extended data Suppl Fig.1).**

We validated the relationship between STM subpopulations and resolution using stringent Boolean criteria for disease remission in RA[28]. Among 36 RA patients in clinical DAS28-defined remission, 11 met 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, and an increased proportion of CD163-expressing STMs as compared to similar populations in sustained DAS28-defined remission (**Fig.1g-j and extended data Suppl Fig.2**).

Next, we investigated the temporal relationship between STM phenotypes and persistence of remission and occurrence of flare. All RA patients enrolled in the study achieved sustained clinical and ultrasound remission through the same treatment (MTX plus TNF-inhibitor; **Suppl. Tab.1**). Among them 22 RA patients tapered first and then discontinued the treatment. A prospective study found that those who flared (n=11) had, at the time of synovial tissue biopsy, a lower proportion of MerTK/CD206^{pos} STMs and correspondingly higher MerTK/CD206^{neg} compared to those who maintained sustained remission despite treatment change **(Fig. 1k-m and extended data Suppl Fig.2)**. Therefore, STM subpopulation profile may provide prognostic biomarkers predictive of disease flare after treatment change. To test this, logistic regression analysis (**Suppl Fig.2**) demonstrated (**Fig.1m**) that a proportion of MerTK/CD206^{pos} STMs $\leq$47.5% or the MerTK**/**CD206^{pos}

to MerTK/CD206^{neg} ratio ≤2.5 are independent factors predicting disease flare after treatment tapering and discontinuation in RA patients in sustained clinical and ultrasound remission [Odds ratio:13.5 (95%CI: 2.26–80.79) and 16.2 (95%CI: 2.61–100.45)] respectively) **(Fig.1l, extended data Supp Fig1).**

Next, we explored the localization of MerTK^{pos} STMs in synovial tissue. We used IHC/IF to demonstrate that MerTK^{pos} STMs are localized mainly in the lining layer in disease remission and in active RA (**Fig.1n**). In accordance with flow cytometry data, the majority of STMs (CD68^{pos}) cells of RA patients in sustained remission are MerTK^{pos} while in active RA the CD68^{pos} cells had heterogeneous expression of MerTK in the lining layer with many areas lacking any MerTK expression (**Fig.1n)**

Together, these data suggest that MerTK/CD206^{pos} STMs characterize synovial tissue of RA patients with high likelihood of sustained remission persistence after drug withdrawal.

scRNAseq of STMs revealed heterogeneity within MerTK/CD206^{pos} and MerTK/CD206^{neg} populations associated with joint homeostasis, progression and resolution of RA synovitis

To better understand the heterogeneity and changes in the phenotypes of human MerTK/CD206^{pos} and MerTK/CD206^{neg} STM populations during development and resolution of arthritis, we performed unbiased molecular profiling (scRNAseq) of broad STMs compartment (CD11b/CD64^{pos}) from naïve to treatment RA (n=5), resistant to treatment RA (n=6) and RA in sustained remission (n=6). As comparators we included synovial tissues of healthy (n=4) and autoantibody negative (ACPA-negative) Undifferentiated Peripheral Arthritis (UPA, n=4), which do not fulfill any classification criteria for definite arthritis [29] **(Suppl Table 3-4)**. We characterized the transcriptome of >5,000 cells per condition (total 32,141) to reveal 9 distinct STM phenotypes (clusters/subpopulations) **(Fig.2a and Suppl Table 5)** each uniquely characterized by 63-432 genes **(Fig.2b-c, and extended data Suppl Table 6)**. Firstly, we examined the potential developmental relationship between the 9 STM clusters and subsequently their connections with populations defined by MerTK expression in Figure 1. Diffusion map **(Fig.2d)**, cell trajectory analysis **(Suppl Fig. 3)** and hierarchical clustering analyses **(Fig.2e)** revealed 4 main STMs subsets: (*i*) TREM2^{pos} STMs containing two phenotypes distinguished by TIMD4 and degree of TREM2 expression, (*ii*) FOLR2^{pos} STMs containing 3 distinct subpopulations categorized by their top marker genes as either ID2^{pos}, LYVE1^{pos} or ICAM1^{pos}. As illustrated on umap and violin plots, all of these STMs express MerTK at the single-cell level suggesting that TREM2 *(i)* and FOLR2 *(ii)* subsets constitute MerTK^{pos} STM, which we confirmed by flow cytometry. In contrast, the distinct STM subsets (*iii*) and (*iv*) are all MerTK^{neg}. Subset (*iii*) is defined by high expression of HLA molecules and contains STMs with two distinct phenotypes distinguished by either the IFN signature (ISG15^{pos} cluster) or the transcriptomic signature of antigen presenting cells (CLEC10A^{pos}). The latter resembles CD1c^{+} dendritic cells (DC)[30] and likely represents synovial tissue resident DCs. CD52^{pos} STMs with transcriptomes

enriched in either alarmins (S1001A12^{pos} cluster) or osteopontin (SPP1/CD9^{pos} cluster) are main constituents of subset (*iv*). The two clusters of MerTK^{neg} STMs population (*i.e.* the SPP1^{pos} and the ISG15^{pos} clusters) were previously noted in the synovium of active RA[18] thus validating our analysis strategy (**Fig.2i**).

