Efferocytosis by Synovial Fibroblasts in PTOA is Mediated by the AXL Receptor

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INTRODUCTION: Effective clearance of apoptotic cells, termed efferocytosis, is essential in restoring tissue homeostasis after injury. Efferocytosis by macrophages has been well established. However, no evidence exists regarding stromal cells undertaking efferocytosis in the context of joint injury or OA. We have made the striking observation that synovial fibroblasts (SFs), specifically lining SFs, perform efferocytosis following joint injury. Herein, we employed a bone marrow chimera model to empirically demonstrate endogenous efferocytosis by SFs, a novel fibroblast function for regulating inflammation, followed by investigation of the role of Axl, a tyrosine kinase receptor, in mediating efferocytosis.

METHODS: ***Mice:*** All experiments were IACUC approved. *Bone marrow chimera model*: C57Bl6/J mice (8 wk old) were irradiated (2 x 550 cGγ, 4 h apart). $5x10^6$ tdTom+ murine bone marrow cells were transplanted into irradiated mice. Engraftment was confirmed 5 weeks later. *PTOA model*: Chimeric mice (13 wk old) underwent non-invasive anterior cruciate ligament rupture (ACLR) to induce PTOA with Sham (anesthesia/analgesia only) as a control. Synovia from Sham, 3d, 7d, and 28d post-ACLR chimeric mice (n=6, 3M+3F per group) were harvested for flow cytometry. Efferocytosing lining SFs were identified as tdTom+ITGA6+THY1-CD31-CD45- cells. *Axl inhibition in PTOA:* (1) Local inhibition: 1 ug of Axl-specific inhibitor (R428) or vehicle was injected into knees of chimeric mice for 5 consecutive days, first dose 2 days pre-ACLR. (2) Systemic inhibition: 100 mg/kg of R428 or vehicle was given to chimeric mice via oral gavage for 5 consecutive days, first dose given 2 days pre-ACLR. Synovia (n=6, 3M+3F per group) were harvested for flow cytometry to assess efferocytosis. ***IHC****:* Hindlimbs from chimeric mice 3d ACLR were collected, fixed, and decalcified. Cryosections (10 µm) from these samples were stained for PRG4 (lining SF), LAMP-1 (lysosome), and DAPI. ***Spatial transcriptomics:*** Xenium 5K was performed on Sham and 3d ACLR knee joints. ***In Vitro***: *Inflammatory model*: Primary SFs (pooled from 3M+3F) were treated with IL-1β (10 ng/mL) for 48 h then camptothecin-induced apoptotic neutrophils (apoNeu) labelled with eFluor670 were added to the culture at 1:5 SF:apoNeu ratio as an *in vitro* efferocytosis model. After 20 h, cells were harvested for flow cytometry to quantify efferocytotic SFs (eFluor670+Ly6G-CD45- cells). *TAM receptor inhibition*: Primary murine or human SFs (from arthroplasty patients, n=4 donors) were pre-treated with pan-TAM, Axl-specific, and MerTK-specific inhibitors, followed by addition of apoNeu. Cells were collected 20 h later for flow cytometry. ***ELISA:*** After 20 h efferocytosis, apoNeu were removed and fresh media was replaced. Conditioned media was collected 24 hrs later for ELISA of chemokines and cytokines. ***scRNA-seq:*** SFs pre-treated with R428 (AXL inhibitor) or vehicle for 30 min, followed by an addition of growth media (control) or apoNeu (1:10 ratio of SF:apoNeu), were sorted to eliminate remaining apoNeu after 20 hrs of efferocytosis. Samples were preprocessed and integrated in Seurat v5. Pathway analysis was performed using Gene Set Enrichment Analysis (GSEA).

RESULTS: In a tdTom+ bone marrow chimera in which joint-infiltrating immune cells are tdTom+, ITGA6+ lining SFs performed efferocytosis after ACLR, evidenced by flow cytometric measurement of tdTom+ lining SFs. This peaked during acute inflammation (3d ACLR), decreased at 7d ACLR and maintained until 28d ACLR (**Fig. 1A**). Given our rigorous flow gating strategy that excludes immune cells and doublets, this is robust evidence of lining SFs internalizing recruited tdTom+ immune cells. Fluorescent microscopy confirmed this, with tdTom+ signal co-localized to lysosomal LAMP-1 inside PRG4+ lining SFs (**Fig 1B, green + red = yellow**). SFs also undertook efferocytosis *in vitro,* and their efferocytic ability was impaired by IL-1β (**Fig. 1C-D**). Among the TAM receptors, which mediate efferocytosis, high-dimensional, single-cell spatial transcriptomics identified and pinpointed high *Axl* expression in lining SFs, which dramatically increased in the injured joint (**Fig. 1E**). AXL inhibition by R428 induced the strongest blockade of efferocytosis in human and mouse SFs *in vitro*, compared to blocking other TAMs (**Fig. 1F-G**). AXL inhibition also ablated the efferocytic increase in CCL-2, CXCL-5, CXCL-1, and IL-6 secretion by mouse SFs (**Fig. 1H**). Pharmacological inhibition of AXL via local and systemic R428 treatment reduced efferocytic capacity of lining SFs, confirming the *in vivo* role of AXL in SF efferocytosis (**Fig. 1I**). scRNA-seq demonstrated that efferocytosis in SFs *in vitro* activated multiple inflammatory pathways, including inflammasome (**Fig. 1J**). The proportion of proliferative SFs exposed to apoNeu was significantly reduced by AXL inhibition (**Fig. 1K-M**), suggesting a role for SF efferocytosis in mediating lining hyperplasia. AXL inhibition also activated lysosome organization, inhibited cell proliferation, and induced apoptosis in the proliferative cell cluster, whereas intact efferocytosis upregulated chemokines and cell adhesion genes (**Fig. 1N-O**).

