

Supplemental information

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

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Supplemental figures

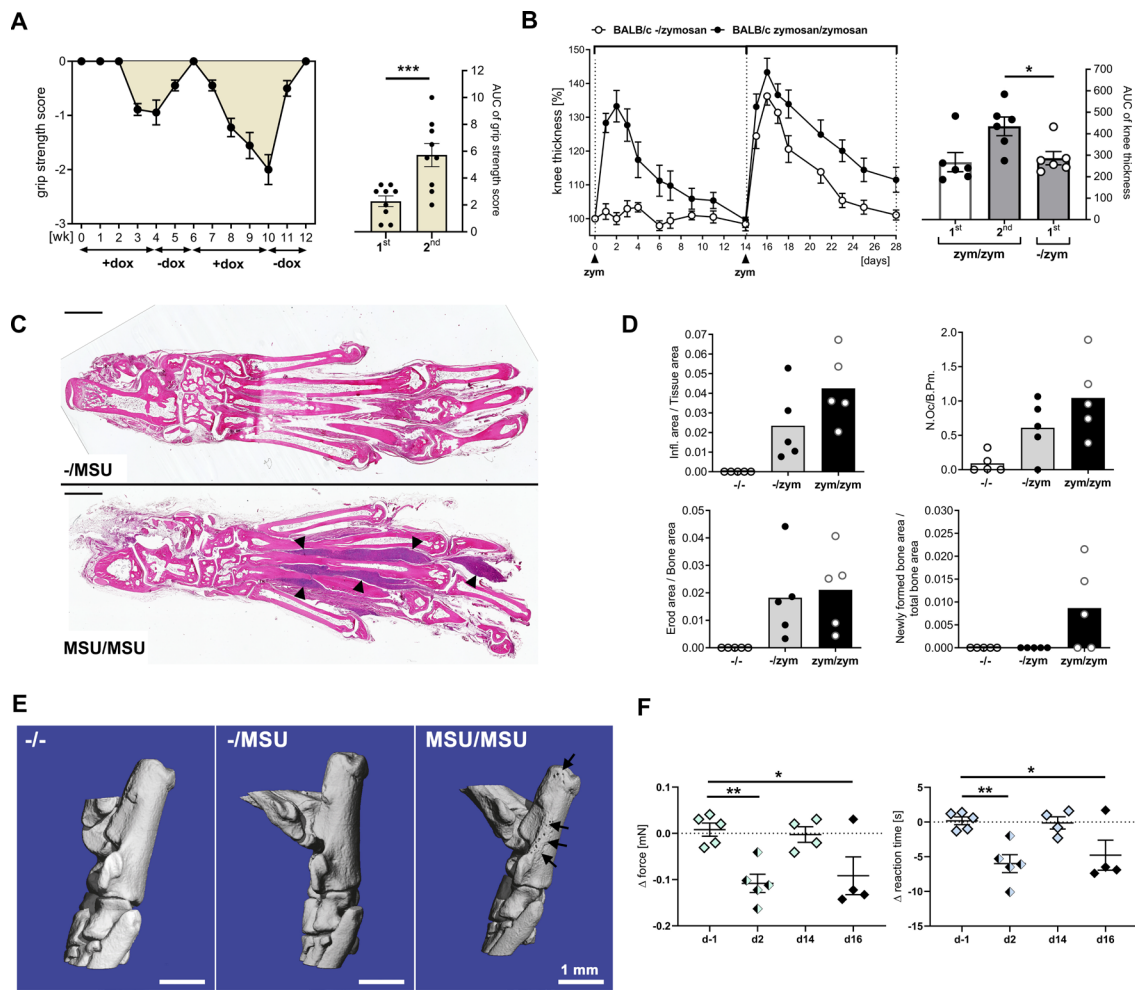


Figure S1, related to main Fig. 1. Inflammatory tissue priming is associated with exacerbated inflammation, bone remodeling, and hyperalgesia. (A) Time course of grip strength reduction and areas under the curve (AUC) of the 1st and the 2nd episode of arthritis induced by doxycycline (dox) in inducible 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.

