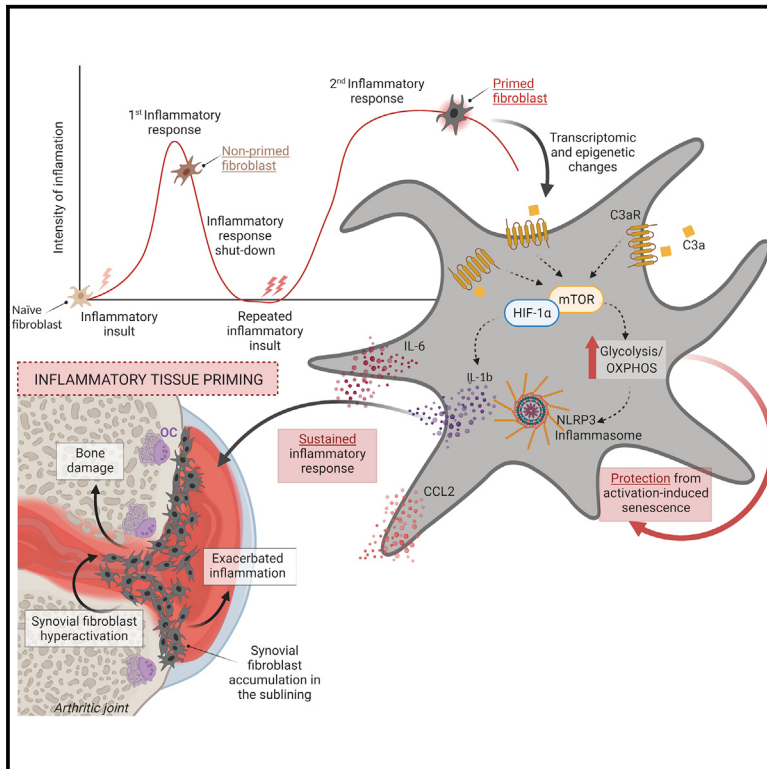


The complement system drives local inflammatory tissue priming by metabolic reprogramming of synovial fibroblasts

Graphical abstract



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In brief

Inflammatory diseases typically involve recurrence and progressive worsening at specific predilection sites, but the underlying mechanisms remain unclear. Friščić et al. reveal that at the center of this inflammatory tissue priming are fibroblasts, which undergo metabolic reprogramming and a shift to a pro-inflammatory state including inflammasome activation.

Highlights

- Synovial fibroblasts mediate tissue priming after repeated inflammatory challenge
- Tissue priming depends on intracellular complement C3 and C3a receptor expression
- Intracellular C3 and C3a receptor drive metabolic reprogramming of fibroblasts
- A metabolic shift promotes inflammasome activation and protects from cell senescence



Article

The complement system drives local inflammatory tissue priming by metabolic reprogramming of synovial fibroblasts

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SUMMARY

Arthritis typically involves recurrence and progressive worsening at specific predilection sites, but the checkpoints between remission and persistence remain unknown. Here, we defined the molecular and cellular mechanisms of this inflammation-mediated tissue priming. Re-exposure to inflammatory stimuli caused aggravated arthritis in rodent models. Tissue priming developed locally and independently of adaptive immunity. Repeatedly stimulated primed synovial fibroblasts (SFs) exhibited enhanced metabolic activity inducing functional changes with intensified migration, invasiveness and osteoclastogenesis. Meanwhile, human SF from patients with established arthritis displayed a similar primed phenotype. Transcriptomic and epigenomic analyses as well as genetic and pharmacological targeting demonstrated that inflammatory tissue priming relies on intracellular complement C3- and C3a receptor-activation and downstream mammalian target of rapamycin- and hypoxia-inducible factor 1 α -mediated metabolic SF invigoration that prevents activation-induced senescence, enhances NLRP3 inflammasome activity, and in consequence sensitizes tissue for inflammation. Our study suggests possibilities for therapeutic intervention abrogating tissue priming without immunosuppression.

INTRODUCTION

Although being usually self-limiting, inflammation can recur under certain circumstances leading to chronic inflammatory disease (Schett and Neurath, 2018). Such recurrence (or flare) of

inflammation mostly involves specific sites which have been previously affected and for some reason are more prone to be revisited by inflammation. The underlying mechanism for this “inflammatory tissue priming” is currently unknown. Nonetheless, it is a highly conserved process and a common phenomenon

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in inflammatory diseases. For instance, the chronic inflammatory phase in rheumatoid arthritis (RA) is often preceded by a period where bouts of inflammation alternate with episodes of non-active disease (McInnes and Schett, 2011; Scott et al., 2010). This shift between active and silent periods is also well-known in gouty arthritis, which typically involves spurious inflammatory attacks revisiting the same joints before the condition becomes a persistent and disabling arthropathy in those not receiving adequate therapy (Richette and Bardin, 2010). Hence, understanding how inflammatory tissue priming controls the transition from acute self-limiting to chronic persistent inflammation is of great importance.

Previous findings suggest that tissue priming is closely related to the exposure of innate tissue-resident cells to danger signals. In gout, for example, tissue-resident phagocytes are activated by monosodium urate crystals (MSU) to produce interleukin (IL)-1 and attract neutrophils that mount inflammation (Martinon et al., 2006; Maueröder et al., 2015). Furthermore, repeated exposure of innate immune cells to danger-associated molecular patterns (DAMPs) causes their epigenetic and metabolic rewiring and enhances responses to repeated stimuli. This so-called “trained immunity” is antigen-independent and primarily serves the aim to more effectively defend against recurrent infections (Crowley et al., 2017; Domínguez-Andrés et al., 2020; Heremans et al., 1990; Netea et al., 2011). However, trained immunity is systemic in its nature and cannot adequately explain site-specific inflammatory flares.

Based on the above findings, we hypothesized that repeated exposure to danger signals may induce augmented activation of resident cells. To delineate the mechanisms of inflammatory tissue priming we have used a combination of experimental models of resolving and persistent arthritis. We show that repeated inflammatory insults induced functional changes in synovial fibroblasts (SFs) *in vivo* (“priming”) that were associated with their metabolic reprogramming and inflammasome activation. These changes required complement factor 3, complement C3a receptor (C3aR) and downstream mammalian target of ra-

pamycin (mTOR) and hypoxia-inducible factor (HIF) 1 α signaling in SF that trigger a sustained inflammatory response accompanied by articular bone damage. We demonstrate that inflammatory tissue priming promotes site-specific recurrence of inflammation.

RESULTS

Tissue priming develops after repeated inflammatory challenge

To investigate whether recurrent exposure to inflammatory stimuli leads to more pronounced and persistent arthritis we set up models in which rodents were repeatedly challenged either systemically or locally. Inflammation after repeated injection of arthritogenic K/BxN serum was prolonged as compared to the response after the first injection (Figure 1A). Similarly, re-induction of arthritis in inducible human tumor necrosis factor transgenic (ihTNFtg) mice resulted in an increased number of swollen digits and concomitant functional impairment (Figures 1B and S1A). To more precisely assess the role of local tissue, we sequentially injected inflammatory triggers directly into one hind paw (Figure 1C). After repeated injection of rat paws with zymosan (Zym), prolonged arthritis developed, indicating local tissue priming, while injection into the previously non-injected (naive) contra-lateral paw did not cause prolonged arthritis (Figure 1D). Very similar results were obtained after intra-articular injection of Zym into mouse knees (Figure S1B) or MSU, an alternative danger signal, into mouse paws (Figure 1E). Of note, MSU also primed the tissue for Zym (Figure 1F).

Clinical signs of prolonged arthritis in the re-exposed primed tissue went along with enhanced inflammation, assessed by magnetic resonance imaging (MRI, Figures 1G and 1H) and histology (Figures S1C and S1D). Moreover, local structural bone remodeling linked to arthritis (Czegley et al., 2018; Tuncel et al., 2016) was enhanced in repeatedly injected paws (Figures 1I, 1J, and S1E). Finally, tissue priming was also reflected by hyperalgesic responses (Figure S1F).

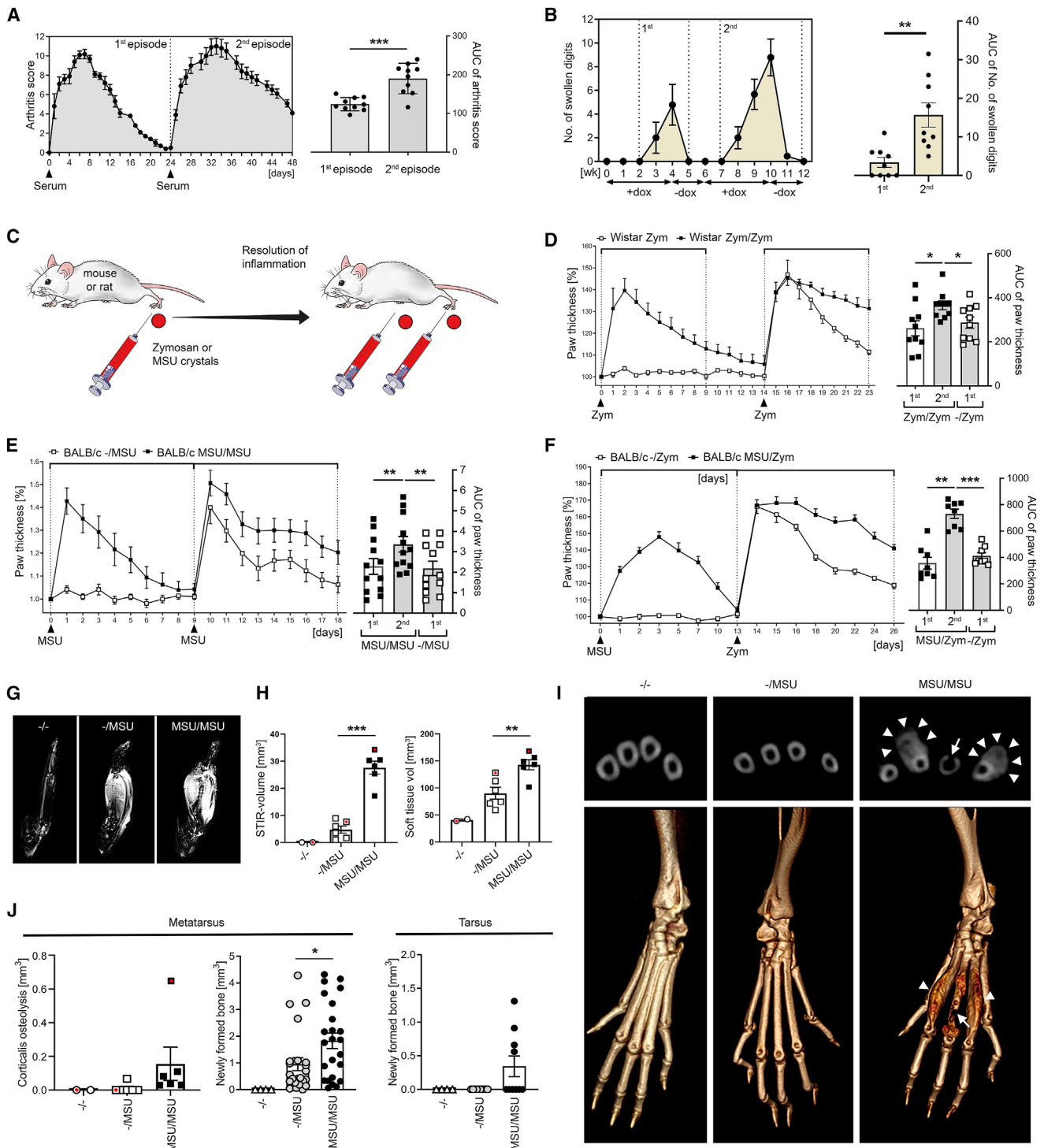


Figure 1. Repeated exposure to an inflammatory trigger causes prolonged arthritis

(A) Time course of arthritis and area under the curve (AUC) after repeated intraperitoneal injection of K/BxN serum (n = 10).
 (B) Arthritis after repeated administration of doxycycline (dox) to iHTNFTg mice (n = 9).
 (C) Injection scheme for the inflammatory tissue priming model of arthritis.
 (D) Time course and AUC of relative thickness of Wistar rat paws injected once (-/Zym) or twice (Zym/Zym) with zymosan. N = 10.
 (E) Time course and AUC of relative thickness of BALB/c mouse paws (n = 12) injected once (-/MSU) or twice (MSU/MSU) with MSU crystals.
 (F) Time course and AUC of relative thickness of BALB/c mouse paws (n = 8) after injection with MSU and re-injection of both paws with Zym.
 (G and H) Analysis of inflammation by MRI in non-injected paws of BALB/c mice (-/-), or 9 days after the 1st or 2nd injection of MSU.
 (G) Increased tissue water content visualized as hyperintense areas on representative short tau inversion recovery (STIR) MRI images.

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Thus, local inflammatory tissue priming is consistently observed in different species, joints, and with various inflammatory triggers.

SF mediate inflammatory tissue priming

Tissue priming by Zym or MSU was maintained in severe combined immunodeficient (SCID) and *Rag1*^{−/−} mice (Figures 2A and 2B and S2A and S2B), which are devoid of functional T- and B cells (Bosma et al., 1989). Nonetheless, tissue priming also occurred in antigen-induced arthritis, a T cell-dependent model (Brackertz et al., 1977) (Figures S2C and S2D). Therefore, tissue priming can be induced by both innate and adaptive immune pathways that trigger inflammation.

We hypothesized that in paws pre-exposed to inflammation, joint-resident cells might have undergone adaptation processes that prime subsequent local responses. Since SF are key players in arthritis (Bartok and Firestein, 2010), we cultured SF from mouse paws injected with MSU once (−/MSU SF) or twice (primed MSU/MSU SF) and from naive paws (Figure S2E) and transferred them into an MSU-based arthritis model. Transfer of MSU/MSU SF promoted a significantly prolonged course of arthritis following subsequent injection of MSU (Figure 2C), suggesting that SF transfer inflammation-induced tissue priming. In contrast, the arthritis upon transfer of −/MSU SF was only mildly exacerbated, though still higher than after transfer of −/− SF. Thus, priming might be a cumulative effect intensified with repeated stimulation.

MSU- or Zym-primed SF also more effectively promoted the differentiation of monocyte precursors into large, multinucleated tartrate resistant acid phosphatase (TRAP)⁺ osteoclasts (OC) (Figures 2D and 2E) and showed more invasive properties (Figure 2F), while *in vitro* cell proliferation was similar (Figure S2F).

During arthritis, sublining SF expand (Croft et al., 2019) and lining layer synovial tissue macrophages (STM), the other major cellular component of the synovium, are dispersed (Culemann et al., 2019). To address whether STM are involved in inflammatory tissue priming, we performed iterated MSU-induced arthritis in mice in which STM were depleted (*Cx3cr1cre*:iDTR mice) before re-injection of MSU (Culemann et al., 2019). The absence of STM caused slightly enhanced arthritis but did not affect tissue priming (Figure 2G). While this is in accordance with the anti-inflammatory action of STM (Alivernini et al., 2020; Culemann et al., 2019), their lack of effect on tissue priming underlined the key role of SF in this process.

To investigate the impact of tissue priming on the synovial lining and sublining structures, we analyzed SF in 3D synovial organ cultures (Figure 2H) (Kiener et al., 2010). Naive SF established a single-layered structure reminiscent of the synovial lining in healthy joints. In contrast, −/Zym SF formed a thickened lining comparable to the hyperplastic synovial lining in inflammatory arthritis. Primed Zym/Zym SF, however, lost their capacity to

form a lining, but instead built a densely interconnected cellular network within the extra-cellular matrix (ECM) of the organ culture. This aberrant behavior of primed SF was also replicated by sustained co-incubation of naive SF with Zym during the formation of the organoid (Figure 2H).

Spontaneous release of IL-6, a fibroblast-derived cytokine with a key role in arthritis (Schett, 2018; Wolf et al., 2014), was elevated from both primed and −/Zym as compared to −/− organoids. Upon re-exposure to Zym *in vitro* IL-6 production was further increased in primed as compared to −/Zym SF (Figure 2I).

Disintegration of the synovial lining and increased accumulation of SF in the sublining and the infrapatellar fat pad was also found *in vivo* by 3D light-sheet microscopy of mouse knees repeatedly exposed to Zym (Figures 2J–2M). Moreover, morphological changes and increased formation of cell-cell-connections were also observed by scanning electron microscopy of primed SF (Figure S2G).

These findings indicate the development of an inflammatory sensitization in fibroblasts, which is gradually established by repeated or sustained inflammatory activation.

Inflammatory Thy1⁺ SF expressing complement components are enriched in primed tissue

To delineate the mechanisms and consequences of fibroblast priming, we performed bulk RNA-seq on cultured −/−, −/MSU and primed (MSU/MSU) fibroblasts (Figures 3A–3E). Gene set enrichment analysis (GSEA) identified several differentially active pathways, among them some connected to inflammation (e.g., IL-6 and the complement system) and canonical metabolic pathways (Figure 3A). Ingenuity pathway analysis (IPA) corroborated metabolic changes (including glycolysis and glutaminolysis) and inflammatory activation with the complement system being specifically upregulated in primed SF (Figure 3B; Table S1).

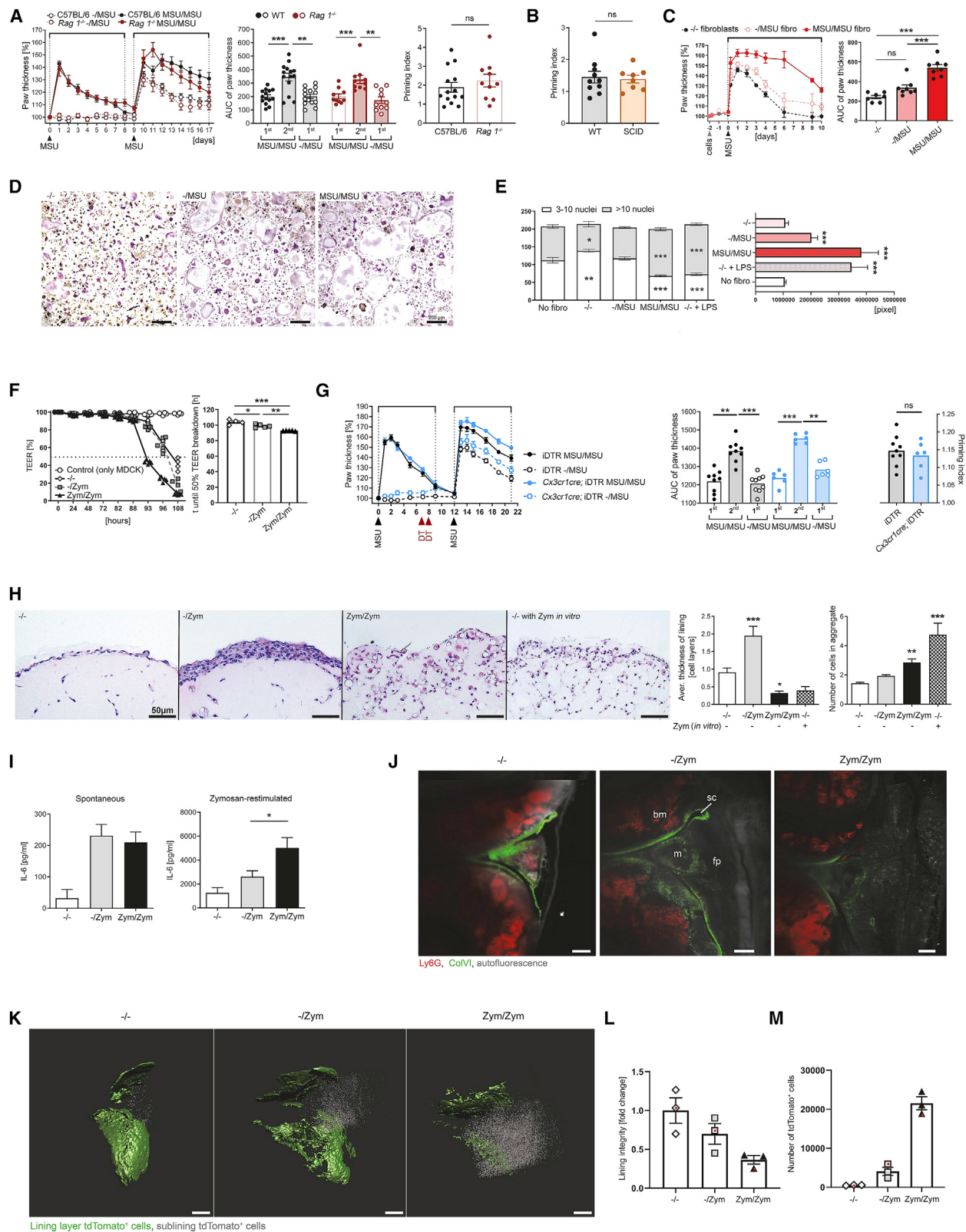
To confirm our findings, we repeated bulk RNA-seq with a validation set of samples (n = 3) and a targeted mRNA enrichment protocol enabling an improved capturing of expression profiles. The results showed that −/MSU and primed SF are distinct populations (Figure 3C) and confirmed upregulation of the complement system in primed SF, together with pathogenic fibroblast markers such as Thy1, fibroblast activation protein (FAP) and Cadherin 11 (Cambré et al., 2019; Chang et al., 2011; Croft et al., 2019; Kiener et al., 2009; Lee et al., 2007), inflammatory cytokines and chemokines, arthritis-associated DAMPs such as biglycan (*Bgn*) (Cambré et al., 2019), and key metabolic pathways (Figures 3D and 3E and S3A–S3C; Table S2). Upregulation of complement components and *Il6* in primed SF was further confirmed by qPCR (Figure 3F). Furthermore, we observed upregulation of key mediators of OC differentiation (e.g., *Tnfrsf11*, *Csf1*, *Tnf*, *Il6*) and enzymes involved in cartilage and bone destruction in primed SF, while, with the exception of *Tnfrsf11b*

(H) Quantification of volumes of hyperintense areas (STIR volume) and soft-tissue volume.