Changes in the relative proportions of the 9 clusters in human synovium revealed condition-specific patterns and genes indicative of mechanisms of pathogenesis and homeostasis (**Fig.2g-l and Suppl Fig.4)**. Healthy synovium lacks MerTK^{neg} STMs, in agreement with flow cytometry data **(Fig.1).** Their STMs form one MerTK^{pos} population composed of MerTK/TREM2^{pos} and MerTK/FOLR2^{pos} subsets. Pathway analysis revealed that these subsets have prominent upregulation of the complement and defensin pathways as compared with MerTK^{neg} clusters, suggesting efferocytosis and anti-microbial functions (**Suppl Fig.5a and Suppl Table 7).** Their broader transcriptome suggests additional immunoregulatory functions. For example, in contrast to MerTK^{neg} clusters, they express key genes of retinoic acid production (*ALDH1A1, RBP4*) which drives regulatory T cells differentiation[31], and high expression of the B7-related co-inhibitory molecule *VSIG4* which inhibits effector T-cells[32]. In addition, high expression levels of *MerTK* and *LILRB5* which inhibits TLR/cytokine[33] and integrin/FcγR[34] driven activation, respectively suggest a role in resolving inflammation **(Suppl Fig.5a).** These MerTK^{pos} STMs from healthy synovium have the highest proportion of the TREM2^{high} cluster as compared to other conditions. This is consistent with recent findings in the healthy mouse synovium where the investigators propose that TREM2^{pos} cells build a protective lining layer[8]. In addition, human TREM2^{high} STMs have a distinctive transcriptome specializing in phagocytosis *e.g.* high expression of scavenger receptors (*e.g. TIM4, MARCO*) and lipid (e.g. cholesterol) binding proteins (*APOE, APOC1, FABP5*), and the components of phagosome suggesting a role in clearing microbes, apoptotic cells and cholesterol (**Suppl Fig.5a**).

Interestingly, synovitis of very early undifferentiated arthritis (UPA) is characterized by an increase in the proportion of the MerTK^{pos} -TREM2^{low} STM cluster (**Fig.2i**). This cluster is closely related to TREM2^{high} cluster in terms of transcriptomics and cell trajectory **(Fig.2e)**, and may represent early activation phenotype of protective TREM2^{high} lining layer macrophages as manifested for example by an increase in oxidative phosphorylation pathway (**Suppl Fig.5a**) and activation of the cytoskeleton (**Suppl Fig.5a).**

In contrast to healthy and UPA, in active RA, both the treatment-naïve and treatment-resistant groups had increased proportions of MerTK^{neg} -CD52**/**SPP1^{pos} cluster. The treatment-resistant RA group also had an increased proportion of MertK^{neg} -CD52/S100A12^{pos} cluster (**Fig.2i**). The transcriptomes of these clusters suggest pro-inflammatory phenotypes *e.g.* increased expression of glycolytic enzymes (*LDHA, ALDOA, PKM, ENO1*; **Suppl Fig.5b**) indicating that their activation is fueled by glycolysis. The top marker of SSP1^{pos} cluster (osteopontin) has multiple pro-inflammatory and bone-resorbing properties[35] and high levels of cytoskeletal proteins and integrins

suggesting a migratory phenotype. (**Suppl Fig.5b**). In contrast to SPP1 cluster that was reported previously in active RA[18], the S100A12^{pos} STM cluster is a novel finding of importance because of their substantial numbers in active RA and the high levels of *S100A8/9/12* transcripts suggest that this cluster specializes in alarmin production. These alarmins are chemoattractant for neutrophils and monocytes and, through binding to RAGE/TLR4 on fibroblasts and monocytes, can induce pro-inflammatory cytokines (IL-6, TNF)[36]. Thus, our analysis strategy comparing different clinical categories provided higher resolution of STM complexity and defined new STM pathogenic phenotypes. A subsequent analysis of these signatures within the independent Pathobiology of Early Arthritis Cohort (PEAC), which gathered total biopsy RNA from 90 RA patients[37,38], confirmed that top markers of the two MerTK^{neg} clusters SPP1 and S100A9 are enriched in myeloid synovial pathotype and their expression correlates positively with disease activity **(Fig.2j-k).**

In patients who achieved sustained remission, the MerTK^{neg}- CD52/SPP1^{pos} cluster resolves, yet the MerTK^{neg}- alarmin expressing cluster (CD52/S100A12^{pos}) can persist. This uncovers the identity of persisting MerTK^{neg} cells in remission depicted by flow cytometry analysis in Figure 1. In addition, in contrast to active RA and in accordance with flow cytometry analysis (Fig.1) remission RA patients show an increase in the MerTK^{pos} cells that scRNAseq analysis identified as FOLR2/LYVE1^{pos} cluster. Their specific expression of LYVE1 suggests they are perivascular tissue macrophages[39]. 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[40], TGFBI[41]*), antiprotease enzymes *(e.g. A2M),* coagulation factors *(F13A1*) and regulators of VEGFR on endothelial cells (*SERPINF1*) (**Suppl Fig.5a**), together suggesting their role in controlling the interstitial synovial tissue remodeling and homeostasis.

To validate scRNAseq based classification of STM phenotypes with flow cytometry, we tested the newly identified cluster markers in conjunction with MerTK on STMs from additional biopsies from RA patients with active disease and remission (**Fig.2l-m**). We focused on predicted MerTK^{pos} STM clusters marker TREM2 and FOLR2 and showed that they were exclusively expressed on MerTK^{pos} STMs, which validated the scRNAseq analysis strategy. In addition, this analysis revealed that not only FOLR2^{pos} but also TREM2^{pos} subsets are increased in patients in disease remission as compared to active RA (**Fig.2n**), revealing that both subsets contribute to an increase in MerTK^{pos} STM population observed in patients in disease remission (Fig.1).

Among MerTK^{neg} cells, CLEC10a^{pos} which are the only non-MerTK^{pos} cells present in healthy synovium, remains in frequency mostly unchanged between conditions. This cluster is enriched in antigen presentation pathway and DC markers [30] and DC transcription factors (*e.g.* NR4A3[42]) clearly representing resident synovial tissue dendritic cells **(Fig.2i).** Interestingly, this cluster contains recently described myeloid phenotype expressing HBEGF, EREG and PLAUR[21] that potentially promotes synovial fibroblasts invasiveness in active RA. We confirmed that expression of HBEGF

in this cluster is increased in early inflammation as compared to healthy and resolved arthritis **(Suppl Fig. 4b).**

Together, these data revealed complex transcriptomic heterogeneity in MerTK^{neg} and MerTK^{pos} STM populations across different joint immune-conditions suggesting potentially distinct inflammatory and regulatory functions that may actively contribute to RA synovitis or disease remission.