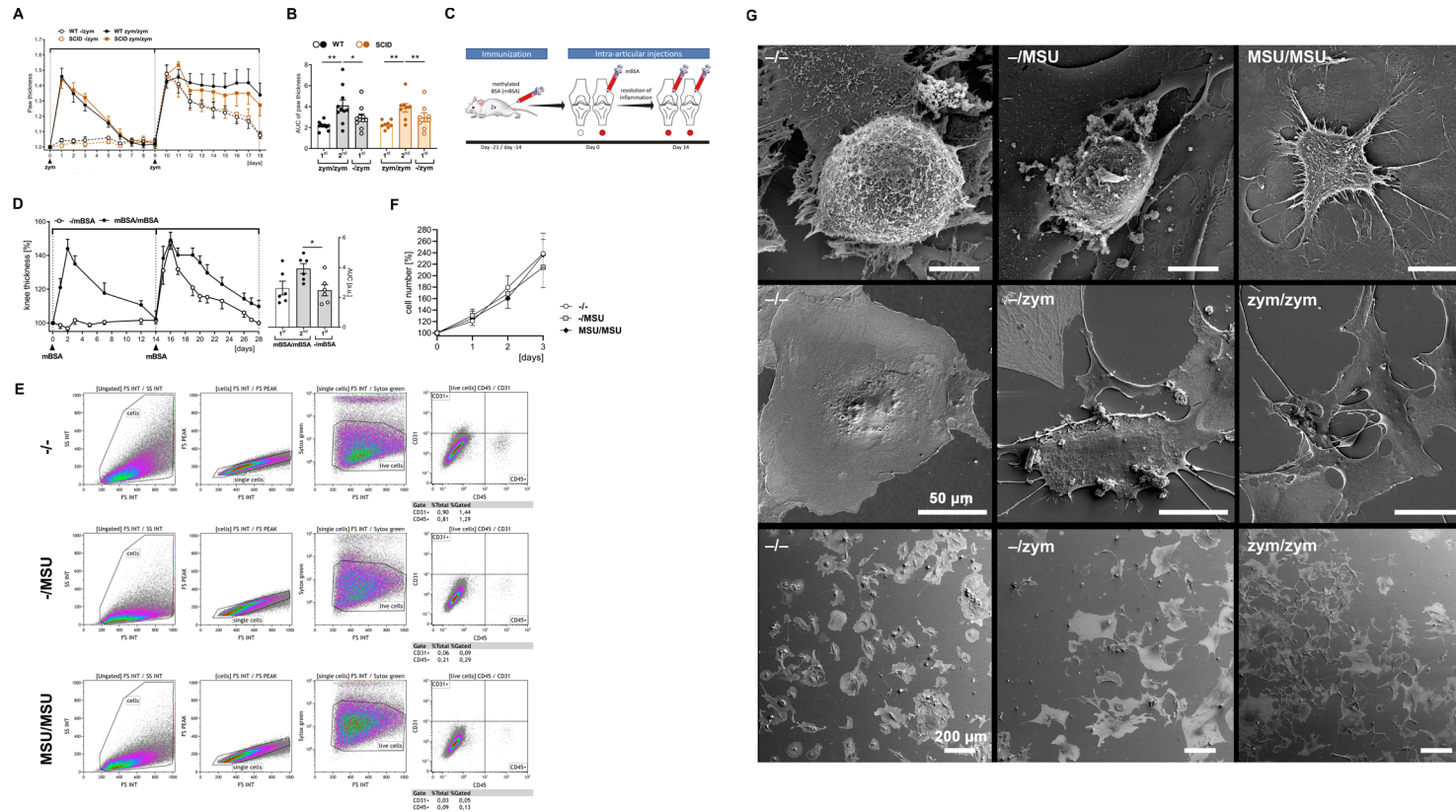


Figure S2, related to main Fig. 2. Inflammatory tissue priming is independent of adaptive immunity, yet can be induced by adaptive immune pathways. (A, B) Arthritis in SCID mice and BALB/c wild type (WT) mice after repeated injection of monosodium urate (MSU) crystals. Shown are **(A)** time course of 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)**.