(I) Representative micro computed tomography (μCT) slice images (upper panel) and 3D surface renderings (lower panels) in naive paws and paws 9 days after 1st or 2nd MSU injection, respectively, showing osteolysis of the corticalis (arrow) and newly formed bone (arrowheads) in MSU/MSU paws.

(J) Volumes of areas of lowered bone density in the cortical bone of the metatarsals (n = 2–6) and of newly formed bone along the metatarsals (n = 4–24) and the tarsals (n = 4–10), as evaluated by μCT.

A, B, D–F means ± SEM. Representative samples shown in (G) and (I) are indicated with a red dot. Paired t test (A, B, H); ANOVA with Tukey's test (D–F); Mann Whitney U test (J). See also Figure S1.



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(OPG) and *Il33*, negative regulators of OC differentiation (such as Interferons, *Il3*, *Il4*, or *Il10*) exhibited low or no expression. Although both RANKL and OPG were upregulated, the RANKL/OPG ratio was markedly elevated in primed SF (Figure 3G). In addition, molecules involved in enhanced bone remodeling in the context of arthritis (such as *Tgfb1*), and ECM components such as collagens (Figures 3E and S3A) exhibited enhanced expression in primed SF.

The inflammatory phenotype of SF during RA is controlled by epigenetic changes (Ospelt et al., 2017; Ostuni et al., 2013). To integrate the expression changes in primed mouse SF with chromatin accessibility we performed ATAC-seq (Buenrostro et al., 2015) (Figures 3H–3K and S3D). Regarding genome-wide chromatin accessibility, naive and -/MSU SF formed a combined cluster, while MSU/MSU SF were markedly distinct (Figure 3H). Primed SF were characterized by increased chromatin accessibility at the transcription start sites (TSS) of genes connected to inflammation, bone remodeling, and metabolism, among them *C3* and *Nlrp3* (Figures 3I–3K and S3D).

To link our findings to subpopulations of cells, we next used single-cell (sc) RNA-seq of CD45⁺ cells from MSU-injected paws (Figures 3L and 3M; Table S3). Cells with high expression of complement components, such as *C3*, *C4b*, and *C1s1* were part of a fibroblast cluster showing enrichment of markers previously found upregulated in the sublining layer (SL) of the synovium (e.g.; *Clec3b*, *Gsn*, and *Apod*) that are connected to inflammation and vasculogenesis (Croft et al., 2019). Enumeration of cell subpopulation frequencies from bulk RNA-seq by CIBERSORTx (Newman et al., 2019) revealed strong expansion of this SL subset in primed paws (Figure 3N). These transcriptomic data were corroborated by flow cytometry, showing that Pdpn⁺Thy1⁺ SL fibroblasts were enriched among cultured SF from primed paws (Figure S3E). Furthermore, *ex vivo* analysis of fresh paw tissue demonstrated that a population of Pdpn⁺Thy1⁺ SL SF with increased expression of *C3* was prominently expanded together with CD45⁺ cells in primed paws already early after the second MSU injection (Figures 3O–3Q, S3F, and S3G). Thus, inflammatory tissue priming is associated

with enrichment of an aggressive sublining *C3*-expressing SF subset.

Expression of complement in SF mediates functional changes and inflammatory tissue priming

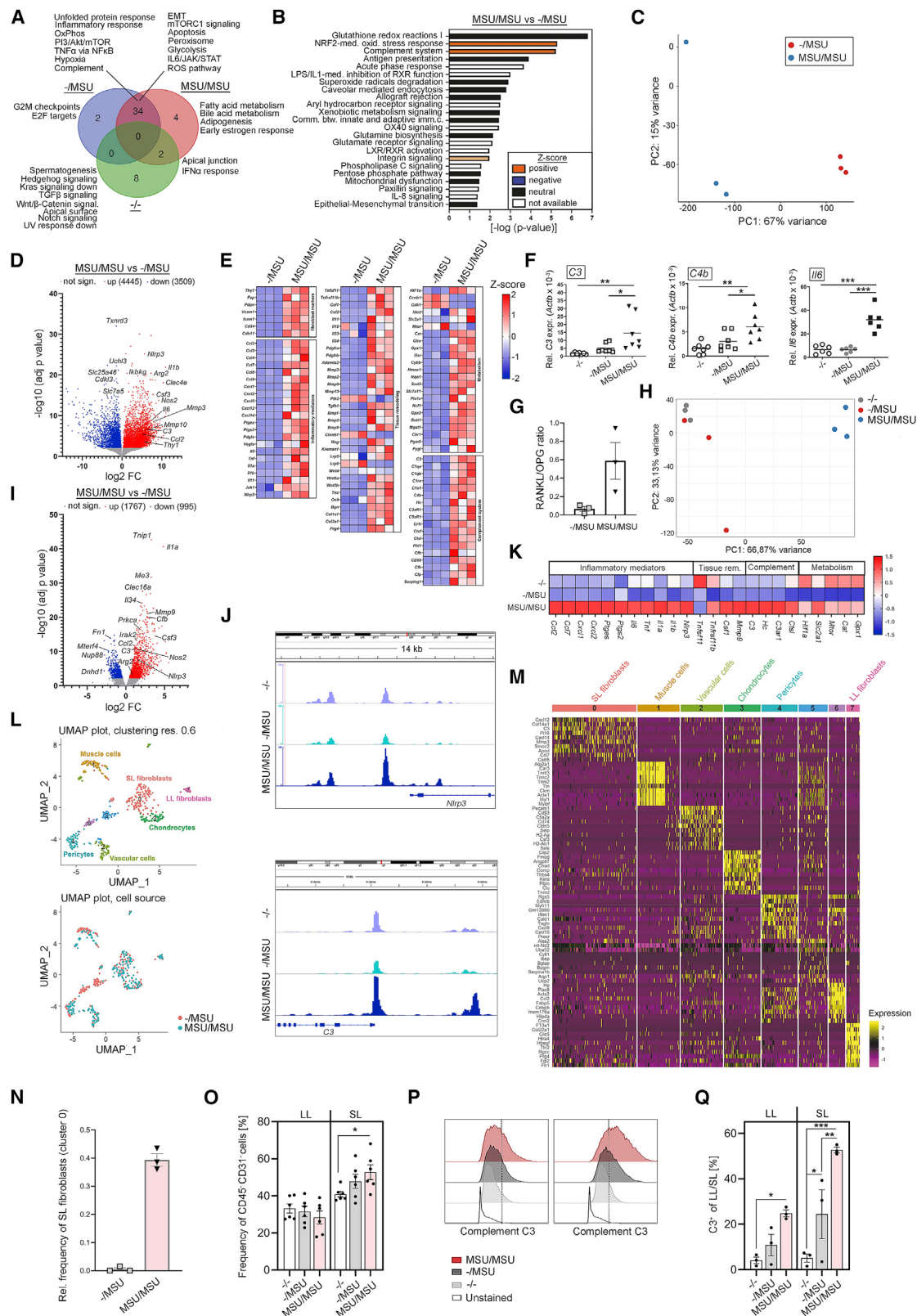
Since the complement system was upregulated in primed SF, we examined its impact on tissue priming. The three major routes of complement activation converge on the central component *C3*. Downstream of *C3* cleavage, the opsonin *C3b* propagates the complement cascade via *C5*, culminating in formation of the membrane attack complex and phagocytosis, while the soluble anaphylatoxin *C3a* is an important modulator of the innate immune response (Coulthard and Woodruff, 2015; Laumonnier et al., 2017; Liszewski et al., 2013). Confocal microscopy confirmed strong upregulation of *C3* protein in primed SF (Figure 4A). *C3* was expressed in large amounts in the cytosol, was not confined to Rab7⁺ endo- and lysosomes, but particularly enriched in organelle structures reminiscent of the endoplasmic reticulum (ER) or Golgi apparatus (Figure S4A). Extracellular stores of *C3* were also observed, suggesting that *C3* may be secreted via the ER, similar to what occurs in pancreatic β cells (King et al., 2019). To assess the impact of complement on tissue priming we induced iterated MSU-induced arthritis in *C3*^{-/-} mice (Figure 4B). Although *C3* had been reported to influence leukocyte accumulation into the peritoneum in response to MSU (Khameneh et al., 2017) the 1st arthritis episode was not different in the absence of *C3*. However, more persistent arthritis upon reinjection only occurred in wild-type (WT) but not *C3*^{-/-} mice.

In contrast, treatment with the *C5*-blocking antibody BB5.1 (Zelek et al., 2020) did not suppress tissue priming (Figure S4B), demonstrating its independence of terminal complement pathway activation. In support, also NOD mice, which, among other inherent immune deficiencies (Kataoka et al., 1983; Pearson et al., 2003; Serreze et al., 1993) do not express *C5* (Baxter and Cooke, 1993), exhibited a certain amount of tissue priming (Figure S4C).

Complement is expressed in many cell types of hematopoietic and non-hematopoietic origin (Lubbers et al., 2017). To assess

Figure 2. Functional changes of primed fibroblasts mediate prolonged arthritis

(A and B) Development of tissue priming is not inhibited in *Rag1*^{-/-} and SCID mice.
(A) Time course, AUC and priming indices of iterated MSU-induced arthritis in *Rag1*^{-/-} (n = 10) and C57BL/6 wildtype (WT) mice (n = 14). Priming index is defined as the ratio between AUC of the 2nd arthritis episode and the 1st arthritis episode in the contralateral paw.
(B) Priming indices of iterated zymosan-induced arthritis in SCID mice (n = 8) and BALB/c WT mice (n = 10).
(C) Time course and AUC of MSU-induced arthritis induced 2 days after injection of cultured -/-, -/MSU or MSU/MSU SF into the paw. N = 7–8.
(D and E) OC differentiation from bone marrow cells in co-culture with -/-, -/MSU, or MSU/MSU SF.
(D) TRAP-stained images representative of 5–7 mice, (E, left) number of small (3–10 nuclei) and large (>10 nuclei) OC, (E, right) total area covered by OC.
(F) Matrix-invasive capacity of -/-, -/Zym and Zym/Zym SF (n = 4–5), shown as % decrease in transepithelial electrical resistance (TEER). Dashed line indicates 50% breakdown of TEER.
(G) Iterated MSU-induced arthritis in STM-depleted *Cx3cr1cre*:iDTR mice (n = 6) and iDTR controls (n = 9) treated with diphtheria toxin (DT) 5 and 4 days before the 2nd MSU injection.
(H) Lining formation and interconnectivity in 3D organ cultures from -/-, -/Zym and Zym/Zym rat SF with or without Zym restimulation *in vitro*. Shown are representative images of H&E stained sections and quantification of lining layer thickness and cell connections in the sublining in organoids from 3–5 mice.
(I) IL-6 concentrations in supernatants from organoids with or without *in vitro* Zym restimulation.
(J) 3D light-sheet fluorescence microscopy (LSFM) images of knee joints of *Col6a1*^{cre}:R26-tdTomato mice. bm, bone marrow; fp, infrapatellar fat pad; m, meniscus; sc, synovial cavity. Scale bars, 200 μ m.
(K) LSFM-derived 3D reconstructions of tdTomato⁺ cells from the knees of *Col6a1*^{cre}:R26-tdTomato mice. Scale bars, 300 μ m.
(L) Quantification of lining integrity in the synovial membrane, normalized to mean values from -/- paws.
(M) Quantification of tdTomato⁺ cells in the sublining of mouse knees during iterated Zym-induced arthritis.
Samples shown in (J, K) are indicated with a red dot. Unpaired t test (A, B, G); paired t test (I), ANOVA with Tukey's test (A, C, F, G); ANOVA with Dunnett's test (E, H). See also Figure S2.



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the importance of C3 in non-hematopoietic cells we created bone marrow chimeras by transplanting WT bone marrow into lethally irradiated $C3^{-/-}$ mice (Figure S4D). After transplantation of WT bone marrow, tissue priming was still significantly reduced in $C3^{-/-}$ as compared to WT recipients (Figures 4C and 4D), suggesting that inflammatory tissue priming is controlled by non-hematopoietic cells.

To determine the effect of C3 specifically expressed in SF, we transferred CD45⁺CD31⁺ WT or $C3^{-/-}$ SF to previously non-injected paws of WT or $C3^{-/-}$ recipient mice (Figure 4E). The comparison with the contralateral paw confirmed that MSU/MSU WT but not $C3^{-/-}$ SF imprinted tissue priming (Figures 4F and 4G). This ability of primed WT SF was also preserved to a certain degree in the paws of recipient $C3^{-/-}$ mice, suggesting that primed SF mediate inflammatory tissue priming independently of other C3-expressing cell types.

SF from chronically inflamed joints exhibit functional changes such as increased proliferation, migration, and invasiveness (Bartok and Firestein, 2010). In line with this, primed SF showed enhanced migration (Figures 4H and S4E) and a higher tissue-invasiveness (Figure 4I), which were both dependent on C3 (Figures 4H, 4I, and S4E).

Thus, inflammatory tissue priming is associated with a pathogenic functional SF phenotype and depends on complement C3.

Metabolic changes in primed SF are dependent on the complement system and promote pathogenic cellular functions

Articular fibroblasts undergo metabolic adaptations during arthritis, including altered glucose and lipid metabolism as well as mitochondrial changes (Falconer et al., 2018), which are connected to pathogenic cell function (Folmes et al., 2012). To assess if functional changes of SF and tissue priming are associated with metabolic rewiring and if such alterations are connected with the complement system, we analyzed the cellular metabolism of SF. We observed increased activity of both

glycolysis and respiration in primed MSU/MSU as compared to $-$ /MSU SF (Figures 5A–5G). Accordingly, we detected enhanced glucose consumption (Figure 5A), accompanied by elevated surface expression of the glucose transporter GLUT1 (Figure 5B). This observation was in line with the enhanced gene expression of key glycolytic enzymes such as *Hk2*, *Eno1*, *Ldha*, *Pdk1*, and *Pkm* (Figure 5C). Metabolic flux analyses confirmed higher basal glycolytic activity and basal respiration in primed as compared to naive SF (Figure 5D), indicating a general increase of metabolic activity in primed SF. Notably, metabolic flexibility of MSU/MSU compared to $-$ /MSU SF was enhanced in terms of glycolysis (increased glycolytic capacity and reserve, Figure 5E), but diminished in terms of mitochondrial respiration (elevated proton leak and reduced spare respiratory capacity, Figure 5F). Overall, the metabolic phenotype of primed SF shifted toward aerobic glycolysis (Figures 5G and 6A).

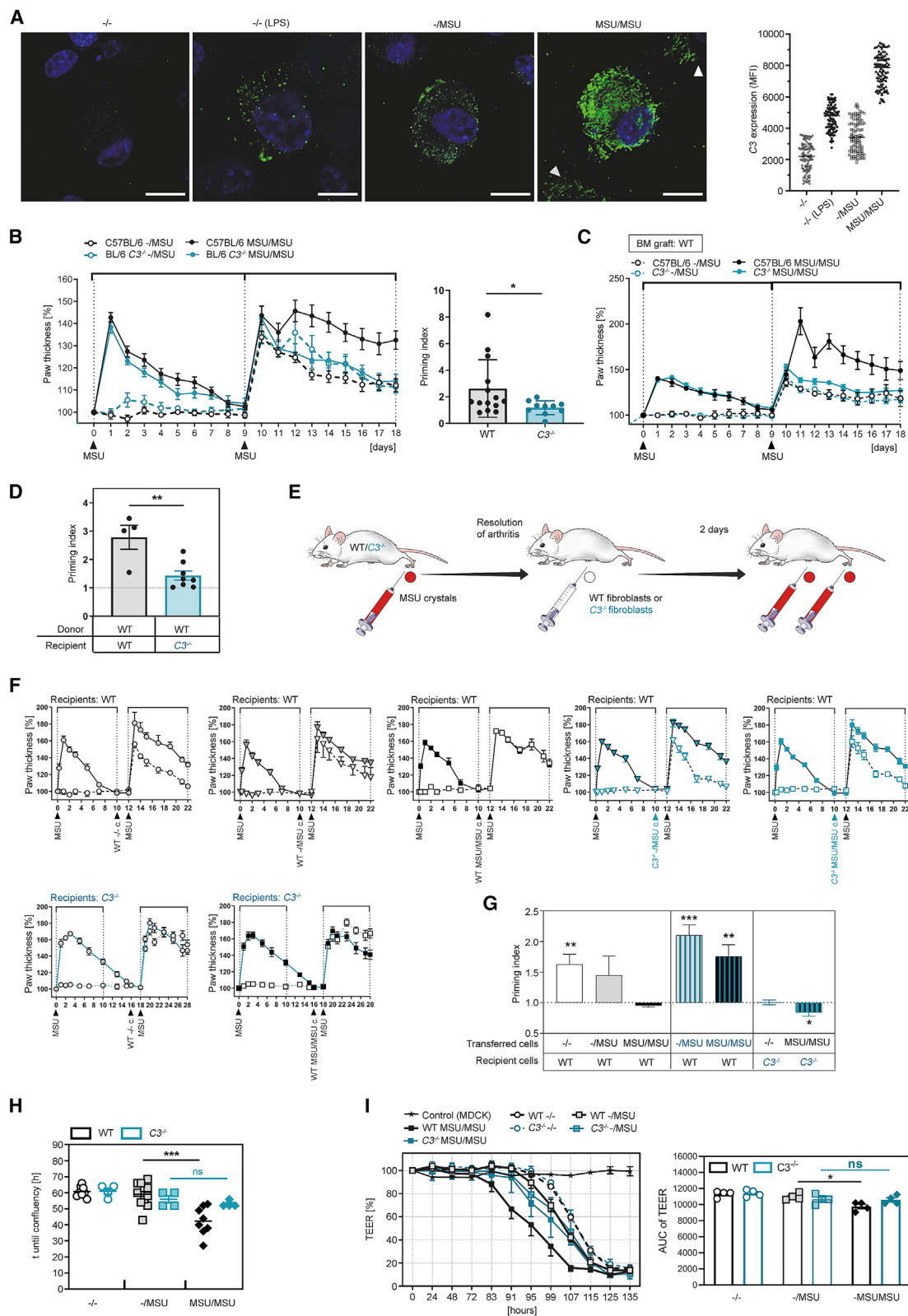
Blocking glycolysis *in vitro*, using the pharmacological hexokinase 2 inhibitor 2-deoxy-D-glucose (2-DG), antagonized the increased migration and invasiveness of primed SF (Figures 5H and 5I) and abrogated their increased production of TNF α , CXCL12, and IL-6 upon re-stimulation *in vitro* (Figure 5J). Treatment with 2-DG *in vivo* already diminished the first bout of arthritis but also completely abrogated priming (Figure S5). Thus, tissue priming is closely related to enhanced glycolysis in primed SF.

While $-$ /MSU SF from WT and $C3^{-/-}$ mice were bioenergetically similar, the increase in metabolic activity upon priming was restricted to WT cells (Figure 6A), corroborating the complement system's role in driving the intrinsic metabolic adaptations during priming. In fact, metabolic changes related to priming were reverted below $-$ /MSU SF without C3 (Figures 6B–6D).