MerTK^{neg} STMs produce pro-inflammatory cytokine and alarmins while MerTK^{pos} STMs produce inflammation-resolving mediators

To dissect the functions of MerTK^{pos} and MerTK^{neg} STM populations, we evaluated how they respond to the inflammatory environment of the RA synovium, and whether the response of MerTK^{pos} STMs from patients in remission differ from those of active RA. To address this, we FACS-sorted both MerTK/CD206^{pos} and MerTK/CD206^{neg} STMs from patients with active RA, and MerTK/CD206^{pos} STMs from RA patients in disease remission, and compared their response to *ex vivo* stimulation with LPS (to mimic 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 significantly differ in their response to TLR4 stimulation (**Fig.3b-d**). MerTK^{neg} STMs produced significantly higher levels of IL-6, TNF, IL-1β, CCL2 and CCL3 as compared to MerTK^{pos} STM from both active RA and RA in remission, suggesting that this population is the primary source of pro-inflammatory cytokines in the RA synovium. Both populations produce comparable concentration of tissue remodeling cytokines (IL-13) and cytokines involved in immune response against pathogens (e.g. IL-8, IL-12p70, IFNα2) upon stimulation. In contrast to cells from active RA, MerTK^{pos} STMs from patients in remission did not spontaneously release any of the mediators tested, and the concentrations of those produce upon stimulation was lower than from MerTK^{pos} STMs from active RA. Interestingly following LPS stimulation, IL-10 was produced in similar concentrations by all STM populations regardless of clinical status. ScRNAseq/flow cytometry analysis (Fig.2) revealed the identities of subpopulations forming MerTK^{pos} STMs in remission that included TREM2^{pos} STM, which in contrast to other STM clusters is enriched in pathways involved in production of lipid mediators (Suppl Fig.5a). Thus, we tested production of the inflammation resolving lipid mediator, resolvin D1 by MerTK^{neg} and MerTK^{pos} STMs. In contrast to pro-inflammatory cytokines, resolvin D1 was released only by MerTK^{pos} STMs, and the concentrations were strikingly higher in culture supernatants of STMs from RA in remission (**Fig.3d**). This, together with the high IL-10 to TNF ratio produced by MerTK^{pos} STMs, suggest that MerTK^{pos} STM functions include protecting the joint from excessive inflammation and to support resolution.

As expected, there was no immunomodulatory effect of GAS6 on its 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, instead GAS6 reduced further the low production of LPS-induced pro-inflammatory cytokines, especially IL-6, by MerTK^{pos} STMs from patients in remission. The difference in response to GAS6 between MerTK^{pos} from active RA and RA in remission could be attributable to the lower surface density of MerTK on MerTK^{pos} STMs from active RA, particularly those with high disease activity (**Fig.3e**).

Together, these data revealed that MerTK^{neg} STMs have an 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 experiment also revealed the identity of MerTK^{neg} STMs that can persist in RA patients in remission. This were MerTK^{neg}-CD52/S100A12^{pos} cluster expressing inflammation triggering alarmins (Fig.2i) that if released upon stimulation may contribute to disease flares. Thus, to test whether MerTK^{neg} STMs release the alarmins and to compare the production of alarmins by MerTK^{neg} STMs from RA patients in remission with those with active RA, we investigated 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) that are similar to those 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 that was similar to those of RA patients with treatment-naïve active RA **(Fig.3f-h).** This suggests that MerTK^{neg}-CD52/S100A12^{pos} cluster, if present in patient in clinical and ultrasound remission, has the same potency to produce inflammation triggering alarmins as equivalent cells from patients with active RA.

In summary, these data demonstrate that MerTK^{neg} and MerTK^{pos} STM populations have distinct pro-inflammatory and resolving properties, respectively. MerTK^{neg} macrophages from patients in remission can produce pro-inflammatory alarmins upon stimulation and if not counterbalanced by MerTK^{pos} STM regulatory functions this may contribute to disease flare upon treatment modifications.

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

To dissect the molecular signature underlying the resolving phenotype of MerTK^{pos} clusters TREM2^{pos}, TREM2^{high} and FOLR2^{high}/LYVE^{pos} in remission we performed comparative analysis of their transcriptomes between conditions. This revealed the transcriptomic trajectory these cells undergo from health, through the joint inflammation then to resolution. This program included set of pathways induced during RA inflammation (*e.g.* glycolysis: *ALDOA, ENO1*) that resolve in disease

remission or if inhibited by inflammation return to levels similar or equivalent to those of healthy synovium (*e.g.* scavenger receptors *MARCO*, and leukotriene and resolvin regulator *ALOX5AP*, **Suppl Fig 6 and Suppl Table 8**). However, they also undergo transcriptomic changes during inflammation that do not return to normal levels in remission. These include sustained upregulation in antigen presentation pathway and sustained repression of the regulatory signature typical of healthy state (*e.g.* retinoic acid pathway [31] and a B7-relate co-inhibitory molecule *VSIG4*[32]), which suggests long-term epigenetic imprinting triggered by inflammation that do not resolves **(Fig.4a-b).**