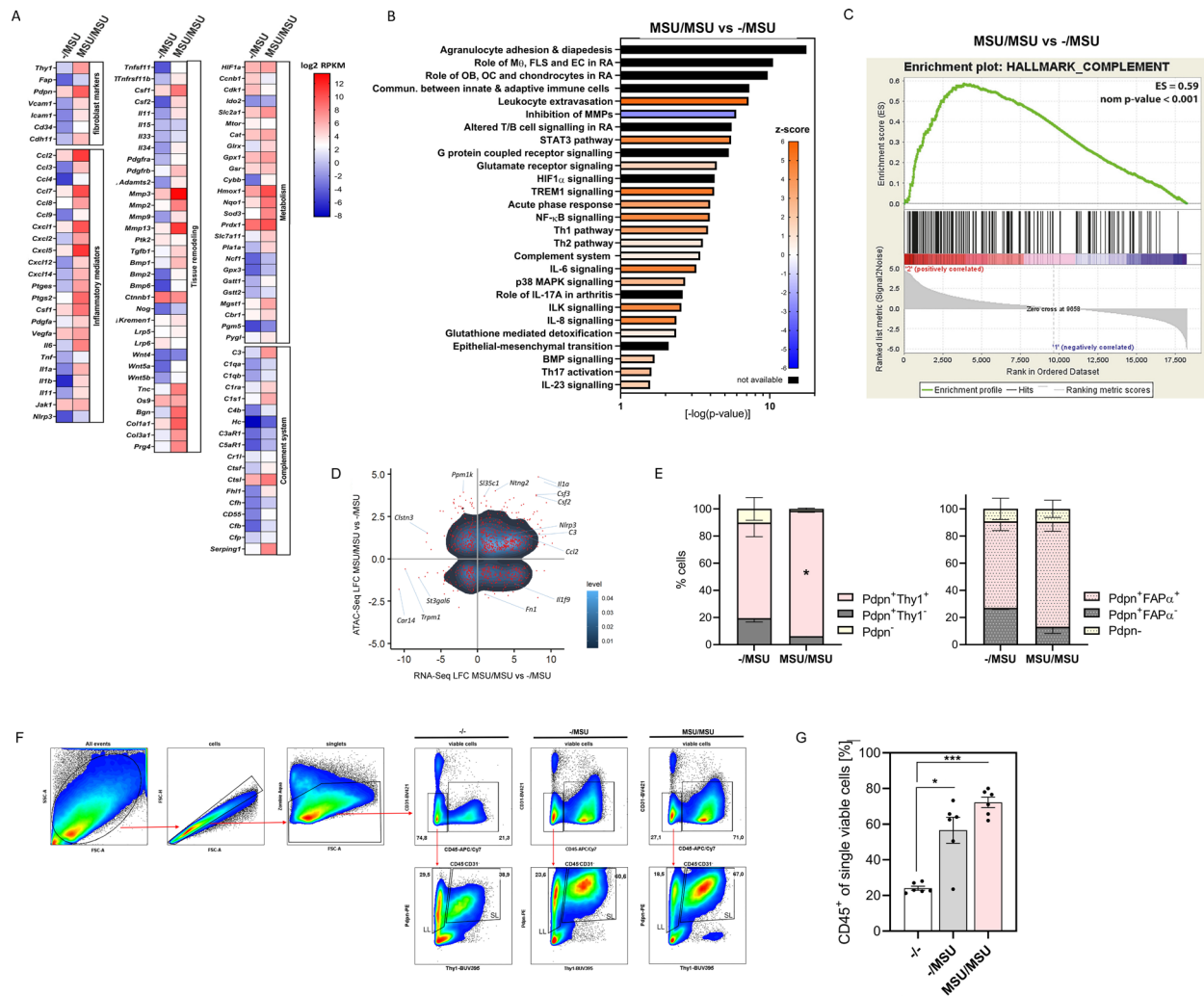


Figure S3, related to main Fig. 3. Inflammatory Pdpn⁺Thy1⁺ fibroblasts expressing complement components are enriched in primed tissue. (A) Mean log₂-transformed RPKM values of selected genes from paws injected once (-/MSU) or twice (MSU/MSU) with monosodium urate (MSU) crystals, generated from bulk RNA 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.