This downregulation of metabolic activity in $C3^{-/-}$ MSU/MSU SF indicated that these cells might have become metabolically exhausted or senescent. Indeed, aged cultured $C3^{-/-}$ SF demonstrated increased accumulation of senescence-associated β -galactosidase (SA- β -Gal), especially when repeatedly

Figure 3. Profiling of primed fibroblasts

- (A and B) Bulk RNA-seq of cultured SF using an rRNA depletion method ($n = 3$).
 (A) Venn diagram of differentially regulated pathways of GSEA.
 (B) IPA showing canonical pathways differentially regulated in MSU/MSU as compared to $-$ /MSU SF.
 (C) Principal component analysis (PCA) of cultured $-$ /MSU and MSU/MSU SF after bulk RNA-seq using mRNA enrichment.
 (D) Volcano plot of differential gene expression in MSU/MSU as compared to $-$ /MSU SF ($n = 3$).
 (E) Expression Z-scores of selected genes from bulk RNA-seq.
 (F) Confirmatory qPCR analysis in cultured SF determining expression of *C3* ($n = 7$), *C4b* ($n = 7$), and *Il6* ($n = 6$). Plotted are individual values and means.
 (G) RANKL/OPG ratios calculated from bulk RNA-seq.
 (H) PCA of SF subsets based on ATAC-seq peak location and intensity.
 (I) Volcano plot of gene-annotated open chromatin regions $\pm$ 3kbp from the TSS in MSU/MSU as compared to $-$ /MSU SF ($n = 3$).
 (J) Normalized ATAC-seq signals around the TSS of *C3* and *Nlrp3*. Each lane shows pooled data from 3 SF cultures.
 (K) Mean Z-scores of selected genes generated from ATAC-seq counts of 3 replicates.
 (L and M) Differential gene expression between clusters from sc RNA-seq of CD45⁺ cells from MSU-injected paws. The UMAP plots (L) depict the initial automatic cluster assignments from Seurat. The lower panel shows the same UMAP plot colored by origin from $-$ /MSU and MSU/MSU paws. The heatmap (M) shows the (row-scaled) expression of the top-10 (by fold change) significant marker genes for each cluster (FDR, Benjamini–Hochberg adjusted) in the downsampled integrated dataset comprising the $-$ /MSU and the MSU/MSU data. Each column represents a single cell and each row shows the given gene. SL, sublining layer SF; LL, lining layer SF.
 (N) Relative frequencies of SL fibroblasts in MSU/MSU and $-$ /MSU SF, deconvoluted from bulk RNA-seq data using sc RNA-seq reference profiles.
 (O–Q) Flow cytometric characterization of SF marker expression in naive paws and in paws two days after the 1st or 2nd MSU injection.
 (O) Percentages of LL (CD45⁺CD31⁺Pdnp⁺Thy1⁺) and SL (CD45⁺CD31⁺Pdnp⁺Thy1⁺) SF ($n = 6$).
 (P and Q) Representative histograms of mean fluorescence intensities of C3 (P) and quantification of C3⁺ cells in LL and SL (Q). Every symbol represents pooled cells from 2 mice. $n = 3$.
 Dashed lines in (P) indicate cutoff values for C3⁺ cells depicted in (Q). ANOVA with Tukey's test (F, O, Q); see also Figure S3.



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challenged with TNF α *in vitro* (Figures 6E and S6A). Also, the senescence markers *p15* (*Cdkn2b*), *p16* (*Cdkn2a*), and *p21* (*Cdkn1a*) were upregulated in C3^{-/-} MSU/MSU SF, while *Il6* expression was abolished (Figure 6F). Proliferation in unstimulated WT and C3^{-/-} SF was not different, but it was found reduced in C3^{-/-} cells after two rounds of stimulation with TNF α (Figure S6B). This suggests that in the absence of C3, repeatedly stimulated SF fail to be primed and instead adopt a senescent immune-regulatory phenotype (Montero-Melendez et al., 2020).

In human T cells, intracellular C3 is cleaved by Cathepsin L to C3a which subsequently engages the C3aR in lysosomes, resulting in tonic activation of mTOR and enabling sustained glycolysis for cell survival (Kolev et al., 2015; Laumonnier et al., 2017). Upon T cell stimulation C3 activation is increased and C3a is shuttled to the cell surface (West et al., 2020). Somewhat similarly, C3aR expression was enhanced in primed SF and localized to the cell membrane (Figures 6G and S6C). These data from confocal microscopy were corroborated by transcriptomic and epigenetic analyses from bulk RNA-seq and ATAC-seq, respectively, which showed inducible expression of *Ctsl* and *C3ar1* mRNAs and increased openness in the TSS of these genes in primed SF (Figures 3E, 3F, and 3K).

To assess if C3aR is instrumental for tissue priming, we performed iterated MSU-induced arthritis in C3aR^{-/-} mice (Humbles et al., 2000). Comparably to C3, priming was significantly reduced in the absence of C3aR (Figure 6H). The metabolic changes seen in cultured primed WT SF were not seen in C3aR^{-/-} SF (Figures 6I, 6J, S6D, and S6E). Rather the metabolic profile of MSU/MSU C3aR^{-/-} SF was reminiscent of C3^{-/-} SF, with a marked shift toward a quiescent phenotype. In line, MSU/MSU C3aR^{-/-} cells had a significantly reduced capacity to transfer tissue priming (Figures 6K and S6F). These data suggest that C3aR is essential for C3-mediated metabolic reprogramming during inflammatory SF priming.

To further investigate metabolic reprogramming downstream of C3 and C3aR, we assessed mTOR activity *ex vivo* in CD31⁺CD45⁻ cells from paws two days after the 1st or 2nd injection of MSU, respectively (Figure 6L). Phosphorylation of mTOR and of S6 kinase and 4EBP1, two translational regulators activated downstream of mTOR, was increased in primed SF. Additionally, we observed increased expression of HIF1 α , a transcription factor that mediates glycolysis and survival in human SF also under normoxic conditions (Del Rey et al., 2017), in MSU/MSU but not -/MSU SF (Figure 6M). Phosphorylation of mTOR and HIF1 α expression was also increased in cultured

primed WT, but not C3^{-/-} or C3aR^{-/-} SF (Figure S6G). Functionally, inhibition of mTOR with rapamycin and treatment with KC7F2, a translational inhibitor of HIF1 α , which also represses 4EBP1 activation (Narita et al., 2009), abrogated glycolysis upon repeated TNF α stimulation *in vitro* and increased senescence (Figures 6N, 6O, S6H, and S6I). Overall, bioenergetics and metabolic dependencies of repeatedly TNF α -stimulated SF were skewed toward aerobic glycolysis (Figure S6H). This metabolic shift was abrogated by treatment with rapamycin or KC7F2.

Metabolic changes modulate the activity of inflammasomes (Arbore and Kemper, 2016). *Nlrp3* was one of the highest upregulated genes in bulk Seq data from primed SF (Figures 3D–3F), which was confirmed by qPCR in WT but not C3^{-/-} cells (Figure 6P). Furthermore, the activity of caspase 1 was enhanced in primed SF (Figure 6Q). Accordingly, primed SF released more IL-1 β into the cell culture supernatants (Figure 6R). *In vitro* treatment with 2-DG diminished *Nlrp3* expression in both -/MSU and MSU/MSU SF (Figure 6S), suggesting a direct connection between glycolysis and the inflammasome in these cells. Finally, to test the effect of NLRP3 inhibition on inflammatory tissue priming, we used MCC950, a small molecule that specifically inhibits canonical and non-canonical activation of the NLRP3 inflammasome (Coll et al., 2015). MCC950 not only dampened paw swelling, but also abrogated tissue priming and bone damage (Figures 6T, S6J, and S6K).

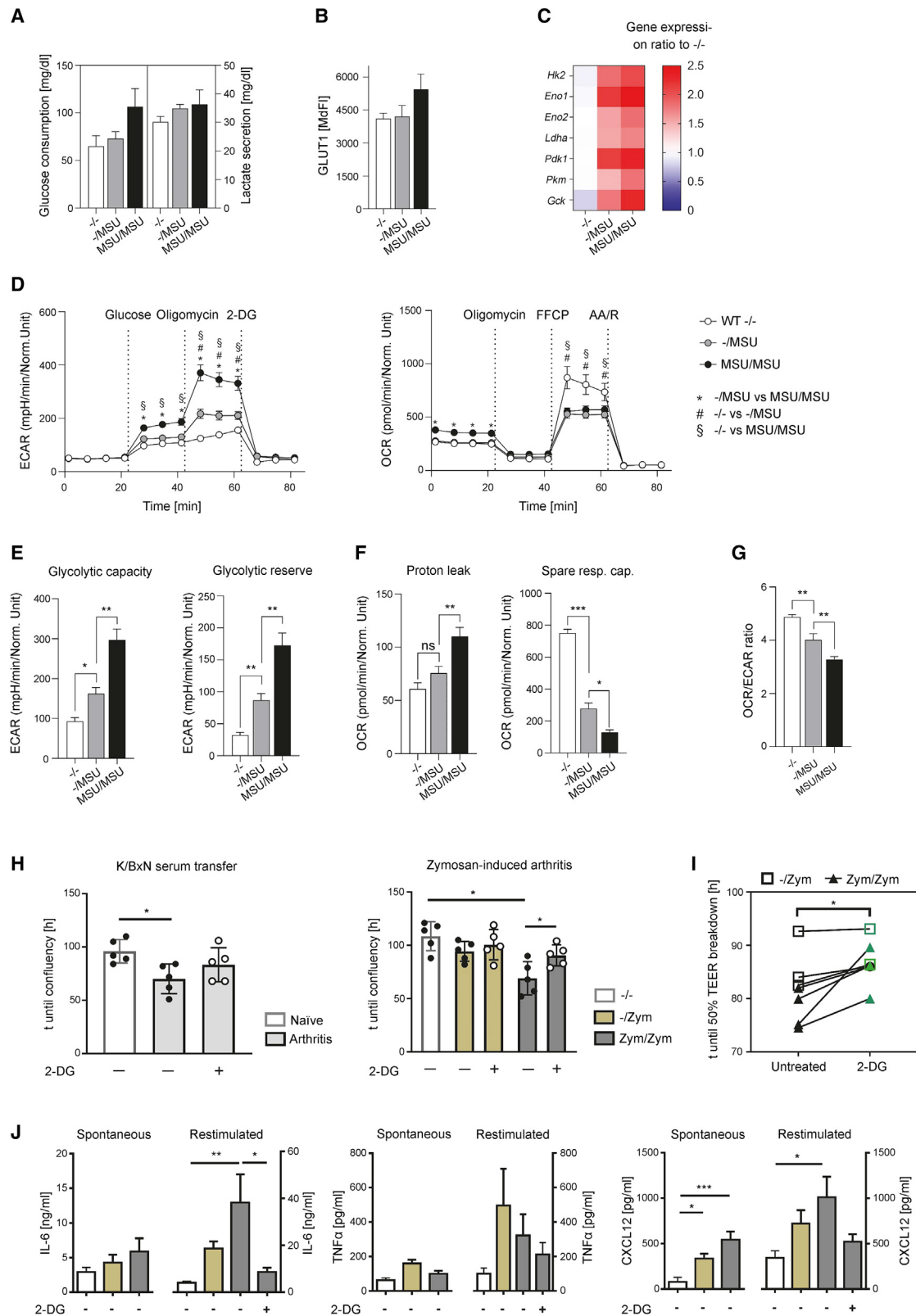
To assess if C3-associated metabolic reprogramming in SF also affects bone destruction, we first performed expression analysis of key mediators of OC differentiation. The enhanced expression of *Tnfsf11*, *Tnf*, *Il1b*, and *Il6* in primed SF was reduced to normal amounts upon treatment with 2-DG or in the absence of C3 (Figure S6L). *Csf1* (M-CSF) and *Tnfrsf11b* (OPG) expression was only diminished in C3^{-/-} mice. The RANKL/OPG ratio was consistently decreased upon 2-DG treatment and in C3^{-/-} SF, both on mRNA level and in supernatants (Figure S6M). Treatment with recombinant OPG, the IL-1 receptor antagonist anakinra and the TNF α decoy receptor etanercept strongly abolished OC differentiation induced by primed SF (Figure S6N).

Taken together, these data indicate that inflammatory tissue priming is based on a bioenergetic switch of fibroblasts toward a more energetic phenotype, with a skewing toward aerobic glycolysis in response to C3-C3a-C3aR axis activation. This metabolic switch fundamentally changes the SFs' functional phenotype, increases the impact of recurrent inflammation, and mediates inflammatory bone destruction which is driven

Figure 4. Complement C3 upregulation mediates pathogenic SF function and inflammatory tissue priming

- (A) Confocal microscopy images and quantification of intracellular C3 expression in 100 SF per condition. Arrowheads, extracellular C3. Scale bars, 5 μ m. Images are representative of 2 experiments with 100 cells each.
- (B) Paw swelling and priming indices of iterated MSU-induced arthritis in C57BL/6 WT and C3^{-/-} mice (n = 10–13). Mann-Whitney U-test.
- (C and D) Paw swelling (C) and priming indices (D) of iterated MSU-induced arthritis in irradiated WT and C3^{-/-} mice (n = 4–8) after stable reconstitution with WT bone marrow. Unpaired t test.
- (E–G) Induction of tissue priming by injection of sorted cultured CD45⁺CD31⁻ -/-, -/MSU, or MSU/MSU SF from WT or C3^{-/-} mice into naive WT or C3^{-/-} paws.
- (E) Injection scheme of SF transfer model.
- (F) Course of paw swelling.
- (G) Priming indices. n = 3–8, one sample t test. c., cells.
- (H) Quantification of migration of WT and C3^{-/-} SF (n = 4–12). ANOVA with Tukey's test.
- (I) Matrix invasion of WT and C3^{-/-} SF, as analyzed by decrease of TEER indicating disturbance of an MDCK epithelial cell layer (n = 4).

See also Figure S4.



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by TNF, IL-1, and disturbance of the balance between RANKL and OPG (Armaka et al., 2008).

C3 is expressed in a distinct population of human sublining SF implicated in arthritis

To translate our observations in animals to human disease, we assessed complement expression and metabolism in SF from the joints of RA patients. These cells have been shown to synthesize several complement components, among them C3, C1r and C1s (Katz and Strunk, 1988; Neumann et al., 2002). Using publicly available scRNA-seq data of human synovial tissue samples (Donlin et al., 2018; Zhang et al., 2019) we found C3, C1R, C1S, CFB, CFH, CFP, SERPING1, and the C3-cleaving protease CTSL specifically upregulated in CD45-Pdpr⁺ SF from leukocyte-rich RA synovial tissue (Figure 7A), which is characterized by higher inflammation scores (Zhang et al., 2019). In contrast, C4A and CD55 exhibited lower expression in SF derived from patients with leukocyte-rich RA than with leukocyte-poor RA or osteoarthritis (OA). The C3AR1 had a generally low expression among SF, without significant differences between groups.

Transcriptomic analysis by scRNA-seq (Figure 7B) and *in situ* hybridization (Figure 7C) showed that C3 expression is confined to distinct fibroblast (scF1,2,3) and macrophage (scM1,2) subsets in the human joint. In line with previous studies, C3-expressing macrophages and SF were located in the sublining and absent from the lining layer (Croft et al., 2019; Donlin et al., 2018; Zhang et al., 2019).

Upregulation of C3 in leukocyte-rich RA suggested that C3 expression is connected to synovial disease activity. To assess if C3 expression is induced by inflammatory triggers we repeatedly stimulated cultured RA-SF with TNF α . Analysis by western blots revealed proteolytical activation of C3 in SF (Figure S7A), as has been described in T cells (Liszewski et al., 2013). Furthermore, C3 expression was upregulated gradually after repeated stimulation with TNF α (Figures 7D and S7B).

To date, functional studies on SF have mostly been performed on cultured cells isolated from individuals with established RA. Therefore, the contribution of SF to the transition from early arthritis to established RA has remained relatively unexplored. We therefore compared SF from very early anti-citrullinated peptide antibody-positive RA (VERA; symptom duration under 3 months) with samples from established RA (symptom duration 6–36 months) and non-inflamed control subjects (NHD). SF from established RA were characterized by increased invasiveness as compared to SF from VERA or NHD (Figure 7E). Samples from RA but also from other chronic arthritides with active synovitis

(gout and psoriatic arthritis) (Cimmino et al., 2011; Fromm et al., 2019) also exhibited increased cell mobility in comparison to SF from VERA and NHD (Figure 7F). Both invasiveness and migration showed trends to correlate with erythrocyte sedimentation rate and C-reactive protein serum concentrations (Figure S7B), establishing a connection between systemic inflammation and altered SF function. Finally, we also observed a trend toward a glycolytic shift in SF from established RA (Figure 7G), similar to primed murine SF (Figure 5G).

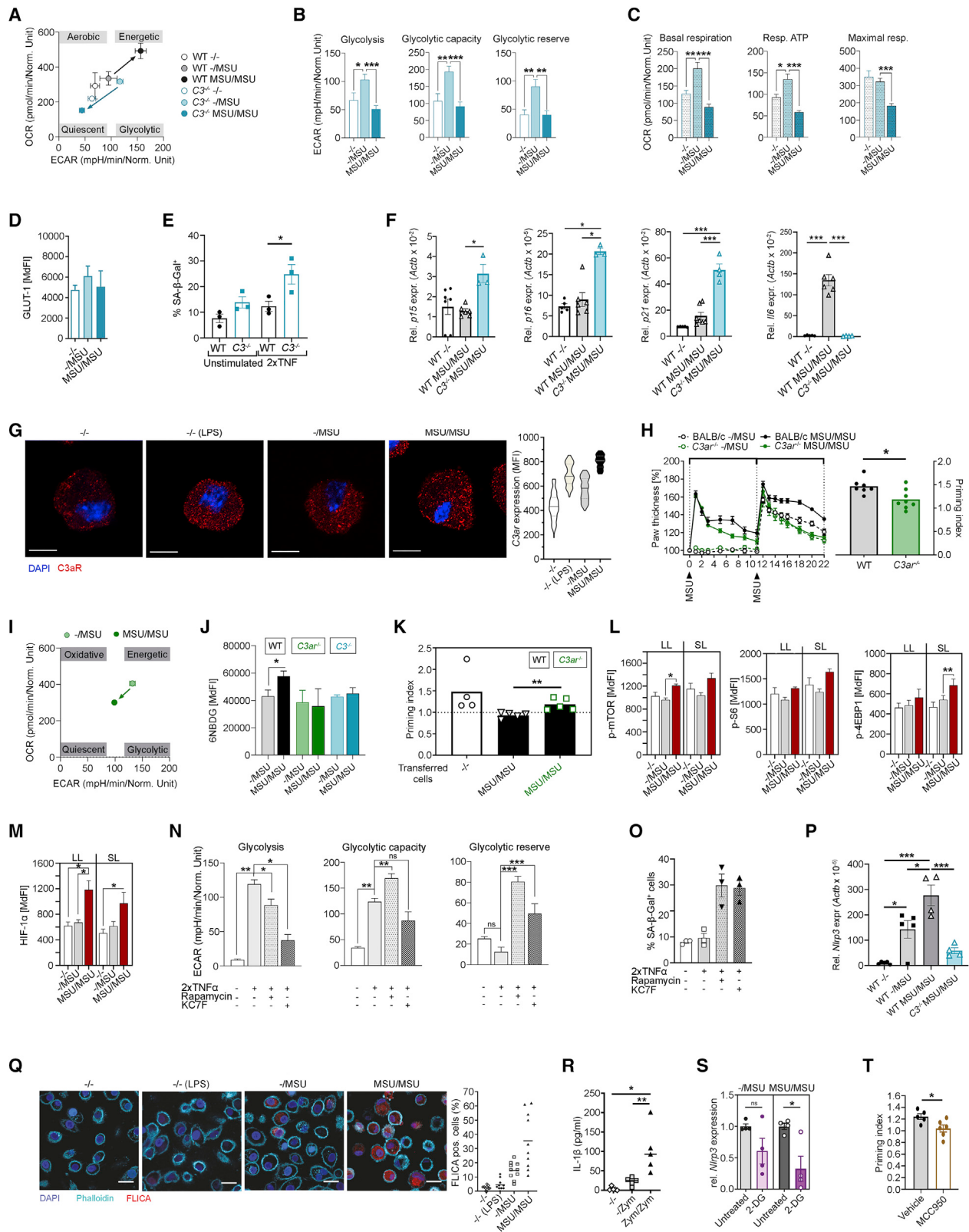
Hence, many of the functional changes observed during the transition between very early and established arthritis in human SF resemble those occurring during priming in rodent fibroblasts.