Interestingly**,** in addition to the transcriptomic sets of pathways shared with either cells of healthy or patients with inflamed joints, MerTK^{pos} clusters: TREM2^{pos}, TREM2^{high} and to a lesser extend MerTK^{pos} cluster expressing FOLR2/LYVE1 gain in remission a unique regulatory transcriptomic signature that is different from regulatory transcriptome of homologous cells in healthy **(Fig.4a and c).** This signature is characterized by upregulation of a set of transcription factors (KLF2, KLF4, NR4A1, NR4A2 and ATF3) and dual-specificity phosphatase 1 (DUSP1) that 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 reinstate tissue homeostasis. DUSP1 drives destabilization of pro-inflammatory mRNA transcripts[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 determined that this remission-specific transcriptomic signature are regulators linked to upstream activation of MerTK by demonstrating *in vitro* that their expression was reduced by MerTK inhibitor **(Fig 4d).** A subsequent analysis of this signature within the independent PEAC cohort [37], confirmed that at least KLF4 and NR4A2 expression in RA synovium is enriched in myeloid synovial pathotype (KLF4) and correlates negatively with disease activity **(Fig.4e)**. In summary, these data suggest that in RA disease remission regulatory functions of MerTK^{pos} STM clusters are underlined by unique set of transcription factors.

Macrophage MerTK controls activation of synovial fibroblasts

Next, we tested whether MerTK-expressing macrophages can modulate the synovial stromal environment. To address this, we used direct-contact and trans-well macrophage-fibroblast (fibroblast-like synoviocytes; FLS) co-cultures to mimic myeloid-FLS interactions in the synovium. We used three models of MerTK-expressing macrophages (*i*) with MCSF-driven expression of MerTK, (*ii*) treated with LPS to generate low MerTK pro-inflammatory macrophage, and (*iii*) treated with LPS plus dexamethasone (Dex) to boost MerTK expression and generate regulatory macrophages **(Fig.5a and Suppl Fig.7a)**. To test the role of MerTK in macrophage/synovial

fibroblast interactions, macrophages were pre-incubated with MerTK-selective inhibitor[49]. After direct co-culture, the FLS and macrophages were FACS-sorted using specific markers. mRNA expression of MMP1 and IL-6 were evaluated in sorted FLS by qPCR. Adding LPS-pretreated macrophages to FLS increased their expression of MMP1 and IL-6. Additional pre-treatment of these inflammatory macrophages with MerTK inhibitor further increased the macrophage-mediated FLS expression of MMP1 and IL-6. Regulatory macrophages (pretreated with LPS plus Dex) did not affect the low constitutive expression of MMP1 and IL-6 in FLS. However, inhibition of MerTK in LPS-Dex macrophages increased MMP1 and IL-6 expression in FLS suggesting that MerTK was responsible for their anti-inflammatory phenotype **(Fig.5b).** These findings were supported by measuring increased concentrations of MMP1, MMP3 and IL-6 in supernatants of co-cultures of pro-inflammatory or regulatory macrophage that were pre-incubated with MerTK inhibitor as compared to the respective controls **(Fig.5c-d).** Other soluble mediators *e.g.* MMP2 and macrophage-derived MMP9 were not affected by MerTK inhibition **(Suppl Fig.7).** Subsequent experiments using the trans-well system **(Fig.5e-f)** showed that direct contact between macrophages and FLS was not required for macrophage MerTK modulation of fibroblasts. All together, these findings suggest that MerTK-expressing macrophages limit the proinflammatory and tissue-destructive properties of synovial fibroblasts.

To further explore the phenotype(s) of FLS potentially altered by contact with MerTK-expressing macrophages, we compared the transcriptome of FLS **(Suppl Table 9)** co-cultured with LPS pre-treated macrophages with FLS co-cultured with macrophages pretreated with LPS and MerTK inhibitor. These revealed 82 differentially expressed genes **(Suppl Table 10)** enriched in two pathways: String pathway analysis highlighted the cytokine pathway (15 of 216 pathway genes, $p=4.10^{-15}$) and the chemokine pathway (9 of 48 pathway genes, $p=1.79^{-12}$). For example, the lack of inhibitory MerTK on macrophages permits increased FLS-expression of numerous chemokines recruiting neutrophils (CXCL8, CXCL1, CXCL2, CXCL5, GCF3), monocytes (CCL3, CXCL3) and T-cells (CCL20, CXCL10). Increased expression of COX2 (PTGS2) generates PGE_2 that mediates pain and blood vessel permeability. In contrast, remodeling mediators (FGF14) and extracellular matrix (COL21a) were downregulated **(Fig.5g-h).** Together these data propose that MerTK^{pos} macrophages may re-instate joint immune-homeostasis by actively limiting the inflammatory and tissue remodeling functions of FLS.

Lining layer synovial fibroblasts in sustained disease remission show a decrease in mediators regulated by MerTK expressing macrophages.

To investigate whether lining-layer FLS of patients in disease remission compared with active RA show transcriptome indicative of local interaction with MerTK^{pos} STMs and to identify the potential local stomal source of MerTK ligand GAS6, we performed scRNA sequencing (10xGenomics) on synovial fibroblasts FACS-sorted from dispersed biopsies from RA patients in sustained remission

and from patients with active RA. Unsupervised clustering of 13,949 FLS confirmed the existing classification of FLS[17-19] that distinguished lining-layer FLS clusters expressing MMPs, and 4 sublining-layer clusters; HLA/COMP^{pos}, Thy1^{high} , Thy1/C3^{pos} and CD34^{pos} expressing collagens and immune-mediators **(Fig.6a-e and Suppl Table 11).**

There were no differences in the relative proportion of clusters between active RA and RA in sustained remission; however, their transcriptome differed between conditions **(Suppl Table 12)**. The top 40 differentially expressed genes in the lining layer FLS of patients in disease remission are presented on **Fig.6f-h.** Among these we observed a decrease in mediators that we identified to be under control of macrophages' MerTK: 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 show a transcriptomic signature of cell with resolved phenotype that could result from an interaction with MerTK^{pos} STMs.