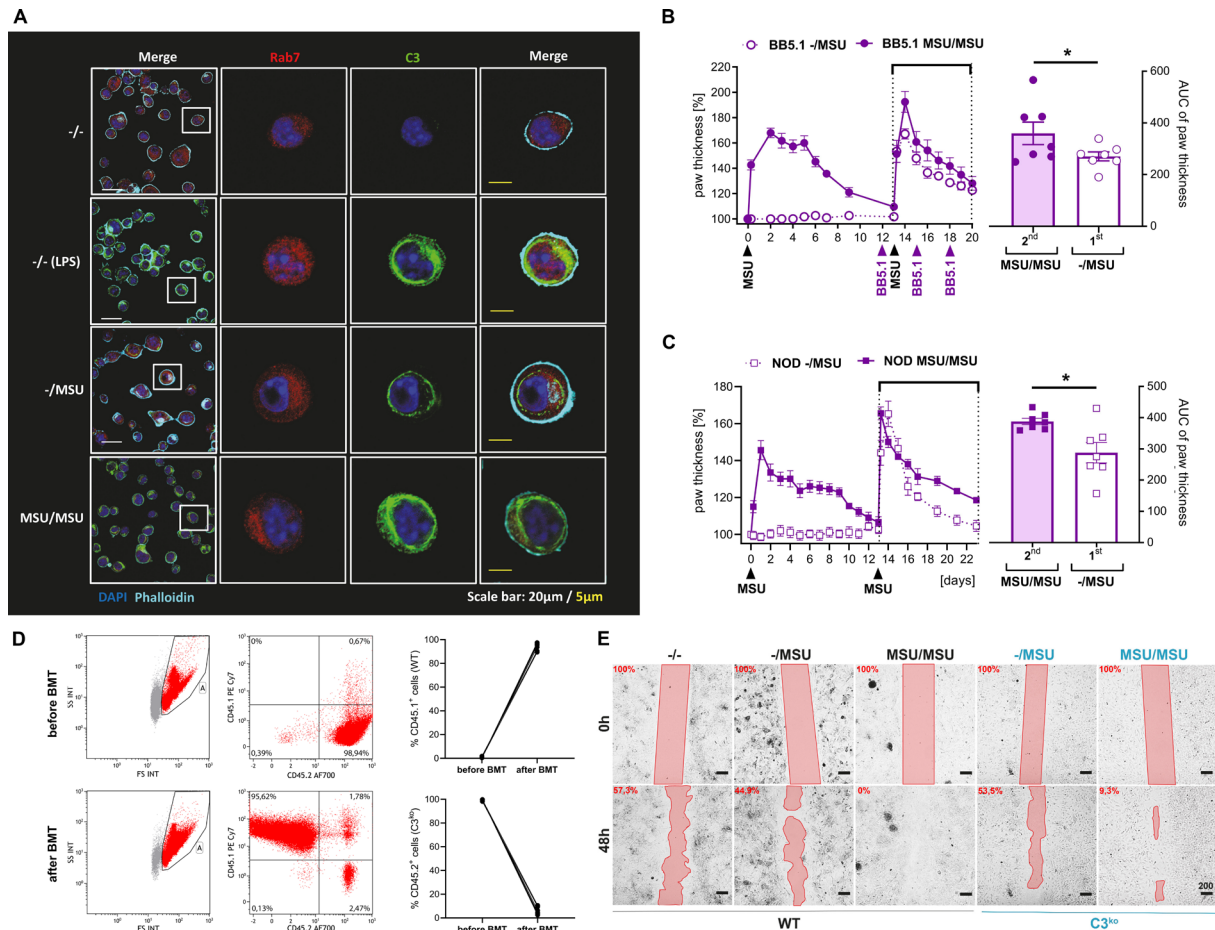


Figure S4, related to main Fig. 4. Expression of complement in synovial fibroblasts (SF) mediates functional changes. (A) Analysis of subcellular localization of complement component C3 assessed by confocal microscopy. Confocal microscopy images (representative of 2 experiments with 100 cells each) of 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.