DISCUSSION

A network of SF interacting with resident macrophages, endothelial and infiltrating myeloid cells characterizes inflamed joints. Macrophages can enter a transient state of non-responsiveness upon challenge with LPS or TNF α and protect from inflammation (Culemann et al., 2019; Huber et al., 2017; Seeley and Ghosh, 2017). In contrast, SF gradually acquire a more disease-promoting phenotype during arthritis, characterized by production of inflammatory mediators (Crowley et al., 2017; Klein et al., 2017; Nguyen et al., 2017), a proliferative and invasive cell behavior triggering tissue destruction (Bottini and Firestein, 2013; Buckley et al., 2001; Scott et al., 2010) and promotion of leukocyte trafficking into joints (Buckley, 2017; Filer et al., 2017; Postigo et al., 1993). Modern cellular phenotyping has uncovered that the Thy1⁺CD34⁺ subset of SF, which is found in the sublining regions of the synovium and closely interacts with vascular endothelial cells through NOTCH3 signaling (Wei et al., 2020), is expanded in murine and human arthritis (Croft et al., 2019; Croft et al., 2016; Mizoguchi et al., 2018; Zhang et al., 2019). In our work, we show that this subset expresses high amounts of complement components, undergoes metabolic rewiring, and mediates inflammatory tissue priming. Physiologically, this allows mounting a local alert state in the tissue that enables site-directed more robust responses to repeated challenges. In disease, tissue priming provides an explanation for relapses and for the transition from self-limited to chronic inflammation. This effect goes along with prolonged but not necessarily more intense local inflammation and is different from trained immunity, which is characterized by a more systemic, faster, and stronger rather than prolonged inflammatory response (Netea et al., 2011).

Figure 5. Metabolic shift during establishment of inflammatory tissue priming

- (A) Glucose consumption and lactate secretion by $-/-$ (n = 5), $-/MSU$ (n = 7), and MSU/MSU (n = 9) SF.
 (B) GLUT1 surface expression, as assessed in the same SF samples (n = 7–9) by flow cytometry. (C) Gene expression of key glycolytic enzymes, determined by RNA-seq and calculated as the ratio to the mean normalized counts of $-/-$ SF (n = 3).
 (D–G) Bioenergetics of $-/-$ (n = 3), $-/MSU$ (n = 7), and MSU/MSU (n = 7) SF normalized to background and total protein (D). *p < 0.01 of $-/MSU$ versus MSU/MSU , #p < 0.01 of $-/-$ versus $-/MSU$, §p < 0.01 of $-/-$ versus MSU/MSU . From these data, key glycolytic (E) and respiratory (F) parameters as well as the ratio (G) between OCR and ECAR were calculated. Kruskal-Wallis test.
 (H) Quantitative assessment of migration in naive SF and in SF isolated from K/BxN serum transfer arthritis or zymosan-induced arthritis (n = 5) and treated with 2mM 2-DG. ANOVA with Dunnett's test (left panel) or Sidak's test (right panel).
 (I) Decline of invasiveness of $-/Zym$ (n = 3) or Zym/Zym (n = 4) SF upon treatment with 2-DG. Paired t test.
 (J) Concentrations of IL-6, TNF α and CXCL12 in supernatants of unstimulated mouse SF (spontaneous) and SF re-stimulated *in vitro* with Zym and incubated with or without 2-DG (n = 5). ANOVA with Tukey's test.
 See also Figure S5.



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Metabolic adaptation is a prerequisite for SF to maintain viability and functional competence (Falconer et al., 2018; Fearon et al., 2016; Folmes et al., 2012). Inflammatory mediators such as TNF α promote glycolytic flux (Garcia-Carbonell et al., 2016) and SF in arthritis display a metabolic shift toward glycolysis that correlates with pathogenic function (Falconer et al., 2018; Fearon et al., 2016; Gallagher et al., 2019; McGarry et al., 2017). In particular, hexokinase 2 mediates synovial lining hypertrophy, disease severity and tissue damage during arthritis (Bustamante et al., 2018; Garcia-Carbonell et al., 2016). Metabolic interventions targeting the altered SF metabolism could therefore represent an option for arthritis therapy, similar to the approaches tested in oncology (Tennant et al., 2010).

Our data indicate that activated C3 (C3a) and its receptor are instrumental for the metabolic rewiring underlying fibroblast priming. In T cells, complement C3 and C5 and its receptors are produced intracellularly and induce metabolic changes that in turn activate the cells in an autocrine manner (Arbore et al., 2016; Kolev et al., 2015; Laumonnier et al., 2017; Liszewski et al., 2013). In contrast to T cells, little is known on the function of the complement system in resident tissue. However, recently it has been shown that upregulation of complement in tendons and synovial tissue is associated with exacerbation of arthritis caused by increased mechanical strain (Cambré et al., 2018; Cambré et al., 2019). We show here that C3 and C3aR were both overexpressed intracellularly in primed SF and C3aR increasingly colocalized with the plasma membrane upon priming.

Mechanistically, inflammatory tissue priming involved the mTOR pathway and HIF1 α in SF. An mTOR–HIF1 α axis has previously also been shown to be responsible for a switch to glycolysis in T helper-17 cells (Shi et al., 2011). HIF1 α is a transcription factor causing sustained production of IL-1 β downstream of glycolytic enzymes and metabolites (Tannahill et al., 2013). In SF, HIF1 α is a pro-survival signal that promotes the expression

of inflammatory cytokines and perpetuates the interaction with adaptive immune cells (Del Rey et al., 2017; Hu et al., 2016). In line with this, pharmacological inhibition of the mTOR or HIF1 α reduced priming-induced glycolysis and promoted cellular senescence. This senescent phenotype also occurred in C3 $^{-/-}$ SF after repeated stimulation. Given the regulatory phenotype of C3 $^{-/-}$ cells, the metabolic activation initiated by C3 seems to be crucial for the override of the stimulation-induced shut-down observed in primed SF.

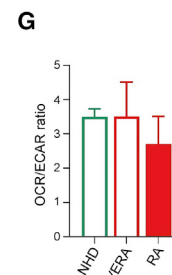
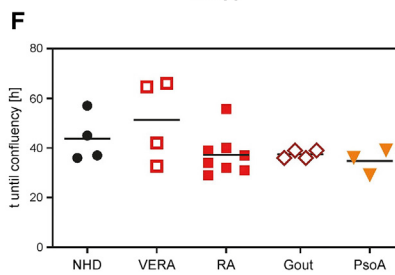
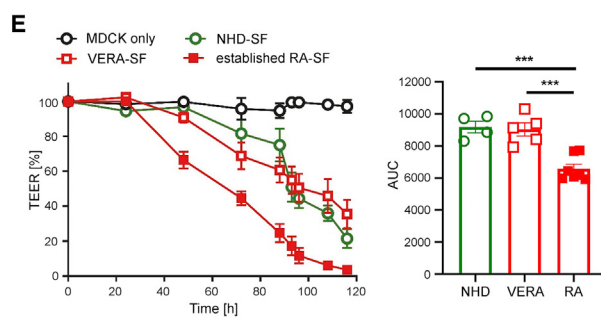
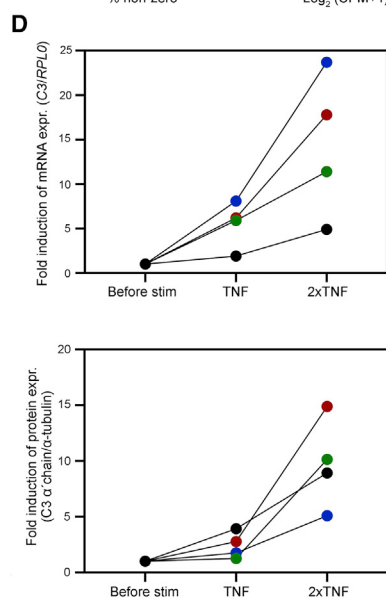
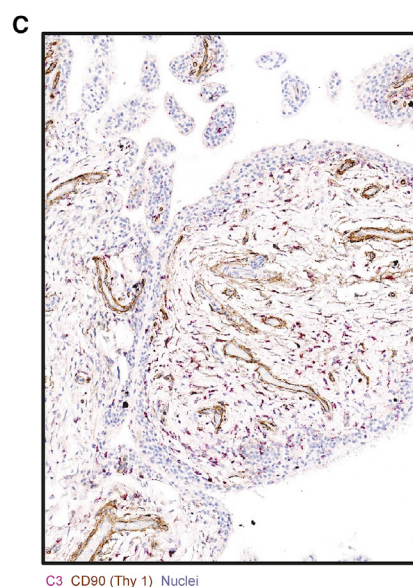
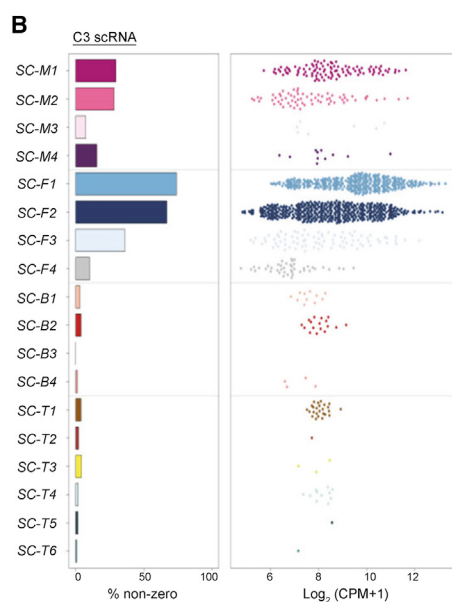
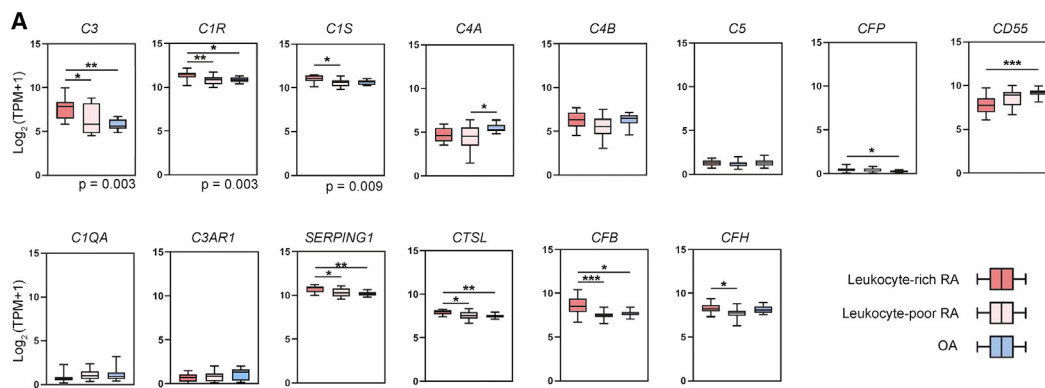
Complement activation and ensuing glycolysis or generation of reactive oxygen species can trigger a local pro-inflammatory environment by inflammasome activation (Arbore and Kemper, 2016; Asgari et al., 2013; Chen et al., 2013; Laudisi et al., 2013; Moon et al., 2015). Hence, C3a and C5a activate the NLRP3 inflammasome and IL-1 β and IL-18 release from monocytes (An et al., 2014; Asgari et al., 2013; Carta et al., 2015; Cumpelik et al., 2016) and intracellular C3 activation contributes to Th1 cell hyperactivity in RA (Liszewski et al., 2013). Thus, a complement–metabolism–inflammasome axis drives recurrent inflammation, yet until our work, evidence that this network is operational in cells other than T cells has been missing and site-directed tissue priming could therefore not be explained.

Our human data strongly support the mechanistic concept developed in rodents. First, we showed that C3 activation is linked to the Thy1 $^{+}$ CD34 $^{+}$ subset of inflammatory sublining SF. Second, C3 expression was associated with a leukocyte-rich more inflammatory phenotype of arthritis caused by inflammatory tissue priming. Finally, SF of patients with established but not early disease acquire a functional phenotype that strongly resembled the changes observed in primed SF from rodent arthritis models.

These findings have fundamental implications for human inflammatory disease. While modern therapy can effectively reduce inflammation in chronic inflammatory diseases, the cessation of treatment usually leads to recurrence, suggesting underlying mechanisms different from the effects of

Figure 6. Complement C3 mediates metabolic changes that propagate tissue priming

- (A) Energy map of SF from WT and C3 $^{-/-}$ mice as assessed by metabolic flux analysis of ECAR and OCR.
 (B and C) Key glycolytic (B) and respiratory (C) parameters calculated from normalized metabolic flux data of C3 $^{-/-}$ SF.
 (D) GLUT1 surface expression in C3 $^{-/-}$ SF (n = 2–4).
 (E) Percentages of SA- β -Gal $^{+}$ SF (passage 8) repeatedly incubated with TNF α or left untreated.
 (F) qPCR analysis of cultured SF (n = 3–7) determining expression of the senescence markers *p15*, *p16*, and *p21*, and of *Il6*.
 (G) Representative confocal microscopy images and quantification of C3aR expression in 100 mouse SF per condition. Blue, nuclei; red, C3aR. Scale bars, 10 μ m. Violin plots show median and quartiles.
 (H) Paw thickness and priming indices of iterated MSU-induced arthritis in BALB/c WT and C3aR $^{-/-}$ mice (n = 7–8).
 (I) Energy map of SF from C3aR $^{-/-}$ mice.
 (J) Median fluorescence intensity (MFI) of the fluorescent nonhydrolyzable glucose analog 6NDGB in SF from WT (BALB/c), C3 $^{-/-}$ and C3aR $^{-/-}$ mice (n = 3–7).
 (K) Priming indices after transfer of $-/-$ or MSU/MSU WT (BALB/c) or C3aR $^{-/-}$ SF into the paws of WT BALB/c mice. N = 4–5.
 (L and M) Flow cytometric analysis of mTOR, 4EBP1 and S6 phosphorylation (L) and HIF1 α expression (M) in CD45 $^{+}$ CD31 $^{+}$ Pdpn $^{+}$ Thy1 $^{-}$ lining layer (LL) and CDC45 $^{+}$ CD31 $^{+}$ Pdpn $^{+}$ Thy1 $^{+}$ sublining (SL) SF from naive paws and from paws 2 days after the 1 st ($-$ /MSU) or 2 nd (MSU/MSU) MSU injection. n = 3 (p-mTOR, p-S6), 6 (p-4EBP1), or 4 (HIF1 α).
 (N and O) Key glycolytic parameters (N) and percentages of SA- β -Gal $^{+}$ cells (O) in naive WT SF (n = 2–3 replicates of pooled cells from 2 mice) after two stimulation rounds with 10 ng/mL TNF α with or without rapamycin (50 μ M) or KC7F2 (30 μ M).
 (P) qPCR analysis in *in vitro* cultured WT and C3 $^{-/-}$ SF determining expression of *Nlrp3* (n = 4–6).
 (Q) Confocal microscopy images and quantification of caspase-1 enzymatic activity in SF by labeling with the fluorescent caspase-1 probe FAM-YVAD-FMK (FLICA). Images are representative of 2 experiments with each time 10 visual fields per condition. Blue, nuclei; turquoise, F-actin (phalloidin); red, FLICA. Scale bars, 20 μ m.
 (R) IL-1 β concentrations in supernatants of SF (n = 5).
 (S) Expression of *Nlrp3* in SF (n = 4) cultured for 24 h with 2 mM 2-DG, as determined by qPCR.
 (T) Priming indices of iterated MSU-induced arthritis upon treatment with the NLRP3 inflammasome inhibitor MCC950 or vehicle.
 Scale bars, 1 mm. All subfigures show means $\pm$ SEM. Unpaired t test (H, K, T); ANOVA with Tukey's test (F, L, M, P, R), with Sidak's test (B–E, J, S) or Dunnett's test (N). See also Figure S6.



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inflammatory effector cytokines (Schett et al., 2016). Proof of efficacy of complement-targeted therapy in human arthritis is still lacking (Holers and Banda, 2018; Trouw et al., 2017). The inhibitory anti-C5 antibody eculizumab and the peptide C5aR inhibitor PMX-53 were both unsuccessful in clinical trials of RA (Vergunst et al., 2007). Of note, both act downstream of C3 cleavage rather than targeting intracellular autocrine C3 function. Furthermore, our results indicate that complement factor C3- or C3a-targeted therapy is expected to prevent flares due to its effect on tissue priming rather than to decrease inflammation by directly interfering with effector cytokines or anaphylatoxins. Hence the design of studies using complement inhibition to control arthritis, i.e., to reverse tissue priming, may need to be re-conceptualized.

Possible intervention strategies could be direct targeting of C3 (Mastellos et al., 2019), the reversal of metabolic rewiring by inhibitors of glycolysis (Akins et al., 2018), and small molecule inhibitors for the NLRP3 inflammasome (Yang et al., 2019). Notably such interventions may not lead to rapid suppression of acute inflammation, such as observed with anti-cytokine drugs, but they may reverse the underlying tissue priming, thus allowing physicians to successfully taper and even stop treatment with anti-inflammatory drugs without provoking disease flares.

Limitations of the study

While our transfer model demonstrated that SF direct inflammatory tissue priming and we excluded a role of tissue-resident macrophages, it might well be that other resident or infiltrating cell types play their part in priming. Furthermore, future studies should address the question how long the observed local priming effect lasts *in vivo* and if there is a connection to the more systemically operating phenomenon of trained immunity. Lastly, due to the differences between the murine and the human complement systems (Neumann et al., 2002; Seya et al., 1998) the full details underlying intracellular C3 cleavage and receptor activation in human SF also need to be elaborated further.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.immuni.2021.03.003>.

Figure 7. The complement system is upregulated in activated sublining SF of human individuals with inflammatory arthritis

(A) Expression (shown as transcripts per kb million + 1) of complement proteins derived from bulk RNA-seq on CD45⁺ Pdpn⁺ SF from synovial tissue of individuals with leukocyte-rich RA (n = 16), leukocyte-poor RA (n = 17), and OA (n = 12). Shown are medians, 25th and 75th percentile (boxes), range (whiskers), and individual values. Kruskal-Wallis test with Dunn's test.

(B) Sc RNA-seq of human synovial membrane showing expression of C3 in fibroblast and leukocyte subsets.

(C) RNAscope *in situ* hybridization of an OA-derived synovium showing accumulation of C3 expressing cells in the sublining.

(D) C3 expression related to expression of *RPLP0* (left) and quantification of C3 protein (α' chain) derived from western blot (right) in cultured SF from 4 individuals with RA before or after repeated stimulation with TNF *in vitro*. Data are normalized to expression before stimulation.

(E) Decrease of TEER in a MATRIN assay using SF from individuals with very early RA (VERA, n = 5), established RA (n = 8) and normal healthy donors (NHD, n = 4). ANOVA with Tukey's test.

(F) Quantification of migration with SF from NHD (n = 4), VERA (n = 4), established RA (n = 7), gouty arthritis (n = 4), and psoriatic arthritis (PsoA, n = 3).

(G) Glycolytic shift of SF from RA patients (n = 3) compared to SF from NHD (n = 2) or VERA (n = 3) as assessed by OCR/ECAR ratio.