Furthermore, a single cell transcriptome analysis revealed that Thy1^{pos} sublining clusters express GAS6 mRNA. In particular, a small Thy1/C3^{pos} cluster showed abundant expression of GAS6 but this did not differ between conditions **(Fig.6e).** However, an increase in GAS6 was observed in Thy1^{high} cluster of patients in disease remission as compared to active RA, suggesting a potential increase in GAS6 levels in specific niches of resolving synovium (**Fig.6i).** The immunohistochemistry confirmed expression of GAS6 protein in the sublining layer **(Fig.6j)** while in vitro cultures of FLS lines derived from biopsies of patients with active RA and RA patient in sustained remission **(Suppl Table 13)** confirmed that FLS release significant amounts of GAS6 that could be further enhanced by anti-inflammatory treatment (dexamethasone) **(Fig.6k)**. Thus, GAS6 derived from sublining FLS could aid regulatory functions of the lining layer MerTK^{pos} STMs.

Discussion

This study provides a comprehensive description of the functional biology of human synovial tissue macrophages in health, autoimmune synovitis and sustained disease remission. This was based on integrated analysis of scRNAseq of >32,000 STMs from 25 synovial biopsies, FACS-phenotyping of STMs from 112 biopsies, and functional analysis of STMs from 47 biopsies. This spanned healthy homeostasis and early undifferentiated joint inflammation (UPA), treatment-naïve early RA, treatment-resistant RA and RA in sustained remission. Multiparameter flow cytometry uncovered that STMs consisted of two main populations; positive and negative for MerTK/CD206. ScRNAseq confirmed that these populations sit on distinct cell trajectory branches, and 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 RA in disease remission while MerTK/CD206^{neg} STMs are enriched in active RA. Their relative proportion in remission is predictive of persistent remission versus flare upon drug withdrawal. Patients in disease remission whose STMs are composed of less than 47.5% MerTK/CD206^{pos} have higher likelihood of flare after treatment cessation. This can be linked to distinct functions of these two populations. The MerTK/CD206^{neg} STMs produce proinflammatory cytokines and alarmins. In contrast, the MerTK/CD206^{pos} cells produce lipid mediators that resolve inflammation and their MerTK pathway controls activation of the stromal compartment indicating that intercellular cross-talk between MerTK/CD206^{pos} and synovial fibroblasts during remission to maintain joint immune-homeostasis.

We uncovered that the regulatory MerTK^{pos} population contains distinct subsets that include *MerTK/TREM2^{pos}* and *MerTK/FOLR2/LYVE1^{pos}* STMs, both increased in remission while *MerTK/TREM2^{pos}* is dominating subpopulation of MerTK^{pos} in health. Their transcriptomes suggest distinct (but complementary) functions controlling the immune-response and tissue homeostasis respectively. Hierarchical clustering of orthologous human and mouse cluster-specific transcripts **(Suppl Fig. 8-9)** suggests that human TREM2^{high} is homologous to mouse lining-layer *Trem2/Cx3cr1*[8] STMs, while human FOLR2/LYVE1^{pos} closely resembles interstitial *Relmα*^{pos} STMs. These murine counterparts differentiate from locally proliferating precursors, and if a similar ontogeny can be confirmed in humans it may be possible to induce proliferation of these subsets to reinstate homeostasis in RA. Furthermore, in disease remission these clusters gain a unique phenotypic and transcriptomic signature that is different from similar cells in active RA and in healthy. Our experimental findings suggest that this is driven by MerTK activation for example by GAS6 locally produced by Thy1^{pos} synovial fibroblasts and include low production of pro-inflammatory cytokines, high production of resolvins and an increase in the expression of the set of transcription factors that inhibit inflammatory response (*KLF2/4, NR4A1/2 and ATF3*). The relative proportion of these anti-inflammatory MerTK/CD206^{pos} STMs in the synovium of RA patients in remission is a

prognostic biomarker predictive of flare after treatment cessation, when the ratio to MerTK/CD206^{neg} is ≤2.5.

Among four pro-inflammatory MertK^{neg} clusters, we identified that the CD52/S100A12^{pos} cluster can persist in patients in disease remission, and that these cells produce high levels of the S100A12 alarmin upon stimulation which may contribute to flare in patients with MerTK/CD206^{pos} to MerTK/CD206^{neg} ration ≤2.5. Thus, sustained remission might be an active process maintained by tissue-resident subsets of MerTK^{pos} STM subpopulations (e.g. *TREM2*^{pos} and *FOLR2/LYVE1*^{pos}) regulating CD52/S100A12^{pos} STMs and synovial fibroblasts to re-instate and maintain homeostasis. Of interest, recent mouse and human studies have suggested that protective TREM2^{+} resident macrophages are present in adipose tissues and counteract inflammation and metabolic deregulation, suggesting a wider regulatory role of TREM2^{pos} tissue resident macropahges[50].

Among MerTK/FOLR2^{pos} subset, relatively small FOLR2/ID2^{pos} and FOLR2/ICAM1^{pos} clusters remained unchanged between different joint immune states. We speculate that FOLR2/ID2^{pos} are human equivalent of mouse M-CSF driven *in situ* precursors that give rise to human FOLR2 (homolog of mouse RELMa^{pos}) STM subset[8]. This is supported by their highest expression of M-CSF-R and ID2, which is a key driver of self-renewing haemopoietic stem cells [51] (Suppl Fig.5a). We also identified a small population of cycling STMs (STMN1 positive cells) that also expressed M-CSF-R, although to a lesser degree than ID2^{+} cells, and cluster together with TREM2^{low} phenotype, suggesting that they might give rise to MerTK/TREM2^{pos} subset of STMs. (Suppl Fig.8-9). The presence of *in situ* proliferating synovial macrophages were recently reported in synovium of patients with inflammatory osteoarthritis [52] supporting the notion of self-renewing STMs in human synovium.