DISCUSSION: Our data empirically confirm that SFs undertake efferocytosis following joint injury. This establishes a novel role for SFs in mediating early inflammation, and acute SF efferocytosis likely perpetuates the nature and degree of downstream synovitis development. Efferocytosis appears to activate a pro-resolution response, which could represent an overlooked mechanism by which SFs restore tissue homeostasis. AXL inhibition impaired efferocytosis, and induced cell autophagy and apoptosis, both of which exacerbate joint inflammation.

SIGNIFICANCE/CLINICAL RELEVANCE: We present the novel paradigm that lining SFs play an important role in early injury-induced inflammation via efferocytosis. This holds extensive clinical relevance as this process can be manipulated to therapeutically modulate joint inflammation and PTOA onset.

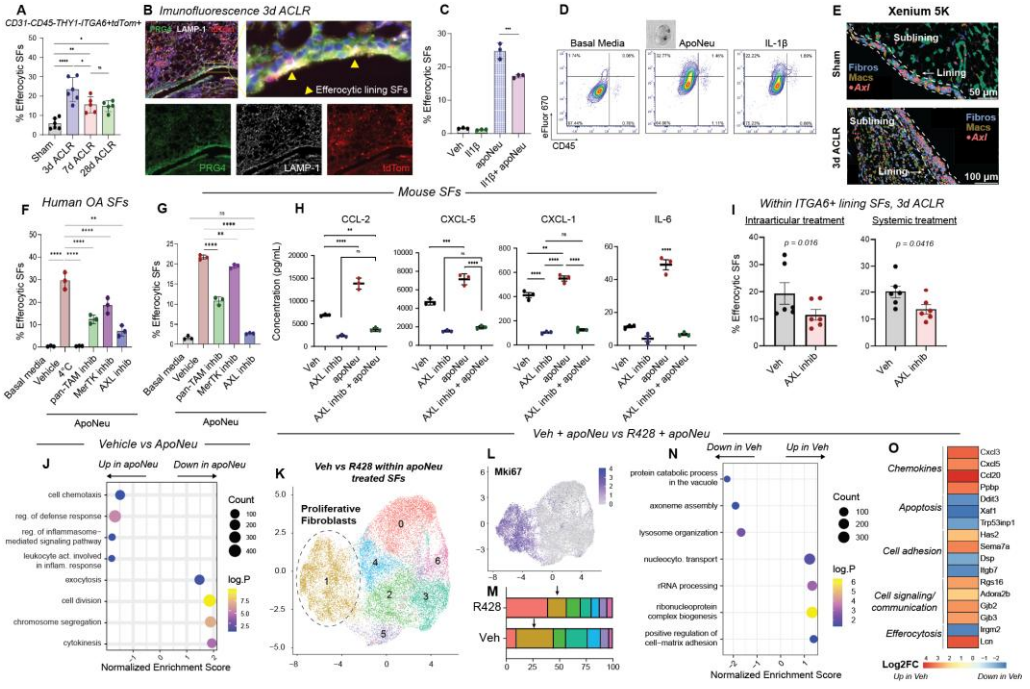

Figure 1. (A) In vivo efferocytosis by ITGA6+ lining SFs. **(B)** Immunofluorescent images of efferocytosing lining SFs in a 3d ACLR synovium. **(C-D)** Impaired *in vitro* efferocytosis after inflammation. **(E)** Xenium 5K showing *Axl* expression in lining fibroblasts of both Sham and 3d ACLR synovium. **(F-G)** Pharmacological inhibition of TAM receptors impacts efferocytosis capability of **(F)** OA human and **(G)** mouse SFs. **(H)** ELISA of mouse SFs post-efferocytosis. **(I)** Efferocytosis capability of lining SFs is impaired by blockade of AXL. **(J)** GSEA pathways comparing SFs with and without apoNeu. **(K)** UMAP of fibroblast subpopulations in Veh vs AXL-inhibition within apoNeu treated SFs. **(L)** Feature plot showing proliferative cluster gene. **(M)** Cell proportion of the clusters. **(N-O)** GSEA pathways and heatmap of genes comparing efferocytosing SFs with and without AXL.