See also Figure S7.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat monoclonal anti-CD45 antibody PerCP Cy5.5, clone 30-F11 anti-mouse	Biolegend	Cat#103132; RRID:AB_893340
Rat monoclonal anti-CD31 antibody AF 647, clone MEC13.3 anti-mouse	Biolegend	Cat#102516; RRID:AB_2161029
Rat monoclonal anti-CD45 antibody APC, clone 30-F11 anti-mouse	Biolegend	Cat#103112; RRID:AB_312977
Rat monoclonal anti-CD31 antibody Brilliant Violet 421™ clone 390 anti-mouse	Biolegend	Cat102423; RRID:AB_2562186
Mouse monoclonal PE-Cy7 anti CD45.1 antibody PE-Cy7, clone A20 anti-mouse	eBioscience	Cat#25-0453-82; RRID:AB_469629
Mouse monoclonal anti 45.2 antibody AlexaFluor700 clone 104 anti-mouse	eBioscience	Cat#56-0454-82; RRID:AB_657752
Rat monoclonal anti-Ly6G antibody AF647, clone 1A8 anti-mouse	Biolegend	Cat#127610; RRID:AB_1134159
Sheep polyclonal anti-CD90-Thy1 antibody, anti-mouse	R&D systems	Cat#AF2067; RRID:AB_11127215
Peroxidase AffiniPure Donkey polyclonal IgG H ⁺ L, anti-sheep	Jackson ImmunoResearch Labs	Cat#713-035-147; RRID:AB_2340710
Rat monoclonal anti-C3, clone 11H9 anti-mouse	Abcam	Cat# ab11862; RRID:AB_2066623
Rabbit monoclonal anti-Rab7, clone D95F2, anti-mouse	Cell Signaling Technology	Cat#9367; RRID:AB_1904103
Goat polyclonal IgG, AF488, anti-rat	Thermo Fisher Scientific	Cat#11006; RRID:AB_2534074
Goat polyclonal IgG, AF 647, anti-rabbit	Thermo Fisher Scientific	Cat#A32733TR; RRID:AB_2866492
Rabbit monoclonal anti-C3, clone EPR2988 anti-human	Abcam	Cat#ab181147
Mouse monoclonal anti α -tubulin anti-human	Abcam	Cat# ab11304; RRID:AB_297909
Peroxidase AffiniPure Goat polyclonal IgG, H ⁺ L, anti-rabbit	Jackson ImmunoResearch Labs	Cat#111035144; RRID:AB_2307391
Peroxidase AffiniPure Goat polyclonal IgG, H ⁺ L, anti-mouse	Jackson ImmunoResearch Labs	Cat#115035003; RRID:AB_10015289
Sheep Polyclonal anti FAP1 α IgG anti-human	R&D Systems	Cat#AF3715-SP; RRID:AB_2102368
Mouse Monoclonal Anti-Goat/Sheep IgG–Biotin antibody, clone GT34	Sigma-Aldrich	Cat#B3148; RRID:AB_258536
Rat monoclonal anti CD45 APC-Cy7, clone 30-F11, anti-mouse	Biolegend	Cat#103115; RRID:AB_312980
Rat monoclonal anti CD90.2 BUV395, clone 53-2.1, anti-mouse	BD Biosciences	Cat#565257; RRID:AB_2739136
Rabbit monoclonal anti Glut1 AlexaFluor 488, clone EPR3915, anti-mouse	Abcam	Cat#ab195359; RRID:AB_2714026
Syrian hamster anti Podoplanin PE, clone 8.1.1, anti-mouse	eBioscience	Cat#12-5381-82; RRID:AB_1907439
Rat monoclonal anti C3 FITC, anti-mouse, clone rmc11 h9, anti-mouse	CEDARLANE	Cat# CL7503F; RRID:AB_10061294

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Rabbit polyclonal (4EBP1 (pT37/47) AlexaFluorTM 594, anti-mouse	Bioss	Cat#bs-3019R-A594
Mouse monoclonal anti mTOR (pSer2448) PE, clone MRRBY, anti-mouse	Thermo Fisher Scientific	Cat# 12-9718-42; RRID:AB_2572724
Mouse monoclonal anti mTOR (pSer2448) PerCP-eFluor780, clone MRRBY, anti-mouse	Thermo Fisher Scientific	Cat# 46-9718-42; RRID:AB_2573895
Mouse monoclonal anti S6 (pSer235, pSer236) APC, clone cupk43k, anti-mouse	Thermo Fisher Scientific	Cat# 17-9007-41; RRID:AB_2573269
Mouse monoclonal anti HIF1alpha APC, clone Mgc3, anti-mouse	Thermo Fisher Scientific	Cat# 17-7528-82; RRID:AB_2802231
Mouse bb5.1 antibody	Zelev et al., 2020	Cardiff University
FcR Blocking Reagent, mouse	Miltenyi Biotec	Cat#130-092-575
Biological Samples		
Serum from arthritic K/BxN mice	Animal facility of the University of Erlangen	https://www.fpz.fau.de/leistungen/transgenic-mouse-facility.shtml
Human synovial tissue specimens from RA patients	Orthopedic Surgery, Schulthess Clinic Zurich	https://www.schulthess-klinik.ch/de
Joint aspirates	University Clinics of Erlangen	N/A
Chemicals, Peptides, and Recombinant Proteins		
Zymosan A	Sigma Aldrich	Cat#Z4250
MCC950	Sigma Aldrich	Cat# PZ0280
Recombinant murine M-CSF	PeproTech	Cat#31502
Recombinant murine RANKL	PeproTech	Cat#31511
Recombinant murine OPG	PeproTech	Cat#450-14
Selective HIF-1 α translation inhibitor KC7F2	Selleckchem	Cat#s7946
BD Matrigel™	BD Biosciences,	Cat#354234
MCC950 (CP-456773 sodium salt)	Sigma-Aldrich	Cat#PZ028
Flash Phalloidin™ Red 594, BioLegend	Biolegend	Cat#424303
ITS supplement	BioWhittaker	Cat#17838-Z
RNA scope C3 probe	Bio-Techne	Cat#430701
Anakinra (Kineret)	SOBI	Cat# 6665823407
Etanercept	Novartis	Cat#49032441
BUV 737 Streptavidin	BDBiosciences	Cat#612775
Critical Commercial Assays		
Zombie Aqua™ Fixable Viability Kit	Biolegend	Cat#423102
Sytox Green Nucleic Acid Stain	Thermo Fisher Scientific	Cat#S7020
ATAC Seq Kit	Active motif	Cat# 53150
TruSeq stranded total RNA kit with Ribo-Zero Globin	Illumina	Cat#20020612
TruSeq stranded mRNA kit	Illumina	Cat#20020594
10x Chromium Single Cell 3c Solution v3	10xGenomics	Cat#1000075
qPCR MasterMix Plus for SYBR® Assay ROX	Eurogentec	Cat#RT-SN2X-03+
Quick-RNA Micro Prep Kit	Zymo Research	Cat#R1050
Wound healing assay kit	Ibidi	Cat#80209
Senescence detection kit	Abcam	Cat#65351
Rat IL-6 ELISA Set	BDOptEIA	Cat#550319
Pierce™ BCA Protein Assay Kit	Thermo Fisher scientific	Cat#23227
Acid Phosphatase, Leukocyte (TRAP) Kit	Sigma-Aldrich	Cat# 387A-1KT

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
RNAscope® 2.5 HD Detection Reagents-RED	Bio-Techne	Cat#322360
FLICA® 660 Caspase-1 assay kit	ImmunoChemistry Technologies	Cat#9122
Mouse TRANCE/TNFSF11/RANK L ELISA	R&D Systems	Cat#Dy462
Mouse Osteoprotegrin Duo set	R&D Systems	Cat#DY459

Deposited data

Mouse bulk RNA Seq data	BioProject	PRJNA714633
Mouse bulk RNA Seq data (validation set)	GEO	GSE163749
Mouse sc RNA Seq data	BioProject	PRJNA714636
Mouse ATAC Seq data	BioProject	PRJNA713761

Experimental Models: Cell Lines

Canine: MDCK-C7	Wunrau et al., 2009	University of Münster
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Experimental Models: Organisms/Strains

MOUSE: C3tm1Crr/J	The Jackson Laboratory	JAX: 003641
MOUSE: CB17/lcr-Prkdcscid	Charles River Germany	Code number: 391
MOUSE: B6.129S7-Rag1tm1Mom/J	University of Erlangen	N/A
MOUSE: ColVlcrr26-tdTomato	University of Erlangen	N/A
MOUSE: hTNFt	Westfaelische Wilhelms-Universität Muenster, Germany	N/A
MOUSE: C3aR-/-	University of Lübeck	N/A
Cx3cr1cre;iDTR	University of Erlangen	N/A
RAT: Wistar WU	Charles River	Code number: 619

Oligonucleotides

Table S5	N/A
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Software and Algorithms

GraphPad Prism 8.3 software	GraphPad	https://www.graphpad.com/scientific-software/prism/
FlowJo V10	BD	https://www.flowjo.com/solutions/flowjo
Osteomeasure	N/A	https://www.osteometrics.com/
R v3.6.1	Butler et al., 2018	https://www.r-project.org/
Adobe Photoshop CS6 software	N/A	https://www.adobe.com/de/
Imaris software, Version 9.3, Bitplane	Grüneboom et al., 2019	https://imaris.oxinst.com/
CIBERSORTx	Newman et al., 2019	https://cibersort.stanford.edu/
Bowtie2 (2.3.4.1)	Langmead and Salzberg, 2012	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
Samtools (1.8)	Li et al., 2009	http://samtools.sourceforge.net/
DeepTools (3.1.3),	Ramirez et al., 2016	https://deeptools.readthedocs.io/en/develop/
Trim Galore (0.6.6).	N/A	https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/
Integrative Genomics Viewer (IGV) (2.8.13)	Robinson et al., 2011	https://software.broadinstitute.org/software/igv/

RESOURCE AVAILABILITY**Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Markus Hoffmann (markus.hoffmann@uk-erlangen.de).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

The accession numbers for the bulk RNA Seq data reported in this paper are Gene Expression Omnibus (GEO) repository: GSE163749 [NCBI tracking system #21564437] and BioProject: PRJNA714633. The accession number for the sc RNA Seq data is BioProject: PRJNA714636. The accession number for the ATAC Seq data is BioProject: PRJNA713761.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human subjects

Synovial tissue samples were collected from knee joints of treatment-naïve patients in the Birmingham BEACON cohort with a recent onset of clinically apparent arthritis and a symptom duration of less than 12 weeks, who at follow up fulfilled RA criteria (VERA), from subjects with established RA or from subjects undergoing exploratory arthroscopy for unexplained joint pain with no macro- or microscopic evidence of inflammation (NHD) (Filer et al., 2017). All of these specimens were sampled from knee joints before initiation of disease-modifying treatment.

Further synovial tissue specimens were obtained from patients fulfilling the criteria for the classification of RA (Arnett et al., 1988) during joint replacement surgery at the Department of Orthopedic Surgery, Schulthess Clinic Zurich, Switzerland. Additional synovial fibroblast samples from individuals with RA, psoriatic arthritis, and gout were grown as described (Stebulis et al., 2005) from joint aspirates obtained for clinical reasons at the University clinics of Erlangen. All participants gave written informed consent as approved by the local ethic committees (recent onset arthritis patients: West Midlands Black Country REC ref 07/H1203/57; Arthroscopy patients: West Midlands Black Country REC ref 07/H1204/191; Punctures No. 334_18B; No. 98_18B). SF were cultured as described (Ospelt et al., 2008) and used between passages 4 to 8 for all experiments. Patient characteristics of samples used are summarized in Table S4.

Leukocyte-rich and leukocyte poor RA in samples from the “Accelerating Medicines Partnership Rheumatoid Arthritis and Lupus” (AMP) consortium (Donlin et al., 2018; Zhang et al., 2019) was defined as described (Zhang et al., 2019) by the Mahalanobis distance from osteoarthritis (De Maesschalck et al., 2000). Whereas leukocyte-rich RA tissues had higher Krenn synovitis scores and substantial infiltration of synovial T cells and B cells, leukocyte-poor RA tissues had cellular compositions more similar to those of OA samples. Synovial monocyte abundances were similar between leukocyte-rich, leukocyte-poor RA and OA samples (Zhang et al., 2019).

Animals

Mice on the BALB/c and C57BL/6 backgrounds and Wistar rats were bred in house and kept in temperature- and humidity-controlled facilities at the University of Erlangen, Germany, the Medical University of Vienna, Austria, or the Faculty of Medicine, University of Belgrade, Serbia, respectively, group housed with free access to food and water and 12 h light/dark cycles. Complement C3-deficient $C3^{tm1Crr}$ mice were bought from Jackson, SCID mice (CB17/Icr-Prkdc^{scid}) and NOD/ShiLtJ were from Charles River Germany. $C3ar^{-/-}$ mice were from Jörg Köhl, University of Lübeck, Germany. $Rag1^{-/-}$ mice and $Col6a1^{cre}$:R26-tdTomato reporter mice, both on C57BL/6 background, were gifts from Mario Zaiss and Clemens Neufert, respectively, both from the University of Erlangen. The inducible hTNF α mice homozygous for the Tg_rTA2S transgene and the human TNF α transgenes have been described previously (Retser et al., 2013). Mice expressing a Cre recombinase under the *Cx3cr1* promoter together with a Cre-inducible diphtheria toxin (DT) receptor (*Cx3cr1cre*;iDTR mice) were generated by breeding C57BL/6-Gt(Rosa)26Sortm1(HBEGF)Awai/J (iDTR) purchased from Jackson with STOCK Tg(*Cx3cr1cre*)MW126Gsat/Mmucd mice (*Cx3cr1cre*) (identification number 036395-UCD) from the Mutant Mouse Regional Resource Center (MMRC), a National Institutes of Health (NIH)-funded strain repository. Resident tissue macrophages become specifically depleted in DT-treated *Cx3cr1cre*:iDTR mice several days after DT injection, since blood monocytes repopulate within 48 h after DT application. Wistar rats were derived from Charles River. The animal studies were approved by the local ethical committees and conducted according to the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA). Healthy adult animals 8–12 weeks old, both male and female were used in studies. All experiments were performed using age- and sex-matched littermate controls. If possible, mice were allocated randomly into groups by a computer-based random number generator (<http://www.randomizer.org>), so that each cage contained animals of every group to compensate for possible cage effects. Power analysis was performed before begin of the experiments on the basis of effect size estimates from previous experience.

Rodent inflammatory arthritis models

Monosodium urate crystal (MSU) induced monoarthritis was induced by injection of 1.4 mg MSU crystals suspended in 70 μ l sterile PBS into the foot pads of the hind paws, between the metatarsals. MSU crystals were generated in house in a lipopolysaccharide (LPS)-free manner as described (Schauer et al., 2014). Solution of 10 mM uric acid and 154 mM NaCl was set to pH 7.2 to initiate crystallization and mixed for 3 days. Formed crystals were washed with ethanol and sterilized at 180°C for 2 h. Zymosan-induced monoarthritis was induced in mice similarly by injection of 300 μ g zymosan (Sigma-Aldrich Z4250) suspended in 70 μ l PBS. Rats received 1.7 mg zymosan suspended in 400 μ l PBS. On day 0, MSU crystals or zymosan were injected only into the left paw while the right paw remained without injection. After resolution of the first arthritis episode (defined as reduction of paw thickness to < 110% of values before injection, normally occurring between day 9 and 14), MSU crystals or zymosan were then injected into both paws. In some experiments complement factor C5 cleavage was blocked using the BB5.1 antibody (Zelek et al., 2020), kindly provided by J. Winter, Cardiff university. To this end, 1 mg BB5.1 per mouse was delivered i.p. in 90 μ l PBS twice a week, starting after

the resolution of the first arthritis episode, 1 day before reinjection into both paws. For analysis of the synovial lining by histology and light sheet microscopy 86 μ g zymosan suspended in 20 μ l PBS were injected intra-articularly into the knee joint. Paw thickness during MSU crystal and zymosan-induced arthritis was monitored with an electronic caliper (Kroeplin) and areas under the arthritis curves (AUC) were calculated. Priming indices were defined as the ratio between the AUC of the second arthritis episode as compared to the AUC of the simultaneously occurring arthritis episode in the contralateral paw. K/BxN serum transfer arthritis (STA) (Christensen et al., 2016) was induced in BALB/c mice by intraperitoneal injection of 100 μ l serum from arthritic K/BxN mice (provided by mice from the animal facility of the University of Erlangen). After resolution of the first inflammatory bout, mice received another injection of serum. Arthritis was scored by measuring paw edema by caliper and additionally by a visual scoring system by assessing swelling and redness in each paw, including the tarsus or carpus joints, as described (Kienhöfer et al., 2014). The following scoring system was used: 0, no erythema or swelling; 1, erythema and swelling in up to two joints; 2, erythema and swelling in more than two joints or slight swelling of the ankle; 3, marked swelling of the ankle; 4, substantial swelling of the whole paw including toes and fingers. Antigen-induced arthritis (AIA) was elicited in C57BL/6 WT mice by two subcutaneous immunizations with 200 μ g sterile-filtered methylated bovine serum albumin (mBSA) emulsified in 100 μ l Complete Freund's adjuvant (CFA) containing 1 mg/mL *M. tuberculosis*, followed by intra-articular injection of 100 μ g mBSA suspended in 25 μ l into the knee joint. To induce hTNF α expression in iTNFTg mice (Retzer et al., 2013), 1mg/mL doxycycline (Sigma) and 5% sucrose was added to the drinking water for the time periods indicated in the figures. Doxycycline-containing water was protected from light and changed twice a week during the course of the experiments. For scoring of arthritis, the number of swollen digits was determined. The grip strength was assessed by placing the mice on the cage grid and using a scoring system ranging from 0 to -4: (0 = normal grip strength, -1 = mildly reduced, -2 = moderately reduced, -3 = severely reduced, -4 = no grip strength). We performed *in vivo* diphtheria toxin receptor (DTR)-mediated cell depletion in *Cx3cr1cre*; iDTR mice by I.P. injection of 500 ng DTX in PBS at day -5 and -4 before the MSU-induced monoarthritis.

Multimodal imaging and analysis of features of murine arthritis models

For histomorphological analysis, paws or knees from naive mice or after one or two injections of MSU crystals or zymosan were fixed overnight in 4% formalin, decalcified with EDTA, and embedded in paraffin. Paraffin sections were stained with hematoxylin & eosin or tartrate-resistant acid phosphatase for assessment of inflammation and osteoclasts, respectively. Analyses were performed on a Nikon microscope equipped with a digital quantification system (OsteoMeasure). Additionally, inflammation and bone remodeling were assessed *in vivo* as described (Czegley et al., 2018) at the Preclinical Imaging Platform Erlangen (PIPE) by a combination of ultra-high field MRI (ClinScan, Bruker, Ettlingen, Germany) and CT using a preclinical scanner (Inveon, Siemens, Erlangen, Germany).

Additional microcomputed tomography (μ CT) of the metatarsal and tarsal areas of mice during iterated arthritis was performed using the cone-beam Desktop Micro Computer Tomograph " μ CT 40" by SCANCO Medical AG, Bruettisellen, Switzerland. The settings were optimized for calcified tissue visualization at 55 kVp, 145 μ A, and 200 ms integration time. For the segmentation of 3D-volumes, an isotropic voxel size of 8.6 μ m was chosen. Using SCANCO's operating system "Open VMS" osteophyte volumes were manually defined as volume of interest (VOI) along the bones. Then greyscale thresholds were set to include all voxels related to bone tissue for the measurement of bone volume.

Isolation and culturing of synovial fibroblasts from rodent paws

SF were isolated from the paws of mice or rats during different stages of K/BxN serum transfer arthritis, MSU crystal-induced arthritis, zymosan-induced arthritis, and from non-injected naive paws. SF cell suspensions of murine paws were prepared by gently rocking dissected and skin-flayed paws for 1 h at 37°C in DMEM containing 2 mg/mL collagenase type IV (Worthington Biochemicals, LS004188). Cells derived from 2 to 3 paws were pooled and cells were then grown in DMEM (GIBCO) supplemented with 10% heat-inactivated calf serum, (glucose 4.5 g/L, L-glutamine 2 mM), penicillin and streptomycin (1% each) antibiotics, and 1% fungizone (Amphotericin B, Sigma Aldrich). Medium was changed twice a week. After 3-4 passages fibroblasts are the dominant cell type, resulting in a homogeneous cell population (Rosengren et al., 2007). Cells between passages 4 and 8 were used for the experiments and contained less than 2% CD45⁺ or CD31⁺ cells (Figure S2G), as determined by flow cytometry with a PerCP Cy5.5 conjugated anti-CD45 antibody (Biolegend 103132) and an AF647-conjugated anti-CD31 antibody (Biolegend, 102516).

RNA Sequencing and qPCR (transcriptomic analysis)

Isolation of RNA from mouse SF was performed using RNeasy kit (QIAGEN 74104). One microgram of total RNA was reverse transcribed and real-time quantitative PCR was performed on a Bio-Rad CFX96 Touch Real-Time PCR Detection System using qPCR Master Mix Plus for SYBR Green (Eurogentec RT-SN2X-03). Normalized gene expression values were calculated as the ratio of expression of the target gene to the expression of the reference gene (*Actb* or *RPLP0*). Primer sequences are given in Supplemental table S5.

For bulk RNA Sequencing of cultured SF sequencing libraries were prepared using either the TruSeq stranded total RNA kit with Ribo-Zero Globin (Illumina, 20020612) from 0.5 μ g RNA or the TruSeq stranded mRNA kit (Illumina, 20020594) from 1 μ g RNA. Libraries were subjected to single-end sequencing (101 bp) on a HiSeq-2500 platform (Illumina). The obtained reads were demultiplexed and converted to FASTQ format using bcl2fastq v2.17.1.14. Quality filtering was performed using cutadapt v. 1.18; total RNA libraries were alternatively filtered against a library of rRNA, tRNA, mt-rRNA and mt-tRNA sequences. Filtered reads were mapped to the mouse reference genome (Ensembl GRCm38) using the splice-aware aligner STAR (total RNA: STAR v 2.4.0i; mRNA: STAR v 2.6.1c). Read counts per gene were obtained by summing up reads for non-overlapping exons using Ensembl gene models (total RNA: HTSeq count, Ensembl annotation v84; mRNA: Subread v1.6.1, Ensembl annotation v96). The subsequent analyses were

performed using the R package DESeq2 (Love et al., 2014) (total RNA: R v3.2, DESeq2 v1.10.1; mRNA: R v3.6.1, DESeq2 v1.24.0).