Of interest, FOLR2/ICAM1^{pos} STMs, which constitutes ~0.025% of STMs and constitutively express high levels of mRNA for pro-inflammatory cytokines (*e.g.*TNF and IL-1β), chemokines and NFKB. They are present in healthy synovium and their frequency did not change upon RA or UPA inflammation or RA disease remission. Their MerTK expression and position on the dendrogram suggest that they are a part of MerTK^{pos} population. These are intriguing cells that may belong to joint first line of defense against pathogens.

In summary, we identified dynamic phenotypic changes in synovial tissue macrophage subsets spanning health, RA inflammation and disease resolution and uncovered the active mechanisms of sustained disease remission driven by tissue resident STMs **(Suppl Fig.10).**

Material and Methods

Patients recruitment and management. One-hundred and twelve patients fulfilling the American College of Rheumatology 2010 revised criteria for RA[53] 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 (SYNGem cohort), Rome, Italy. RA patients were stratified into naïve to treatment (n=45), inadequately responder to Methotrexate (Treatment resistant RA) (n=31) and patients in sustained (for minimum 9 months) clinical and US remission under combination of MTX+TNF-inhibitor (n=36). Ten healthy donors attending for arthroscopy for meniscal tear or cruciate ligament damage, with normal synovium by MRI and macroscopically were included as a control group, at the University of Glasgow. The study protocol was approved by the local Ethic Committee of the Università Cattolica del Sacro Cuore (6334/15) and by the West of Scotland Research Ethical 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 and 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 40 mg/4 weeks or etanercept 50 mg/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 [54].

Patients selection for single-cell RNA sequencing. Seventeen patients fulfilling the American College of Rheumatology 2010 revised criteria for RA[53] (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)[55] 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, 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 immediately below. Four healthy donor tissues were included as control. Demographic, clinical and immunological features of patients and healthy donors' samples used in scRNAseq are summarized in **Suppl Table 3-4.**

Synovial tissue biopsies. All RA and UPA arthritis patients enrolled underwent ultrasound-guided synovial tissue biopsy of the knee following the already published protocol [56] at the Division of Rheumatology of Fondazione Policlinico Universitario A. Gemelli IRCCS – Università Cattolica del Sacro Cuore (the SYNGem cohort), Rome, Italy. All patients underwent ultrasound evaluation of the knee using an ultrasound machine with a multi- frequency linear transducer (MyLab Twice, Esaote). Using the ultrasound view, the best point of entrance for the needle was identified on the lateral margin of the suprapatellar recess. Each patient was provided with a face-mask and cap and the whole procedure was under sterile conditions. Skin disinfection was done with iodine solution (performed twice, starting from the point of needle entrance up to 25 cm 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 10 ml 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 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 3–4 mm. Sections were stained for Haematoxylin and Eosin as follows: sections were deparaffinized in xylene and rehydrated in a series of graded ethanol then 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) and moderate (2) to 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 [57]. For ***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: 3 um sections were prepared from formalin-fixed paraffin-embedded tissue blocks and dried in a 60°C oven for 30 min. Sections were placed in a Bond Max Automated Immunohistochemistry Vision Biosystem (Leica Microsystems GmbH, Wetzlar, Germany) according to the following protocol: firstly, 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 again 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 ***Immunofluorescence****,* formalin-fixed RA synovial tissues were microwaved in a citric acid-pH 9.2 and pre-incubated with phosphate-buffered saline 10% bovine serum albumin (BSA) for 30 min. Sections were then stained with a primary antibody against CD68 (clone L26 mouse anti-human monoclonal antibody, at 1.2mg/ml, Leica Biosystem, Newcastle, UK), and anti-MerTK (rabbit IgG polyclonal Cy3-coniugated antibody anti-human MerTK, clone 5770, BIOSS bs-0182R-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 37˚C, 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 1800 RPM for 10min at 4°C 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. Twenty ul aliquot was used to count the cells. Then cells were centrifuged at 1500rpm for 5min at 4^{o}C. 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) cells were added to 1ml of ice-cold freezing mix [Bambanker (302-14681; Wako)], immediately frozen at -80^{o}C then stored in liquid nitrogen.

Phenotyping and FACS-sorting of STM subsets. Digested biopsies were centrifuged at 1800rpm for 10min, resuspended and washed with FACS buffer, and transferred to a FACS tubes (BD Biosciences) in a final volume of 3ml FACS buffer (PBS/2%FSC/2mMEDTA). An 80 μl aliquot was taken aside for setting up live-dead gates (unstained cells). To the rest of the cells, Fixable Viability Dye eFluor^{TM} 780 (eBioscience) was added at 1:1000 in PBS and incubated for 20min at 4°C. Cells were then washed with FACS buffer. Four tubes were labelled: a) unstained b) the 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 other lineage-positive. Staining was performed in a final volume of 500μl with antibody dilution 1/100 for 30min on ice. All the antibodies are listed in **Suppl Table 2.** Cells were washed twice with FACS buffer and resuspended in finial volume of 500μl, filtered through and Easy Strain 100μM cell-strainer and analyzed or sorted with the use of FACS ARIA III 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). FMO-FITC cells were used to set up a gate for the exclusion of 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. In addition, MerTK/CD206^{pos} and MerTK/CD206^{neg} STM population 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 (Tree Star Inc, OR, USA).