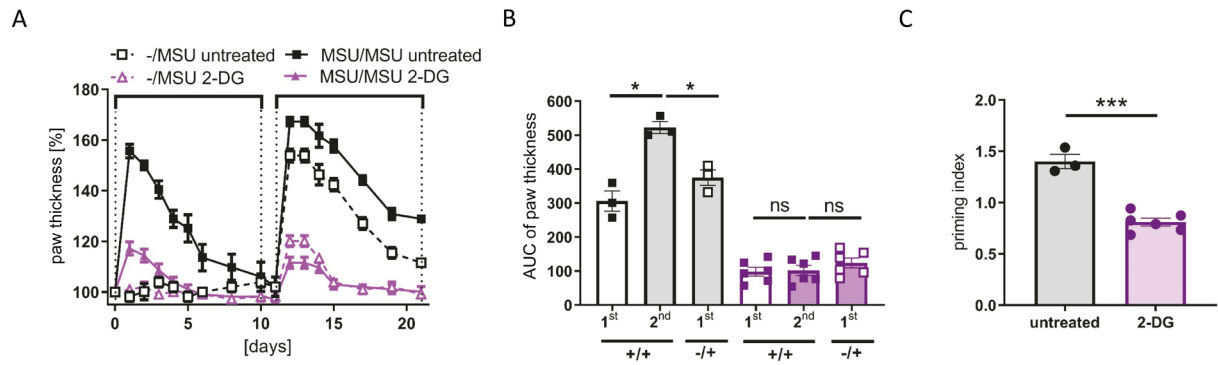


Figure S5, related to main Fig. 5. Treatment of iterated monosodium urate (MSU) crystal-induced arthritis with the glycolysis inhibitor 2-DG abrogates inflammatory tissue priming. Time course of paw thickness **(A)**, areas under the curves (AUC) **(B)** and priming indices **(C)** during iterated MSU 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)**.