For single cell RNA-Seq CD45⁺ hematopoietic cells and dead cells were removed from total cell suspensions of enzymatically digested hind paws of mice at steady state and after a single or repeated injection of MSU crystals on a FACS Aria machine, using an anti-CD45 antibody (Biolegend 103132 and 103112) and Sytox Green (Thermofisher S7020). Remaining viable (Sytox Green⁻) CD45⁻ non-hematopoietic cells were then subjected to 10x Chromium Single Cell 3c Solution v3 library preparation according to the manufacturer's instructions. Library sequencing was performed on an Illumina HiSeq 2500 sequencer to a depth of 200 million reads each. Reads were converted to FASTQ format using mkfastq from Cell Ranger 3.0.1 (10x Genomics). Reads were then aligned to the mouse reference genome (mm10, Ensembl annotation release 93). Alignment was performed using the count command from Cell Ranger 3.0.1 (10x Genomics). Primary analysis, quality control filtering (gene count per cell, unique molecular identifier count per cell, percentage of mitochondrial transcripts), clustering, identification of cluster markers and visualization of gene expression were performed using the Seurat (v3.1.1) package under R v3.6.1 (Butler et al., 2018). To be able to compare gene expression between conditions, datasets were integrated using the shared nearest neighbor approach as implemented in Seurat v3.1.1. Prior to integration, cell counts were equalized by random downsampling of the larger sample.

Cell type abundance was enumerated from bulk RNA Sequencing by CIBERSORTx (Newman et al., 2019), using single-cell reference profiles. First, we used single cell RNA-Seq data to generate a gene signature matrix for each cluster. Then, we utilized S-mode batch correction and 1000 permutations with quantile normalization disabled for analyzing cell type relative fractions.

Assay for Transposase-Accessible Chromatin using sequencing (ATAC Seq)

ATAC-seq library preparation was performed on cultured, FACS sorted CD45⁺CD31⁻ naive, -/MSU and MSU/MSU fibroblasts (35,000 cells per sample) using the ATAC-seq kit from Active motif (#13150). In short, nuclei were isolated by adding 100 μ L ice cold ATAC-lysis buffer to the cell pellet and resuspending cells with a pipette. After centrifugation (500 g, 10 min at 4°C), cells were washed and incubated with the tagmentation master mix in a shaking heat block at 37°C/800 rpm for 30 min. Obtained DNA was taken up in DNA purification buffer, purified using the contained DNA purification columns, amplified for 10 cycles using indexed primers, and size-selected using SPRI bead solution. A quality control (QC) was performed in order to verify the size distribution of the PCR enriched library fragments. To this end, aliquots of the DNA libraries were analyzed on an Agilent Technologies 2100-Bioanalyzer, using a High Sensitivity DNA chip. This Bioanalyzer based QC was for qualitative purposes only. A precisely quantification of the libraries was done using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific).

The bar-coded amplicons from ATAC-Library preparation were sequenced in a HiSeq 2500 platform (Illumina) under a 2 $\times$ 100 pair-ended format. Reads were quality filtered according to the standard Illumina pipeline, de-multiplexed and fastq files were generated. Illumina adaptor sequences were trimmed using Trim Galore (0.6.6). Reads were then aligned to the mm10 version of the mouse genome using Bowtie2 (2.3.4.1) and non-uniquely mapping fragments excluded using SAMtools (1.8). PCR-duplicates and reads mapping to ChrM were removed with SAMtools. Biological replicates from the samples were merged using SAMtools. Normalized BigWigs from merged files were generated for visualization using DeepTools (3.1.3), which were merged for visualization via Integrative Genomics Viewer (IGV) (2.8.13). The peaks from individually biological replicates were called with Genrich (0.6). Peaks found in at least two biological replicates were kept for further analysis as merged peak set. Reads were counted over the merged peak set using Rsubread (2.2.6). Furthermore, significance and fold change were determined using DESeq2 (1.28.1). Quantification involved 3kbp regions around the Transcription start sites.

C3 expression analysis in human rheumatoid arthritis-derived synovial fibroblasts (RA-SF) after stimulation with TNF α

For double stimulation of RA-SF with TNF α , we followed a previously published protocol (Crowley et al., 2017). 24 h after seeding, RASF were stimulated with the first dose of TNF α (10 ng/mL) for 24 h. Afterward, cells were washed twice with PBS, left untreated for 24 h, followed by a second stimulation with TNF α (10 ng/mL) for another 24 h. Cells were lysed for RNA isolation (R1050; Quick-RNA Micro Prep Kit, Zymo Research) or western blotting after a total treatment period of 72 h. For qPCR, total RNA was reverse transcribed, followed by real-time PCR (7900HT real-time PCR system, Life Technologies) using SYBR green (Roche). Dissociation curves and samples containing the untranscribed RNA were measured in parallel. Constitutively expressed human ribosomal protein large P0 (RPLP0) was measured for internal standard sample normalization and relative mRNA expression was calculated by the comparative threshold cycle method ($\Delta\Delta C_t$).

For western blotting, RA-SF were lysed in Laemmli buffer (BioRad). Lysates and sera from the same patient were separated on 10% SDS polyacrylamide gels and electro blotted onto nitrocellulose membranes (Whatman). Membranes were blocked for 1 h in 5% (w/v) non-fat milk in TBS-T (20 mM Tris base, 137 mM sodium chloride, 0.1% Tween 20, pH 7.6) and probed with antibodies against the C3 α chain (EPR2988; Abcam), or α -tubulin (ab11304; Abcam) as endogenous control. As secondary antibodies, horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibodies (2307391 and 10015289 Jackson ImmunoResearch) were used. Signals were detected using the ECL western blotting detection reagents (GE Healthcare) and the Alpha Imager Software system (Alpha Innotech). Expression analysis of specific proteins was performed by pixel quantification of the electronic image.

Functional testing of synovial fibroblasts

Analysis of the invasive capacity of fibroblasts. A Matrix-associated transepithelial resistance invasion (MATRIN-) assay was utilized as described (Wunrau et al., 2009) for testing the SFs' invasive potential into extracellular matrix. A polycarbonate membrane of

a filter mounted in a 6-well-dish was coated with a collagen matrix on one side, while MDCK-C7 epithelial cells (kindly provided by Prof. Thomas Pap, University of Münster, Germany) were seeded onto the other side. Following full establishment of the MDCK-C7 monolayer, SF were seeded onto the collagen matrices. Disturbing MDCK-C7 cell monolayer integrity by invading SF leads to a decrease of the transepithelial electrical resistance (TEER) that is measured with a set of electrodes connected to an ohm-meter. The electrical resistance was measured twice a day over the course of an experiment. All measurements were performed in triplicates and were terminated after total breakdown of the electrical resistance.

Wound healing and migration assay. For analysis of SF migration we used a wound-healing assay with culture inserts (Ibidi, 80209) in 6-well culture dishes. 20,000 SF were adjusted in 70 μ L culture medium per chamber of an insert. After 24 h, the inserts were removed gently and the well was filled with 2 mL culture medium. The change in cell-covered area over time was monitored on a microscope equipped with a temperature and CO₂ control chamber (Keyence) and quantified using Photoshop CS6 software (Adobe). For the assessment of cell proliferation rate we used daily confluence measuring of growing adherent cells in normal culture medium at 37°C and 5% CO₂ for 4 days.

3D organoids. The formation of an organized synovial lining was analyzed in 3D synovial organ cultures (micromasses) as previously described (Kiener et al., 2010). SF were resuspended in Matrigel Matrix (BD Biosciences) at a density of 1×10^6 cells/mL. The obtained Matrigel Matrix cell suspension was plated in 1 mL/well Poly-2-hydroxyethylmethacrylate (poly-HEMA, P3932 Sigma-Aldrich) coated cell culture plates. After 35 min incubation at 37°C growth medium was added to the plate. SF were maintained in culture in DMEM/F12 (GIBCO) supplemented with 10% FCS, 0.1 mM ascorbic acid, ITS supplement (17838-Z, BioWhittaker) and 1% penicillin-streptomycin at 5% CO₂, 37°C for 3 weeks until the organ culture had built a sphere. In some cultures, 50 μ g/mL zymosan (Sigma) was additionally added. For analysis of 3D organ cultures, paraffin-embedded sections were stained with hematoxylin and eosin.

Measurement of interleukin 6 in rat synovial fibroblasts. Concentrations of rat IL-6 in supernatants from 3D micromass cultures were determined by ELISA (550319; OptEIA, BD Biosciences PharMingen, San Diego, CA, US) according to the manufacturer's instructions.

Analysis of senescence. For detection of senescence associated β -galactosidase (SA- β -Gal), aged SF from passage 8 were stimulated with 10 ng/mL TNF α on flat bottom 12-well plates for 24 h. After washing with PBS, cells were left untreated for 24 h, followed by a second stimulation with TNF α (10 ng/mL) for another 24 h. Control cells were incubated without any stimulation with TNF α . SA- β -Gal activity was then determined with a senescence detection kit (Abcam, 65351).

Metabolic mapping of synovial fibroblasts

Glucose consumption and lactate secretion. Supernatants from cultured SF were harvested and made cell-free by centrifugation after 48 h of culture. Concentrations of glucose and lactate were determined using a SuperGL_{Compact} (Hitado, Möhnesee, Germany).

Flow cytometric analysis. Total cells were isolated from paws of mice during different stages of MSU crystal-induced arthritis as well as from non-injected naive paws by collagenase-digestion as described above and either used for *ex vivo* measurements (isolation 2 days after last injection of MSU crystals) or cultured *in vitro* (isolation 9 days after last injection). The *in vitro* cultured samples were re-seeded at a defined density of 10^5 cells per well in 24-well plates 48 h prior to the staining and harvested using Accutase (ThermoFisher Scientific, 00-4555-56). Glucose uptake was analyzed using 6-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-6-Deoxyglucose (6NBDG, ThermoFisher Scientific, N23106) by incubating the samples in glucose-free medium for 15 min with 0,3 mM 6NBDG at 37°C, 5% CO₂.

For the detection of dead cells samples were stained with Zombie Aqua Fixable Viability Kit (Biolegend, 423102) according to the manufacturer's instructions. Surface proteins were stained after Fc γ R blocking for 10 min at 4°C (Miltenyi Biotec, 130-092-575) using following fluorochrome-conjugated antibodies CD31 BV421 (Biolegend, 102516), CD45 PerCP Cy5.5 (Biolegend, 103132), CD45 APC-Cy7 (Biolegend, 103115), CD90.2 BUV395 (BD, 565257), Glut1 AlexaFluorTM 488 (Abcam, 195359) and Podoplanin PE, at pre-titrated dilutions for 30 min at 4°C. To detect FAP α , samples were first incubated with unconjugated, polyclonal sheep anti-FAP α (R&D Systems, AF3715) for 30 min at 4°C, second with biotinylated anti-sheep IgG (Sigma Aldrich, B3148) for 30 min at 4°C, and third (together with the other fluorochrome-conjugated surface antibodies) with BUV737-conjugated Streptavidin (BD, 612775). Intracellular (phospho-) proteins were stained after fixation (BD PhosflowTM Fix Buffer I, 557870) and permeabilization (BD Phosflow Perm Buffer III, 558050) according to the manufacturer's instructions using following fluorochrome-conjugated antibodies; complement C3 FITC (Cedarlane, CL7503F), 4EBP1 (pT37/47) AlexaFluorTM 594 (Bioss, bs-3019R-A594), mTOR (pSer2448) PE (ThermoFisher, 12-9718-42, mTOR (pSer2448) PerCP-eFluor780 (ThermoFisher, 46-9718-42), S6 (pSer235, pSer236) APC (ThermoFisher, 17-9007-41) and HIF1 α APC (ThermoFisher, 17-7528-82), at pre-titrated dilutions for 30 min on ice. Samples were recorded on a FACS Canto II or a LSR Fortessa SORP (both BD Bioscience) and analyzed using FlowJo V10 (FlowJo LLC).

Extracellular flux analysis. Bioenergetics were analyzed using a Seahorse XFe96 (Agilent, Santa Clara, CA) as previously described (Böttcher et al., 2018). Briefly, 3×10^4 SF were seeded per well in heptaplicates into Seahorse XF culture plates and incubated overnight at 37°C, 5%CO₂. Next day, medium was replaced by glucose-free (glycolysis stress test, GST) or glucose-containing DMEM (mitochondrial stress test, MST) with tightly adjusted pH and samples were incubated an additional 1 h in a CO₂-free atmosphere. Samples' extracellular acidification rate (ECAR, as surrogate for aerobic glycolysis) and oxygen consumption rate (OCR, as surrogate for mitochondrial respiration) were recorded after sequential injection of glucose, oligomycin, and 2-DG for the GST or oligomycin, FCCP, and antimycin A/rotenone for the MST. Last, total protein content per well was determined using

the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) to normalize the recorded raw data. Resulting data was converted to csv-files using the Wave Desktop Software and metabolic parameters were calculated with Microsoft Excel. GraphPad Prism 8 was used for visualization and statistical analysis.

Treatment with 2-Deoxy-D-glucose (2-DG) *in vitro* and *in vivo*

For inhibition of glycolysis *in vitro*, isolated SF were cultured with 2mM 2-DG (D8375; Sigma Aldrich) for 24 h at 37°C, 5% CO₂. For inhibition of glycolysis *in vivo*, C57BL/6 WT mice received 2 mM 2-DG in their drinking water. 2-DG treatment was begun one day before the induction of arthritis by injection of MSU crystals and continued until the end of the experiment.

Treatment with rapamycin and KC7F2 *in vitro*

For inhibition of mTOR *in vitro*, naive SF were cultured with 50 nM Rapamycin (Selleck, S1039) for 72 h at 37°C, 5% CO₂. For the translation inhibition of HIF1 α cells were cultured for 72 h with 30 μ M KC7F2 (Selleck, S7946). Cells were stimulated twice with 10 ng/mL TNF, with intervening 24 h rest, as described (Crowley et al., 2017).

Cell sorting of synovial fibroblasts by flow cytometry

Cultured SF were detached at passage 2 from flasks by Corning™ Cell stripper (15313661; Fisher scientific) and stained in FACS buffer (PBS, 10% FCS, 0.5 mM EDTA) with a PerCP Cy5.5 or an APC-conjugated anti-CD45 antibody (Biolegend 103132 and 103112), a BV421-conjugated anti-CD31 antibody (Biolegend, 102423) and Sytox Green (ThermoFisher S7020). After washing Sytox Green⁺ (dead) cells, CD45⁺ and CD31⁺ cells were sorted out on a FACS Aria machine. CD45⁺CD31⁺ remaining SF were used after re-culturing.

Scanning electron microscopy

10,000 mouse SF were cultured for 24 h in plastic flat-bottomed 96-well plates precoated with 50 μ g/mL fibronectin over night at 4°C. After fixation with 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.2) containing 0.01 M CaCl₂, 0.01 M MgCl₂, 0.09 M sucrose, and 2% glutaraldehyde as a fixative. Samples were dehydrated in an ascending ethanol series (2 $\times$ 10 min 30% EtOH, 1 $\times$ 10 min 50% EtOH, 1 $\times$ 10 min 70% EtOH, 3 $\times$ 10 min 100% EtOH), followed by 2 $\times$ 5 min incubation with 99.9% hexamethyldisilazane ReagentPlusR (MilliporeSigma, Burlington, MA, USA) and air drying overnight as described (Nation, 1983). Subsequently, the base of the well was carefully punched out including the cell layer, attached on stubs for electron microscopy, and sputter-coated with Au (Emitech K575X; Quorum Technologies, Laughton, United Kingdom), 2 $\times$ 15 mA, 60 s. The samples were then examined using a Tescan Lyra3 field-emitting scanning electron microscope at 5 kV accelerating voltage.

Bone marrow transplantation

6 weeks old C57BL/6 Complement C3-deficient CD 45.2⁺ recipient mice were irradiated with a total of 10 Gray at the department of Radiation oncology, University of Erlangen, and reconstituted 2 h afterward intravenously with 5 $\times$ 10⁶ bone marrow cells isolated from CD 45.1⁺ C57BL/6 WT donor mice. 6 weeks after transplantation, bone marrow chimerism was verified by detecting CD45.1⁺ and CD 45.2⁺ cells in the peripheral blood of recipient mice by flow cytometry, using a PE-Cy7-labeled anti-CD 45.1 (25-0453-82, eBioscience) and an AlexaFluor700-labeled anti-CD 45.2 antibody (56-0454-82, eBioscience), respectively.

Osteoclast differentiation

Bone marrow cells were isolated from tibia and femur of 8-week-old Balb/C mice as described elsewhere (Steffen et al., 2019). After lysis of erythrocytes with erylisis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7), cells were cultured for 24 h in α MEM supplemented with 10% FCS and 1% Penicillin-Streptomycin. Non-adherent cells (200 $\times$ 10³ well) were added to 96 well plates and co-cultured with naive, -/MSU or MSU/MSU fibroblasts (1 $\times$ 10⁴/well) in α MEM supplemented with 10% FCS, 1% Penicillin-Streptomycin, 30 ng/mL recombinant murine M-CSF (PeproTech, 315-02) and 10 ng/mL recombinant murine RANKL (PeproTech 315-11). In some experiments, the TNF inhibitor etanercept (Enbrel, Pfizer; 25 μ g/mL), the IL-1 antagonist anakinra (Kineret, SOBI; 20 μ g/mL) or recombinant osteoprotegerin (450-14 Peprotech; 20 ng/mL), the decoy receptor of RANKL, were added. After 5 days, cells were fixed and stained for TRAP activity using a commercially available kit (Sigma, 387A-1KT). TRAP-positive multinucleated cells designated as osteoclasts were counted by light microscopy and number of nuclei was determined. Total area covered by osteoclasts was determined by Adobe Photoshop CS6 software.

Determination of RANKL and OPG concentrations in synovial fibroblast-derived supernatants

Mouse SF were incubated in 96 well-plates in 200 μ l culture medium with or without 2 mM 2-DG for 24 h. Concentrations of RANKL and OPG in supernatants were then determined by Mouse RANKL DuoSet (R&D Systems #DY462) and Mouse Osteoprotegerin Duo set (R&D Systems #DY459) ELISA, respectively, according to the manufacturer's instructions.

Induction of inflammatory tissue priming by injection of fibroblasts into the paw

Left paws of mice were injected with MSU crystals. After the resolution of the inflammation, 500 $\times$ 10³ flow cytometry sorted cells were transferred to contralateral paws by subcutaneous injection between the metatarsals. After two days, arthritis was initiated in both paws by the injection of MSU crystals.

Inflammasome inactivation

Balb/C mice received intraperitoneal injections of 10 mg/kg MCC950 (Sigma Aldrich PZ0280) or sterile PBS (vehicle) 2 days before the MSU crystal injection. Treatment was continued every second day until the end of the experiment.

Optical clearing of mouse knee samples

Col6a1^{cre}:R26-tdTomato mice were intravenously administered 2.5 μ g Alexa Fluor 647 anti-mouse Ly6G antibody (Biolegend, 127610) in PBS and sacrificed after 1 h. Perfusion was performed using 5 mM EDTA in PBS and fixation was carried out by perfusion of 4% PFA in PBS (pH = 7.4). Hind limbs were cut and surrounding skin and muscles were removed. Knee joints were fixed in 4% PFA (pH 7.4) for 4 h at 4°C. Tissue dehydration was done by immersing samples in a series of ethanol solutions: 50%, 70% and two consecutive incubations with 100% ethanol at 4°C for 12 h each. Mice knee samples were transferred to ethyl cinnamate and incubated at room temperature for 6 h.

Light sheet fluorescence microscopy (LSFM)

LSFM of optically cleared mouse knee joints was performed with a LaVision BioTec Ultramicroscope II including an Olympus MVX10 zoom body (Olympus), a LaVision BioTec Laser Module, and an Andor Neo sCMOS Camera with a pixel size of 6.5 μ m. For visualization of general tissue morphology, a 488 nm optically pumped semiconductor laser (OPSL) was used to generate autofluorescent signals. For tdTomato excitation, a 561-nm OPSL and for Ly6G-AF647 excitation, a 647-nm diode laser was used. Emitted wavelengths were detected with specific detection filters: 525/50 nm for autofluorescence, 620/60 nm for tdTomato, and 680/30 nm for Ly6G-AF647. The optical zoom factor of the measurements varied from 1.26 to 8 and the light-sheet thickness ranged from 5 to 10 μ m.