Ex-vivo stimulation of sorted STMs. MerTK/CD206^{pos} and MerTK/CD206^{neg} STM were FACS-sorted into complete medium and plated in 96-well of a 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 37°C, 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 Gas 6 (100 ng/ml, R&D Systems, 885-GSB-050), or both in combination or left unstimulated for 24h in 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 macrophages with synovial fibroblasts. a Direct co-culture. 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) or dexamethasone (1μM) or both, in the presence of absence of MerTK inhibitor UNC1062[49] (Aobious; 250μM). After 24h, macrophages were de-attached and labelled with CellTrace Far Red (5 μM, Life Technologies) according to the manufacturer's protocol. These cells were added at $2x10^3$ per 1 well of 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 (5 μM, Life Technologies) 24h before the co-culture with macrophages. After 24 or 48h co-culture, culture supernatant was collected for assay of mediators, and macrophages and synovial fibroblasts were de-attached and stained with antibodies against the synovial fibroblasts' marker podoplanin and the macrophages marker CD64 (both at 1/100 dilution, details in **Suppl Table 2**. 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 -80^{o}C for RNA isolation. ***Trans-well system.*** CD14^{pos} monocytes were plated in a 24-well plate in 3ml complete medium contained M-CSF (PeproTech) at the concentration of 50ng/ml. On day 3, some cells were pre-treated with LPS (1ng/ml) for 4h. For the last 2h, MerTK specific inhibitor, UNC1062[49] (Aobious) was added at the concentration of 100 or 250 μM. Cells were then washed with PBS and Transwell inserts (0.4μm pore size) containing $3x10^5$ RA synovial fibroblasts (Suppl Table 5) was added to the wells to generate a co-culture system to test the effect of soluble mediators without the direct cell contact. After 48h, supernatants were collected while the cells in the macrophage and fibroblast compartments were lysed separately in RLT buffer with 1% β-mercaptoethanol (Qiagen) and stored at -80^{o}C till the RNA isolation step. 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 supernatant from direct and trans-well co-cultures.

Evaluation of GAS6 production by synovial fibroblasts. FLS were derived from biopsies of RA patients' treatment-naïve, treatment-resistant and in sustained disease remission **(Suppl Table 13).** 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

then seeded on 48-wells cell culture plates at a density of 30×10^3 cells/well in the complete medium containing 1% FCS. Cells were stimulated with dexamethasone 1μM or TNF or IL-1β or IL-10 or TGFβ or LPS at the concentration of either 10 or 100 ng/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 with 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.

Single Cell Sequencing of STM and whole synovial tissues. Our sequencing experiments were performed across two different centers. The first set of samples, which we refer to as our 'Discovery Cohort' was sequenced at the Oxford Genomics Centre, Oxford UK. Synovial tissue myeloid cells were sorted before sequencing, isolating cells with positive expression of CD11b and CD64 and negative expression of a range of other lineage specific cell markers (CD3, CD19, CD20, CD56, CD49, CD117 and CD15) as described in section "*Phenotyping and FACS-sorting of STM subsets"* Around 2000-10000 synovial tissue macrophages per sample were sorted into qPCR 0.2ml tubes coated with FSC and containing 10 μl of PBS/0.02%BSA according to 10xGenomics protocol available online. With this data, 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. In addition, we sequenced a second set of samples at Glasgow Polyomics, University of Glasgow, Glasgow, UK; our "Validation Cohort". The patient groups included Undifferentiated Peripheral Arthritis (UPA), treatment-naive active RA, treatment-resistant active RA and RA in sustained remission and the synovial tissue samples were analyzed for both STMs and FLS. We used the 'validation' scRNAseq transcriptomic profile of FACS-sorted synovial myeloid cells sequenced by Glasgow Polyomics to validate the 'discovery' transcriptomic profile of FACS-sorted STMs scRNAseq profile measured at Oxford. Detailed information on both cohorts is provided in **Suppl Table 5 and Suppl Fig.11a-c.** Data from both these cohorts was 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. As our samples were sequenced on 3 separate occasions to increase read depth, we refer to all 3 sets of FASTQ files (per sample) in the cellranger count command to ensure sequencing runs are combined to generate one count matrix per sample for downstream analysis. ***QC & Filtering.*** The Seurat package (3.0.1) *https://www.cell.com/cell/fulltext/S0092-8674(19)30559-8* in R was used for the majority of secondary data analysis because of its suitability for 10X Genomics data. A Seurat object was created for each sample, removing any genes expressed in less than 5 cells. Cell filtering involved removal of cells with less than 500 detected genes. The expression of mitochondrial (MT) genes was illustrated and a threshold was set for each sample, by visual identification of outliers. In addition, a threshold for high levels of gene expression was also set using this approach, ensuring that cells with particularly high gene expression compared to other cells (likely doublets) were removed. Exact values for thresholds set upon cell filtering are included in **Suppl Table 5**. The data was then normalized by using Seurat's standard approach, whereby feature counts are divided by the total counts for that cell and multiplied by a scale factor of 10000. This is then natural-log transformed. For the analysis of synovial macrophages only, these cells were isolated from other cell types in Validation Cohort samples based on expression of CD14, MARCO or LYZ. These STM specific markers were selected based on Discovery cohort data. The top 2000 variable genes were then identified for all samples. ***Integration.*** Sample integration was first performed in a step-wise approach, whereby the samples of each subject group were integrated before performing the final integration of group-specific Seurat objects. Data integration in Seurat (3.0.1) involves pairwise CCA dimensionality reduction and L2-normalization of the datasets. The top 20 dimensions of each pairwise comparison was then used in the identification of anchors, pairs of cells identified with mutual nearest neighbours across the datasets. These anchors represent cell-states present in both samples, with common gene expression profiles. The next step of integration results in the creation of a merged Seurat object containing batch-corrected expression values for each sample. The