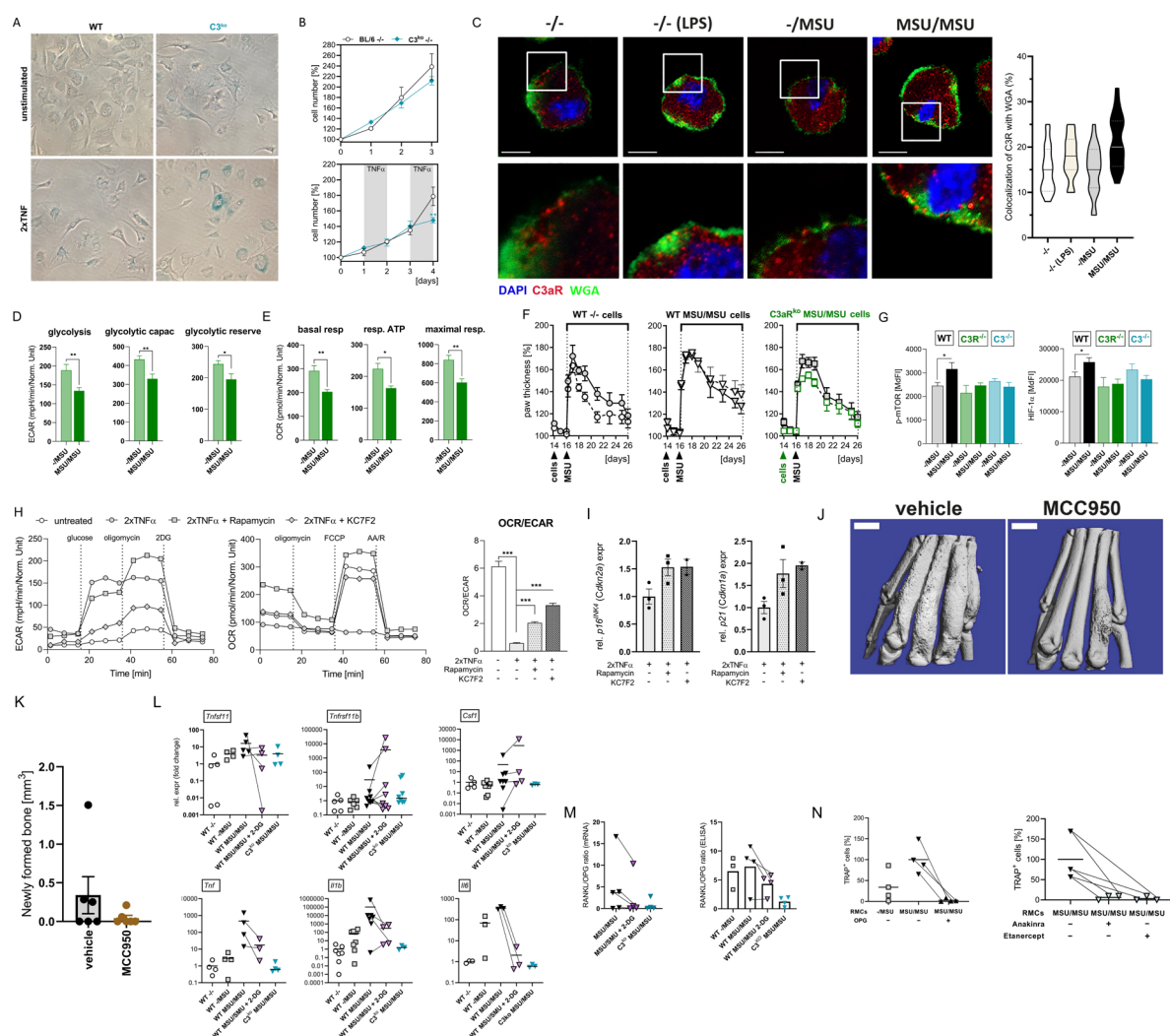


Figure S6, related to main Fig. 6. Complement C3 mediates metabolic changes that mediate inflammatory tissue priming. (A) Representative images from wildtype (WT) or $C3^{-/-}$ synovial fibroblast (SF) subsets (passage 8) either repeatedly incubated with TNF α or left untreated and stained for senescence-associated β -galactosidase (blue). **(B)** Proliferation in naïve WT and $C3^{-/-}$ SF left unstimulated (left) or stimulated twice with 10 ng/ml TNF α (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 α 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.

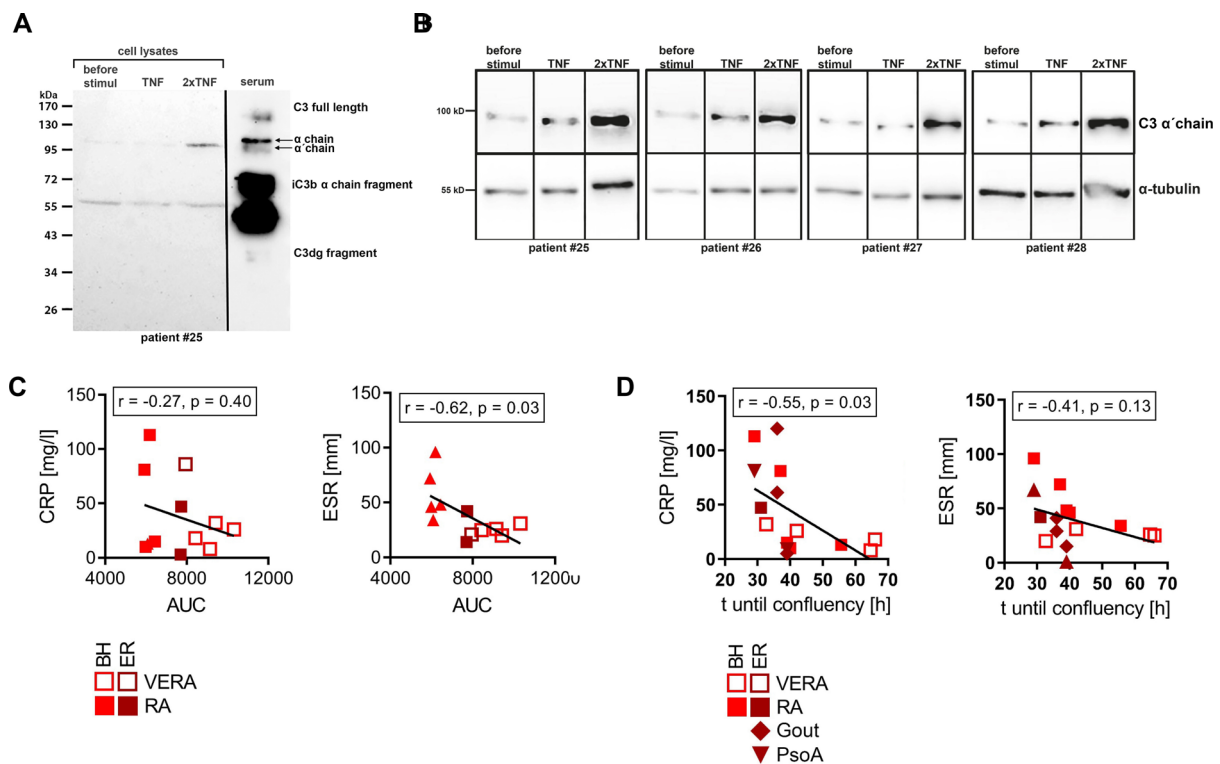


Figure S7, related to main Fig. 7. The complement system is upregulated in activated sublining synovial fibroblast (SF) of human individuals with inflammatory arthritis. (A, B) Analysis of C3 protein expression in human rheumatoid arthritis (RA)-derived SF. **(A)** Representative Western blot showing 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.