3D surface rendering and quantification of lining integrity and cells in the sublining

3D reconstruction of LSFM-scanned mouse knee joints was performed using Imaris software (Version 9.3, Bitplane). The FIJI plugin “Calculator Plus” was utilized to subtract the intensity adjusted autofluorescence (488 nm) from the tdTomato signal (561 nm), improving the signal to noise ratio. For analyzing the synovial lining integrity, the synovial lining was optically separated from the sublining tissue by semi-automated volumetric surface rendering. The ratio of tdTomato⁺ lining SF volume to lining tissue volume was calculated and normalized against the mean of the —/— control group.

The number of tdTomato⁺ cells in the synovial sublining tissue was quantified using the Imaris cell counter module with a size threshold of 5 μ m.

RNAscope and immunohistochemistry for C3 and CD90 in human synovium

RNAscope was carried out on formalin fixed paraffin embedded osteoarthritis human synovium sections according to the manufacturer's protocol. C3 probe (430701) was utilized with the single-color RNAscope 2.5 HD Assay Red kit (322360, both Bio-Techne). The slides were treated for 15 min with the Retrieval Buffer and incubated for 30 min with Protease Plus. Following the RNAscope color development, the slides were washed in distilled water for 5 min and the immunohistochemistry protocol was started. In brief, sections were blocked with Bloxall Endogenous Peroxidase and Alkaline Phosphatase Blocking Solution (SP-6000, Vector Laboratories) for 10 min and with 10% normal horse serum (H0146, Sigma-Aldrich) Tris buffer for 10 min. The sections were then incubated with sheep anti-human CD90 primary antibody (AF2067, R&D Systems) at a dilution of 1:100 at 4°C overnight, followed by incubation with donkey anti-sheep HRP (713-035-147, Jackson Immuno Research), and color development with ImmPACT DAB Peroxidase (HRP) Substrate (SK-4105, Vector Laboratories). The nuclei were stained with Vector Hematoxylin QS (H-3404) and slides were mounted in VectaMount Permanent Mounting Medium (H-5000, both Vector Laboratories). The slides were visualized using Zeiss Axio Scan and analyzed in Zen Blue (both Zeiss).

Confocal microscopy for detection of complement C3 and C3aR expression and inflammasome activation in synovial fibroblasts

For C3 expression analysis, SF were adhered to poly-Lysine slides and fixed with paraformaldehyde (2%), treated with Triton X-100 (Sigma, 0.3%, 10 min on ice) and incubated with anti-C3 (clone: 11H9, Abcam) and anti-Rab7 (clone: D95F2, Cell Signaling Technology) overnight at 4°C in a humid chamber. After incubation with anti-rat IgG F(ab')₂ Fragment Alexa 488 conjugate and anti-rabbit IgG F(ab')₂ Fragment 674 conjugate (11006 and 32722 Thermo Fisher Scientific) for 2 h at 4°C in a humid chamber, DAPI (1 μ g/mL) was added during the last 10 min. In some experiments SF were stained with phalloidin (Flash Phalloidin Red 594, BioLegend) according to the manufacturer's instructions.

For C3aR expression analysis, fixed cells on poly-Lysine slides were incubated with an anti-C3a receptor antibody (1:200, PA5-50364, ThermoFisher) overnight at 4°C in a humid chamber. After incubation with an anti-rabbit IgG F(ab')₂ Fragment 674 conjugate (2 h, 4°C in a humid chamber) staining of the cell membrane and nuclei was performed using Alexa Fluor 488-conjugated Wheat Germ Agglutinin (WGA, 2.5 μ g/mL) (Vector Laboratories, Burlingame, USA) and DAPI (Thermo Fisher Scientific, Waltham, USA), respectively.

Caspase 1-activity was detected using the FLICA® 660 Caspase-1 assay kit (9122 ImmunoChemistry Technologies, Bloomington, USA) according to the manufacturer's instructions. Cells were washed with PBS and incubated with the FLICA® 660-YVAD-fmk re-

agent (1:150, 30 min) at 37°C and 5% CO₂. After this, cells were adhered to poly-Lysine slides and fixed with paraformaldehyde (2%). Cells were stained with DAPI and phalloidin (Flash Phalloidin Red 594, BioLegend) according to the manufacturer's instructions.

Slides were analyzed by confocal microscopy (LSM700, Zeiss) at x630 magnification. Regions of Interest (ROIs) matching fibroblasts were segmented using Zeiss software (ZEN 2.6), and the mean fluorescence intensity (MFI) of C3 or C3aR was assessed for 100 ROIs of each condition. For colocalization analysis, image signals generated by WGA were used to estimate cell membrane borders, which were merged with C3aR images to identify the association between C3aR and the plasma membrane. The percentages of C3aR in the membrane were estimated in 20 SF/condition by calculating the percentage of C3aR voxels in WGA colocalized channels.

Nociception analysis

Tests for mechanical allodynia were performed using a dynamic plantar aesthesiometer (Model 37450, Ugo Basile, and Varese, Italy) according to Von Frey. The animals were habituated to the test situation (wire mesh base plate with 10 × 10 cm transparent animal enclosures, sides neighboring two animals were covered) for 2 h prior to testing. Performing the test, a blunt 0.5 mm Von Frey-type filament exerted an increasing force of 0.2 g/s until the animal twitched or lifted the paw, or until the cut-off value of 8 g was reached. Both paws were tested alternately, with at least 5 min pause between two tests of the same paw. Per animal and paw, 4 tests were performed, and exerted force (g) and paw withdrawal latencies were averaged per animal.

QUANTIFICATION AND STATISTICAL ANALYSIS

Two group comparisons were performed using unpaired or paired 2-tailed Student's t test or, in the case of non-normally distributed data, by 2-tailed Mann-Whitney U test. Gaussian distribution of samples was checked using D'Agostino-Pearson omnibus normality test. Within each set of experiments shown in one graph multiple comparisons of groups were performed by ANOVA and adjusted using Tukey's (if the mean of each group was compared to the mean of every other group) or Sidak's (if the means of selected pairs of groups were compared) multiple comparisons test, or Dunnett's test if several means were compared to a control mean. In case of non-normally distributed data we used Kruskal-Wallis test with Dunn's post hoc test for multiple comparisons. For comparing the mean of every group to a fixed value we used a One sample t test.

Adjusted p values < 0.05 were considered statistically significant. Computations and charts were performed using GraphPad Prism 8.3 software. Levels of significance are allocated as follows throughout the whole manuscript: ns, not significant; * p < 0.05; ** p < 0.01; *** p < 0.001, as stated otherwise in the figure legend. Unless indicated otherwise, figures show means ± SEM.

Supplemental information

The complement system drives local inflammatory tissue priming by metabolic reprogramming of synovial fibroblasts

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Supplemental figures

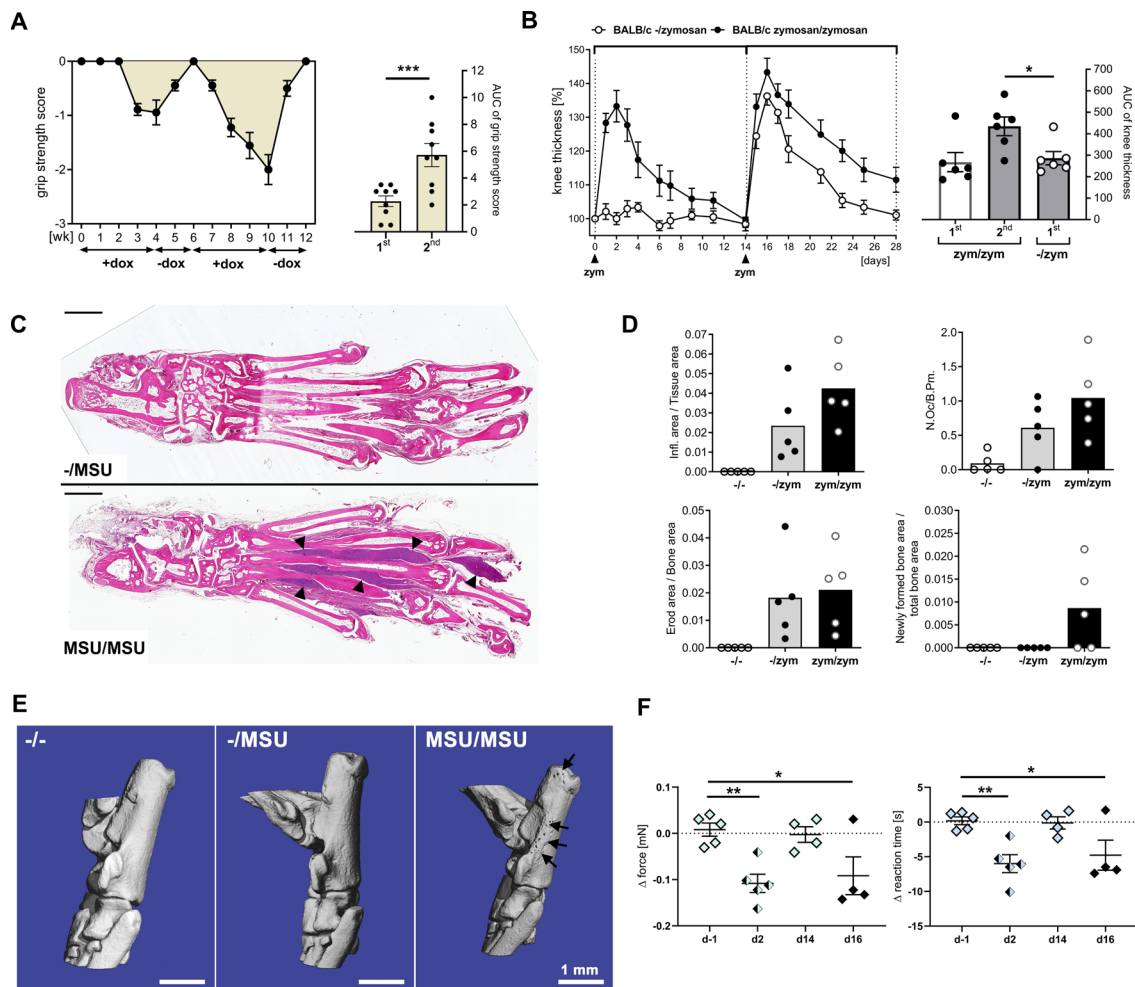


Figure S1, related to main Fig. 1. Inflammatory tissue priming is associated with exacerbated inflammation, bone remodeling, and hyperalgesia. (A) Time course of grip strength reduction and areas under the curve (AUC) of the 1st and the 2nd episode of arthritis induced by doxycycline (dox) in inducible human TNF transgenic mice. Means $\pm$ SEM. N = 9. **(B)** Time course and AUC of knee swelling in BALB/c mice injected intra-articularly once (-/zym) or twice (zym/zym) with zymosan. Means $\pm$ SEM. N = 6. **(C-E)** Inflammatory and bone changes after repeated injection of monosodium urate (MSU) crystals or zymosan. **(C)** H&E staining of sections from mouse paws 9 days after the first (-/MSU) or second (MSU/MSU) injection of MSU crystals between the metatarsal bones. Black arrowheads indicate inflammatory infiltrates. Scale bars, 1 mm. **(D)** Histological quantification of inflammation area, osteoclast numbers per bone perimeter (N.Oc/B.Pm), eroded area, and newly formed bone in sections from -/-, -/MSU and MSU/MSU mouse knee joints. Plotted are means and individual values. N = 5. **(E)** Representative 3D surface renderings from μ CT scans of the tarsal area of -/-, -/MSU and MSU/MSU mouse paws, showing bone destruction and aberrant new bone formation (black arrows) in MSU/MSU paws. **(F)** Increased nociception in mouse paws injected twice with MSU crystals. Differences (Δ) between paws injected twice or once with MSU crystals in withdrawal threshold (left) and reaction time (right) to von Frey filaments. Δ = (MSU/MSU paw) – (-/MSU paw). Day (d) -1, baseline values before MSU crystal-injection; d2, two days after first injection (injected paw is hyperalgesic as compared to non-injected paw); d14, before second injection (arthritis in injected paw resolved); d16, 2 days after injection into both paws (MSU/MSU injected paw is hyperalgesic as compared to -/MSU injected paw). **(A)** paired t-test; **(B)** ANOVA with Tukey's test; **(F)** ANOVA with Dunnett's test.

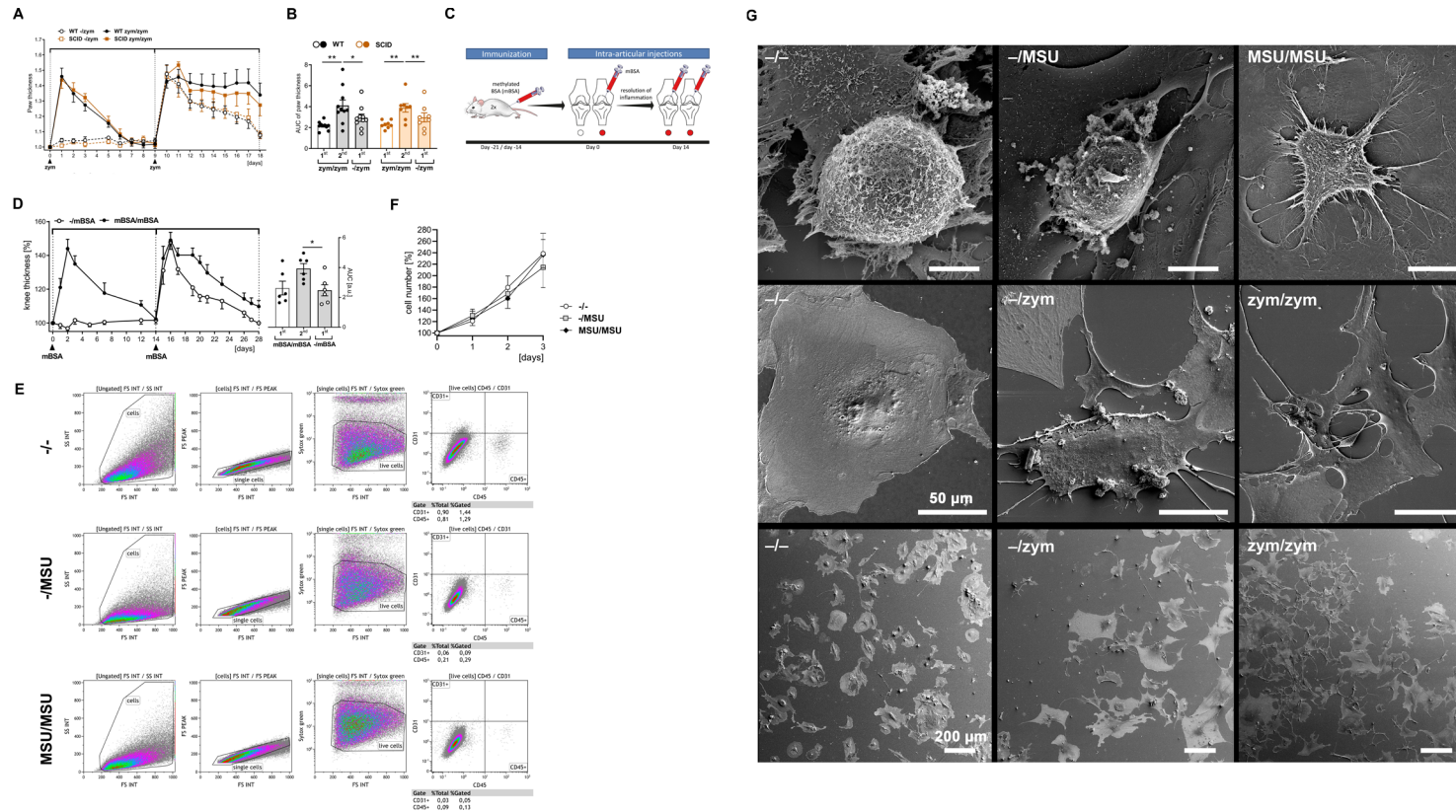


Figure S2, related to main Fig. 2. Inflammatory tissue priming is independent of adaptive immunity, yet can be induced by adaptive immune pathways. (A, B) Arthritis in SCID mice and BALB/c wild type (WT) mice after repeated injection of monosodium urate (MSU) crystals. Shown are **(A)** time course of paw thickness and **(B)** areas under the curves (AUC). N = 8-10. **(C, D)** Model of inflammatory tissue priming in antigen-induced arthritis (AIA). **(C)** AIA was elicited in C57BL/6 mice by two immunizations with methylated bovine serum albumin (mBSA) followed by one or two intra-articular injection of mBSA into the knee. **(D)** Time course (left) and AUC (right) of relative thicknesses of mouse knees injected once (-/mBSA) or twice (mBSA/mBSA). N = 6. **(E)** Purity of synovial fibroblast (SF) cultures. Percentages of CD45⁺ and CD31⁺ cells in representative cultures of -/-, -/MSU and MSU/MSU SF (passage 6), as determined by flow cytometry. **(F)** Assessment of proliferation in -/-, -/MSU and MSU/MSU SF by cell counting over the course of 3 days. All cells were in passage 5. N = 4. **(G)** Representative scanning electron microscopy images of SF isolated from mouse paws during iterated MSU crystal or zymosan-induced arthritis. SF show morphological changes associated with inflammatory tissue priming. Scale bars, 200 μ m. All subfigures show means $\pm$ SEM. ANOVA with Tukey's test **(B, D)**.

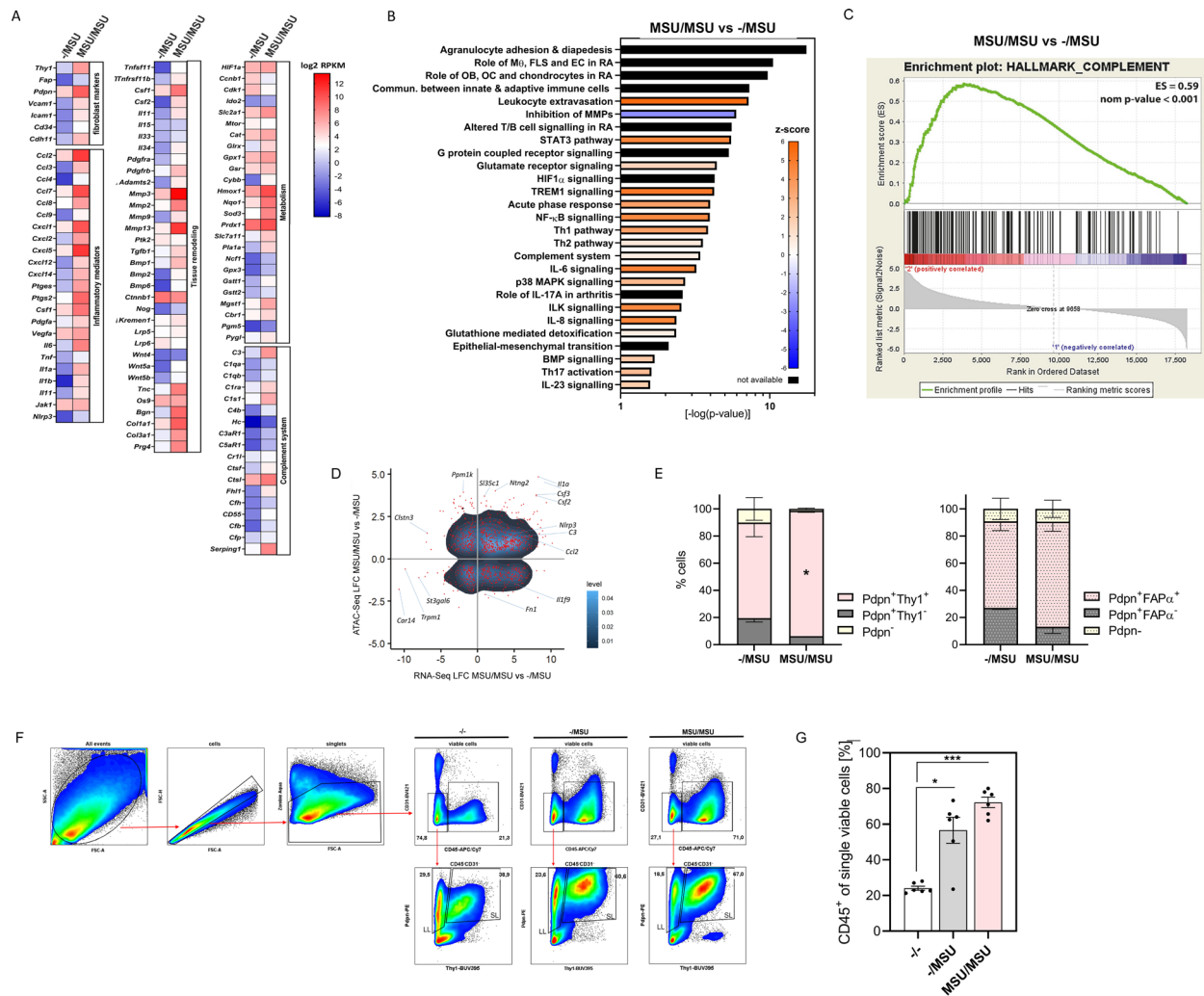


Figure S3, related to main Fig. 3. Inflammatory Pdpn⁺Thy1⁺ fibroblasts expressing complement components are enriched in primed tissue. (A) Mean log₂-transformed RPKM values of selected genes from paws injected once (-/MSU) or twice (MSU/MSU) with monosodium urate (MSU) crystals, generated from bulk RNA sequencing. **(B, C)** Ingenuity pathway analysis (IPA) and Gene set enrichment analysis (GSEA) in primed (MSU/MSU) and non-primed (-/MSU) SF. **(B)** IPA showing canonical pathways differentially regulated in a confirmation set of primed SF (n = 3) as compared to -/MSU SF (n = 3). **(C)** Analysis of RNA sequencing data from MSU/MSU and -/MSU SF by GSEA showing upregulation of complement genes in primed SF when compared to -/MSU SF. ES, enrichment score. **(D)** Density plot of genome-wide differential gene regulation by ATAC Seq and bulk RNA Seq. All genes with adjusted p-value < 0.01 in both RNA Seq and ATAC Seq are shown as red dots. **(E)** Flow cytometric analysis of flow cytometry-sorted (CD45⁻CD31⁻) cultured SF from -/MSU and MSU/MSU paws. Bars show percentages of Pdpn⁺Thy1⁻, Pdpn⁺Thy1⁺ and Pdpn⁻ cells. N = 3. ANOVA with Sidak's test. **(F, G)** Flow cytometric analysis of cell populations in naive mouse paws and in paws 2 days after the first (-/MSU) or second (MSU/MSU) injection of MSU crystals. Shown are gating strategy and representative results **(F)**, and means ± SEM of percentages of CD45⁺ cells **(G)**. One symbol represents pooled cells from two mice. N = 6 replicates of pooled cells.