function is provided with a list of genes to integrate, which were found to be expressed in all samples involved in the integration. These "integrated" batch-corrected values are then set as the default assay and the gene expression values are scaled before running principle component analysis. We stored the batch-normalized counts from both steps of integration in separate assays of the final Seurat object. In addition, we tested the reliability of the output of the 'step-wise' Seurat integrations by comparison with the outputs 'all at once' approach in Seurat and 'step wise' and 'all at once' integration approach with another tool, namely Harmony *https://www.biorxiv.org/content/biorxiv/early/2018/11/05/461954.full.pdf*. To do Seurat 'all at once' approach, the data were integrated using the same Seurat methods described in our first approach, but instead in a random-pairwise fashion, where samples were chosen by Seurat regardless of subject group. In contrast to the Seurat integration method, for integration with Harmony we first merged the datasets of interest before identifying variable genes, scaling the data and performing principle component analysis. Harmony then accepts PCA co-ordinates from merged, unintegrated data and performs fuzzy clustering – running iteratively to adjust cell embeddings for batch effect. This adjusted dimensional reduction produced by Harmony is stored in the merged Seurat object. ***Clustering and Dimensional Reduction.*** Depending on the method of integration, the method of dimensional reduction used in downstream clustering of cells and UMAP visualization differs. Following integration using the Seurat method, UMAP is based on PCA cell embeddings generated from integrated counts batch-normalized by Seurat and the first 12 principle components (PCs) are visualized. Following integration using Harmony, UMAP is based on harmony's corrected PCA embeddings and again, the first 12 PCs are visualized. The same PCs are used in determination of the k-nearest neighbours for each cell during SNN graph construction before clustering at a chosen resolution of 0.5. In addition to UMAP visualization, we also plotted a diffusion map using the Destiny (2.14.0) R package *(https://academic.oup.com/bioinformatics/article/32/8/1241/1744143)*. The diffusion map generated from count matrix of the top 50 variable genes allows us to explore the relationship between the clusters. We also generated a count matrix with the average expression of each of our clusters before using Seurat's PlotClusterTree function to generate a dendrogram. Three out of four integration methods (Seurat 'step wise, Harmony 'step wise and Harmony 'all at once') generated similar results regarding main STM subsets **(Suppl Fig.11 d-f)**. We have chosen Seurat step-wise approach for our further data analysis because it provided better understanding of the complex phenotypes of STMs (9 phenotypes instead 5 provided by other methods) and has been widely used by scRNAseq community. ***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. This allowed us to visualize whether points group by cluster identity or by sample. Based on the result, we decided to remove SA139 and SA225 because of its low sequencing depth and separation from all other samples in the PCA reduced dimensional space, respectively **(Suppl Table 5** and **Suppl Fig.12**). ***Differential Expression Analysis.*** In order to identify cluster

markers and variable genes between conditions of RA, we run differential expression analysis using the MAST method *(https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4676162/)*, which can be implicated using the 'test.use' parameter of Seurat's FindAllMarkers function. For identification of cluster markers, we specify a value of 0.4 for the 'min.pct' parameter – which ensures that any markers identified must be expressed by at least 40% of cells in the cluster. List of genes characterizing each of the cluster is provided in Supplementary Table 8. For differential expression analysis between conditions, we increase this value to 0.6 (or 60%) to reduce the risk of sample bias. We use the default values for all other parameters. Before performing any DE comparisons, we set the default assay of the Seurat object to "RNA" which contains our non-batch normalized counts, as recommended by Seurat. We then return to the batch-normalized, integrated counts for visualization of expression of DE genes. Genes which identified by Seurat's function as DE were manually filtered afterwards to ensure that the p-value adjusted by Bonferroni Correction is significant (p-value < 0.05) List of DE genes is provided in Supplementary Table 9. ***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. We investigated the pathways associated with the positive marker genes identified for each cluster. For the String analysis, we exported a table including significantly enriched Reactome pathways for each cluster. We merged the results of all clusters and reduced this list to include pathways relevant to the disease phenotype. This table includes both the 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 less than 0.05 are listed. These results are shown in **Supplementary Table 7** and is visualized in **Supplementary Figure 5**. *Raw data is accessible at EMBL-EBI with the accession number E-MTAB-8322.*

Trajectory Analysis. The Monocle 2.99 package (implemented in R) was used to construct a single-cell trajectory of our identified synovial tissue macrophage clusters. A downsample of our dataset was created by selecting 10,000 random cells from the Discovery cohort to minimise the effect of batch in the Monocle analysis pipeline. A CellDataSet object was then constructed from the raw counts and metadata contained within the downsampled Seurat object. The single cell trajectory was constructed by performing differential expression analysis between the macrophage clusters identified from the Seurat analysis. Dimensional reduction was performed to generate a DDRTree and the top 1,000 differentially expressed genes between clusters were then used to order the cells along the trajectory. Pseudotime calculations then allowed for identification of genes which are differentially expressed along the trajectory. Hierarchical clustering identifies genes which have similar expression patterns as a function of pseudotime.

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 feature Counts (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.[8] performed single cell transcriptional profiling on murine synovial tissue macrophages from the K/BxN serum transfer induced arthritis model (STIA). We obtained 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. 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 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 was generated before performing hierarchical clustering. A dendrogram was plotted from the result to demonstrate the relationship between synovial macrophage clusters from different species **(Suppl Figure 9-10).**

Analysis of candidate genes in 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[38]. 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. To define the best cut-off value for MerTK^{pos}/CD206^{pos}, MerTK^{neg}/CD206^{neg}, CD163^{pos}/CD206^{pos} and CD163^{neg}/CD206^{neg} ST-derived macrophages associated with disease flare after treatment modification in RA patients in sustained clinical and US remission (n=11 RA patients in sustained clinical and US remission who experienced disease flare and n=11 RA patients in sustained clinical and US remission who did not experience disease flare after treatment modification) ROC analysis was performed for each parameter. Logistic regression model 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 are 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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