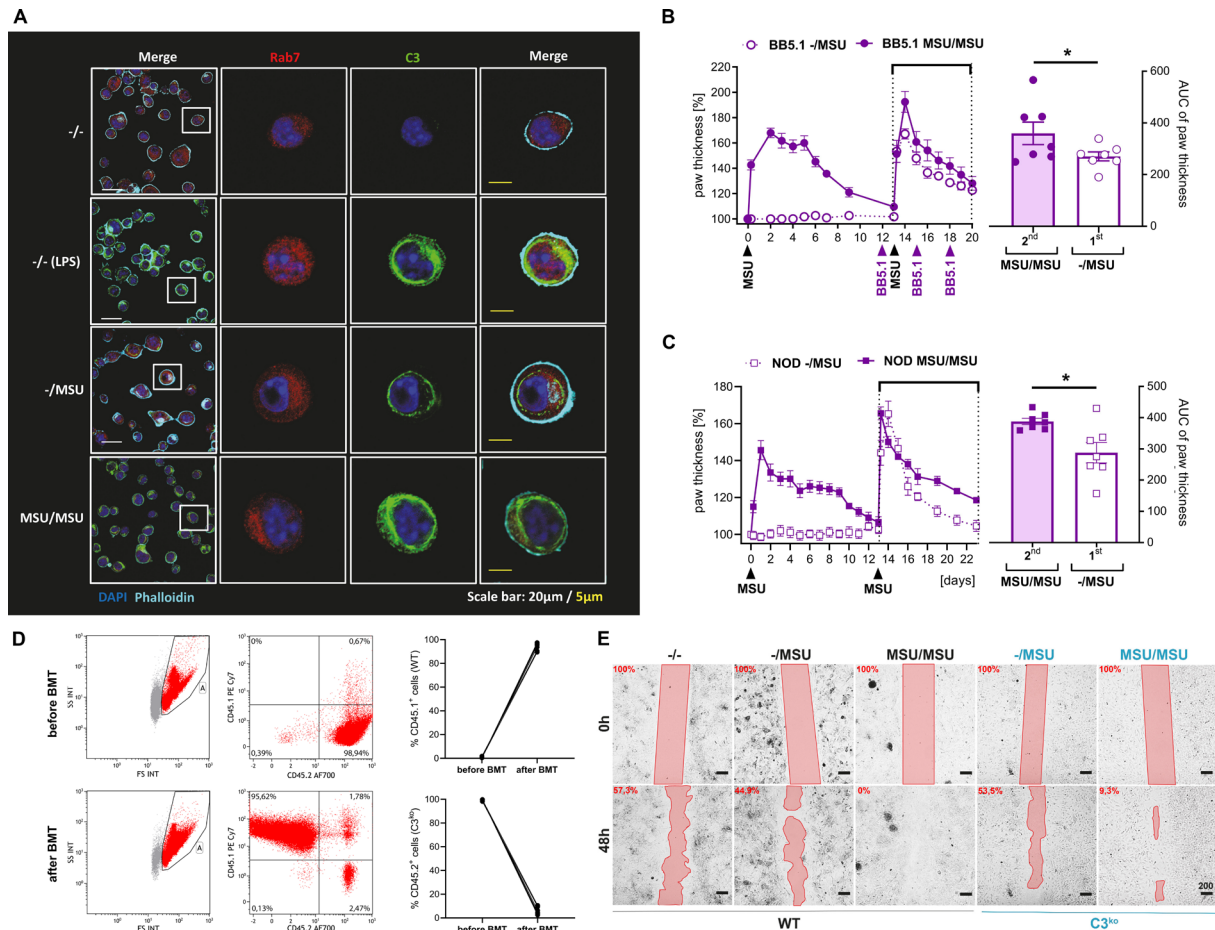


Figure S4, related to main Fig. 4. Expression of complement in synovial fibroblasts (SF) mediates functional changes. (A) Analysis of subcellular localization of complement component C3 assessed by confocal microscopy. Confocal microscopy images (representative of 2 experiments with 100 cells each) of intracellular complement C3 expression in cultured SF from naive or monosodium urate (MSU) crystal-injected paws with or without lipopolysaccharide (LPS) stimulation *in vitro*. C3 (green), F-actin (phalloidin, turquoise), nuclei (DAPI, blue), late endosomes/lysosomes (Rab7, red). Scale bars, 20 μ m (white), 5 μ m (yellow). **(B)** Means $\pm$ SEM of time course (left) and area under the curve (AUC, right) of relative paw thickness in BB5.1 treated BALB/c mice injected once or twice with MSU crystals. N = 7. Unpaired t-test. **(C)** Means $\pm$ SEM of time course of relative paw thickness (left) and AUC (right) in non-obese diabetic (NOD) mice injected once or twice with MSU crystals. N = 7, unpaired t-test. **(D)** Reconstitution of irradiated $C3^{-/-}$ mice ($CD45.1^+CD45.2^+$) with wild type (WT) bone marrow ($CD45.1^+CD45.2^+$). Bar diagrams show values from blood of individual mice and means before and 6 weeks after bone marrow transplantation (BMT). N = 8. **(E)** Increased migration in primed SF depends on complement C3. Representative images of a wound healing/migration assay with SF isolated from naive or MSU crystal-injected paws from C57BL/6 WT or $C3^{-/-}$ mice. Relative areas not covered by SF are highlighted in red.

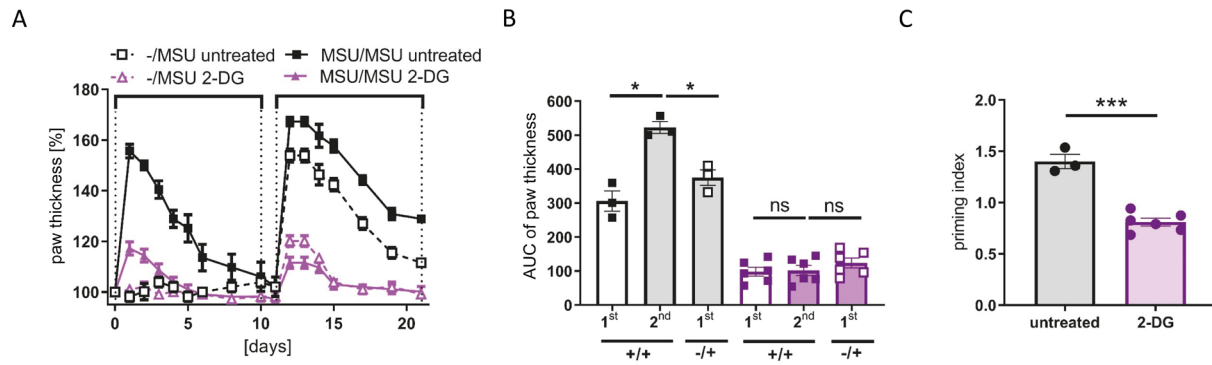


Figure S5, related to main Fig. 5. Treatment of iterated monosodium urate (MSU) crystal-induced arthritis with the glycolysis inhibitor 2-DG abrogates inflammatory tissue priming. Time course of paw thickness (**A**), areas under the curves (AUC) (**B**) and priming indices (**C**) during iterated MSU crystal-induced arthritis in mice treated with 2 mM 2-DG in the drinking water ($n = 6$) or left untreated ($n = 3$). Means $\pm$ SEM are plotted. ANOVA with Tukey's test (**B**), unpaired t-test (**C**).

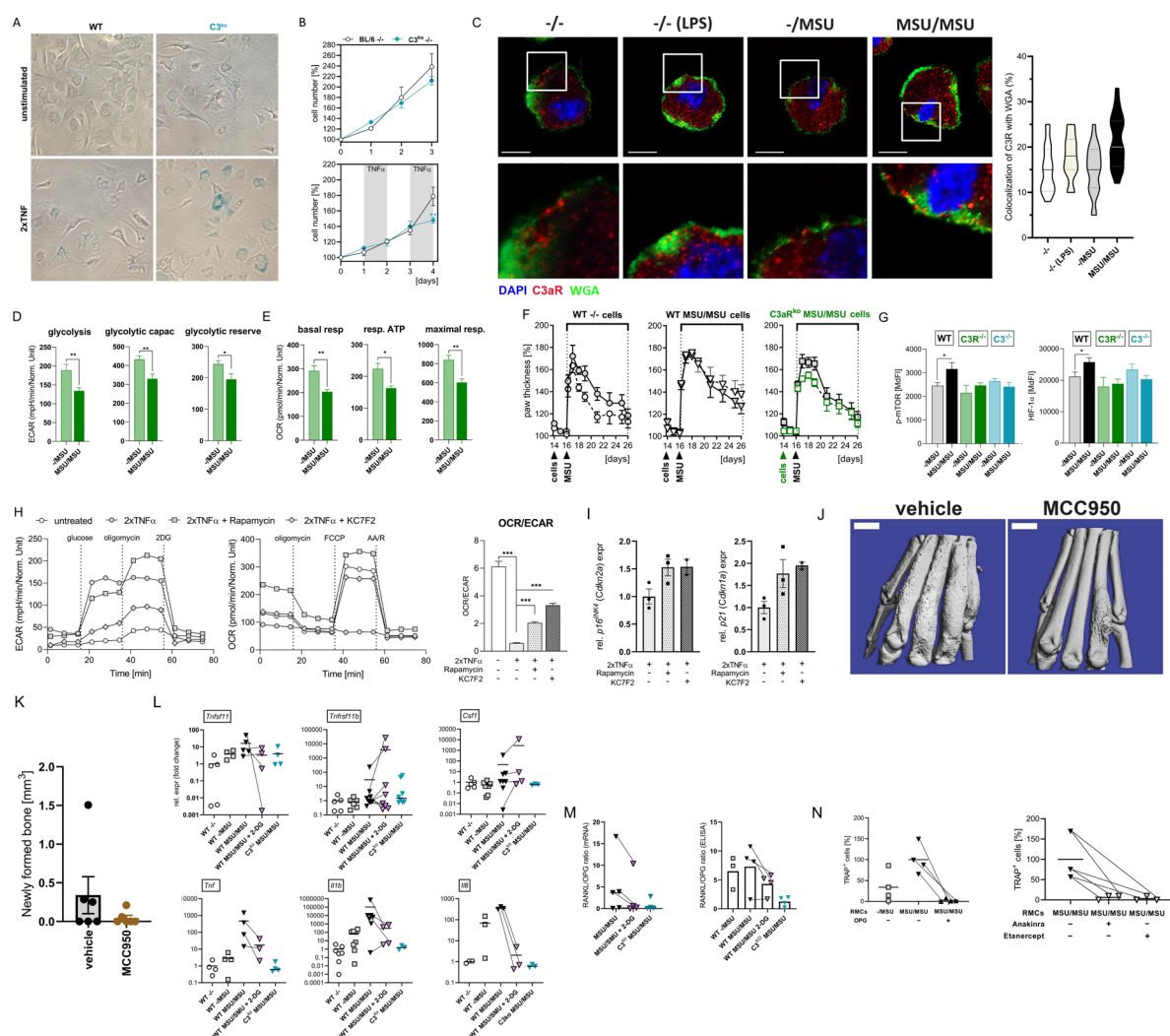


Figure S6, related to main Fig. 6. Complement C3 mediates metabolic changes that mediate inflammatory tissue priming. (A) Representative images from wildtype (WT) or $C3^{-/-}$ synovial fibroblast (SF) subsets (passage 8) either repeatedly incubated with $TNF\alpha$ or left untreated and stained for senescence-associated β -galactosidase (blue). (B) Proliferation in naïve WT and $C3^{-/-}$ SF left unstimulated (left) or stimulated twice with 10 ng/ml $TNF\alpha$ (right). ANOVA with Sidak's test. N = 3-8. (C) Analysis of subcellular localization of C3aR expression by confocal microscopy. Representative images show naïve ($-/-$), $-/MSU$ and primed (MSU/MSU) SF stained with DAPI (blue), an antibody to C3aR (red), and wheat germ agglutinin (WGA) as a plasma membrane marker (green). Scale bars, 10 μ m. Violin plots show medians and quartiles (dashed line) of colocalization between C3aR and WGA in 20 cells. (D, E) Key glycolytic (D) and respiratory (E) parameters calculated from normalized (background and protein) metabolic flux data of $-/MSU$ or MSU/MSU SF from $C3aR^{-/-}$ mice. Means $\pm$ SEM. N = 12-14, paired t-test. (F) Time course of arthritis after transfer of $-/-$ WT fibroblasts (left), MSU/MSU WT fibroblasts (center), or MSU/MSU $C3aR^{-/-}$ fibroblasts (right) into the paws of BALB/c WT mice. (G) Median fluorescence intensities (MdfI) of phosphorylated mammalian target of rapamycin (p-mTOR) and hypoxia-inducible factor (HIF) 1 α in $-/MSU$ and MSU/MSU SF from WT, $C3^{-/-}$ and $C3aR^{-/-}$ mice, as determined by flow cytometry. Means $\pm$ SEM. N = 4-7, ANOVA with Sidak's test. (H) Bioenergetic profiles of naïve WT fibroblasts (n = 3 replicates of pooled cells from two mice) after two stimulation rounds with 10 ng/ml $TNF\alpha$ in the presence or absence of rapamycin (50 μ M) or KC7F2 (30 μ M), as recorded by Seahorse and normalized to background and total protein. ANOVA with Dunnett's test. (I) Expression of the senescence markers $p16$ (*Cdkn2a*) and $p21$ (*Cdkn1a*) in cells stimulated twice

with 10 ng/ml TNF α in the presence/absence of 50 μ M rapamycin or 30 μ M KC7F2, as determined by qPCR. N = 2-3 replicates of pooled cells from two mice. **(J)** Representative 3D rendering from μ CT scans and **(K)** Quantification of newly formed bone 9 days after the second injection of MSU crystals with and without treatment with the inflammasome inhibitor MCC950. N = 6. **(L)** Quantitative RT-qPCR analysis determining expression of mRNAs encoding *Tnfsf11* (RANKL), *Tnfrsf11b* (OPG), *Csf1* (M-CSF), *Tnf*, *Ilb* and *Il6* in -/-, -/MSU and MSU/MSU SF from WT and *C3^{-/-}* mice cultured with or without 2-DG. Shown are individual values and means. **(M)** RANKL/OPG ratio in SF, as determined from mRNA levels and from supernatant concentrations. Graphs show individual values and means. N = 4. **(N)** Differentiation of tartrate resistant acid phosphatase (TRAP)⁺ cells from bone marrow cells in co-culture with -/MSU or MSU/MSU SF with or without inhibition by osteoprotegerin (OPG), anakinra or etanercept. Shown are individual values and means. N = 3-4.

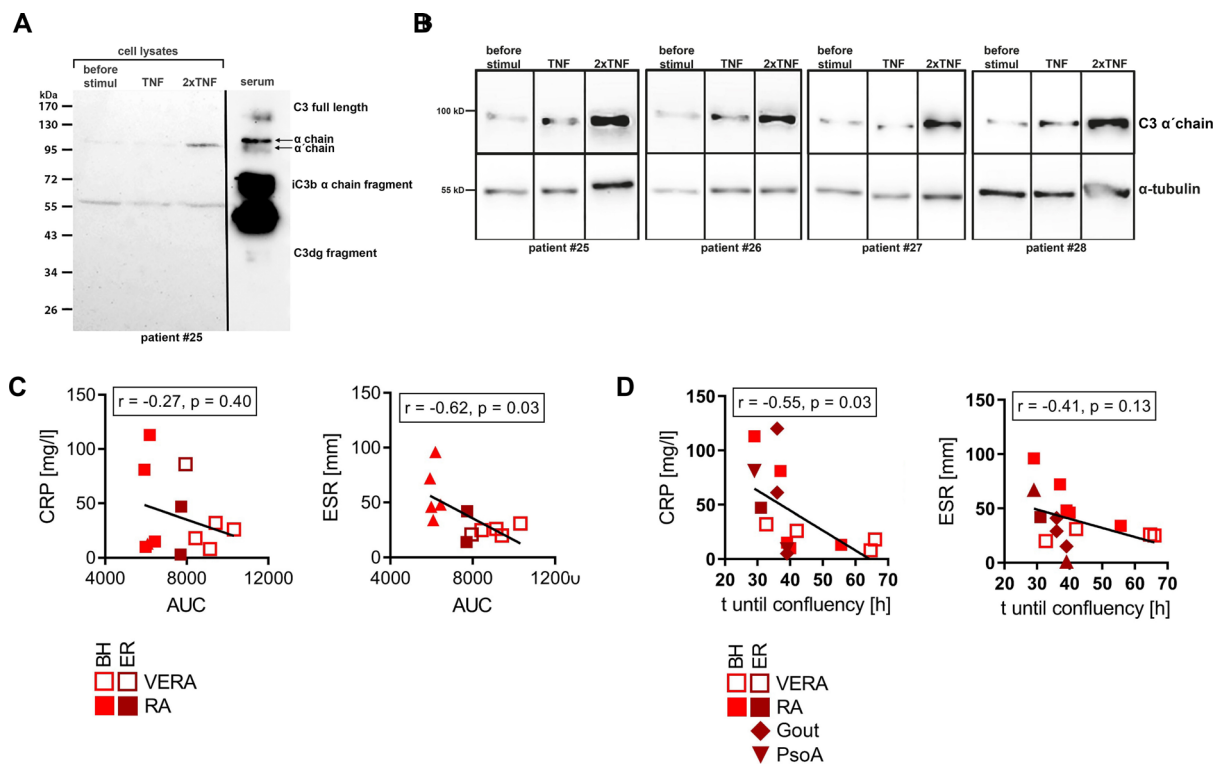


Figure S7, related to main Fig. 7. The complement system is upregulated in activated sublining synovial fibroblast (SF) of human individuals with inflammatory arthritis. (A, B) Analysis of C3 protein expression in human rheumatoid arthritis (RA)-derived SF. **(A)** Representative Western blot showing proteolytic cleavage of C3 α chain into α' chain in SF. **(B)** Protein lysates from four RA patients (patients 25-28) at baseline (before stimulation) and after single (TNF) or a repeated (2xTNF) *in vitro* stimulation with TNFα. α-tubulin served as a loading control. C3 α' chain bands were analysed by densitometry and expression levels normalized to α-tubulin bands (see Fig. 7D, right panel). **(B, C)** Correlation of SF function with systemic parameters of inflammation in human individuals with arthritis. Correlation analysis between SF invasiveness, shown as the area under the curve (AUC) from a MATRIN assay (B) or between wound healing/migration (C) and serum concentrations of C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR) in SF from very early RA (VERA; n = 5), established RA (n = 7), or individuals with gout (C only, n = 3) and psoriatic arthritis (PsoA, C only, n = 2). Pearson coefficients (r) and P values (determined by 2-tailed Student's t test) are depicted within the graphs.