The phenotype and fate of monocyte-derived and tissue-resident synovial macrophages is modulated by injury and obesity in osteoarthritis

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INTRODUCTION: Immune cells are not only the central component of the body's defense mechanisms but also contribute to organ development and adult tissues' homeostasis. Macrophages are the main immune cell population found in synovium and other musculoskeletal tissues, playing a crucial role in maintaining tissue homeostasis as well as inflammation processes occurring in obesity and osteoarthritis (OA). However, the precise contribution of macrophages in the OA disease sequence is still unclear, with recent challenges to the simplified dogma of either M1/M2 macrophage polarization in the synovium. Studies analyzing macrophage subtypes in rheumatoid arthritis (RA) and OA have shown the presence of more defined populations both in mice and humans[1,2]. However, the origin, phenotype, and spatial localization of macrophages within joint tissues during the progression of DMM/injury and obesity-induced OA have not been well characterized. Here, using multicolor flow cytometry, immunofluorescent labeling, and transgenic mouse models, we analyzed the more defined sequence of events during the progression of OA and obesity. **METHODS:** All animal procedures were approved by the IACUC. Wild-type C57BL/6J and CCR2GFP mice strains were used. RosaTd were crossed with either LysMCre, Lyve1Cre, or CX3CR1CreERT2 mice (Jackson Laboratories). Four-week-old mice were fed either a control (10% kcal fat) or high-fat diet (HFD, 60% kcal fat) (Fig.1A). At 16 weeks of age, mice underwent surgical destabilization of the medial meniscus (DMM) on the left knee to induce post-traumatic OA. For fate mapping experiments only, Tamoxifen (TA, 75mg/kg) was injected intraperitoneally for five consecutive days at 14 weeks of age. Mice were sacrificed either at three days or 12 weeks post-surgery. Synovial pouches were collected, and cells were isolated using collagenase digestion. Synovial macrophages were analyzed by multicolor flow cytometry. Knee joints were cryopreserved, cryotome cut, immunofluorescently labeled, and imaged by confocal microscopy. Statistical analysis was performed using 2-way ANOVA with Bonferroni correction at $p<0.05$. **RESULTS:** Our previous scRNA-seq analysis revealed the presence of multiple populations of macrophages in the synovial capsule with unique gene expression profiles. Here, we confirmed the presence of four main macrophage populations based on their CCR2, MHCII, and LYVE1 expression, namely CCR2$^+$ MHCII$^-$ LYVE1$^-$ [**CCR2$^+$MHCII$^-$**], CCR2$^+$ MHCII$^+$ LYVE1$^-$ [**CCR2$^+$MHCII$^+$**], CCR2$^-$ MHCII$^+$ LYVE1$^+$ [**LYVE1$^+$MHCII$^+$**], and CCR2$^-$ MHCII$^-$ LYVE1$^+$ [**LYVE1$^+$MHCII$^-$**] (Fig.1B,C). We illustrated that both injury and obesity significantly modulate the content and distribution of those populations. For instance, **CCR2$^+$MHCII$^-$** macrophages, that reside in the sublining area of the synovium, show an increase in content at three days post-injury, which is significantly more pronounced in obese mice (Fig.2B, 3B). Their presence greatly diminishes at 12 weeks post-injury compared to other populations, with a remaining trend towards higher content in obese mice. The content of synovial lining macrophages defined by **LYVE1$^+$MHCII$^-$** expression significantly decreases in the first three days post-injury and is successfully restored in mice fed the control diet. However, obese mice failed to restore their numbers and synovial lining localization after 12 weeks post-injury. Finally, our lineage-tracing model utilizing CX3CR1-TdT (TdTomato) (Fig.2C, D and Fig. 3C, D) has shown that at three days post-injury there is an increase of TdT-positive cells that are mostly CCR2-positive. Interestingly, at 12 weeks post-injury, their presence diminishes, and the majority of TdT-positive cells are defined by LYVE1 expression. **DISCUSSION:** Our study demonstrates the presence, spatial location, and changes in the content of multiple populations of macrophages in the synovial joint during the progression of obesity and injury-induced OA. We characterized four distinct populations of macrophages in the synovium with unique localization and function. Interestingly, by utilizing the CX3CR1-TdT lineage-tracing model, we showed that in the initial phase after injury, the majority of synovial macrophages are derived from circulating monocytes and co-express TdT and CCR2. These cells are known to contribute to inflammation, be short-lived, and disappear from tissues within a couple of days. Twelve weeks post-injury, the remaining TdT-positive cells mainly express LYVE1$^+$. These results suggest that LYVE1$^+$ cells most likely represent self-renewing, yolk sac-derived tissue-resident macrophages in the synovium. In this study, we also validated a distinctive sequence of macrophages' contribution during the progression of OA that is significantly disturbed in obese animals. Our and other previous studies have shown that obese mice display a significantly higher predisposition to joint damage after DMM. Results presented here indicate that obese mice have a significantly higher influx of monocyte-derived **CCR2$^+$MHCII$^-$** macrophages in the initial phase after injury and additionally fail to restore the protective barrier created by **LYVE1$^+$MHCII$^-$** synovial lining macrophages at 12 weeks post-injury. These data suggest the important contribution of different macrophage populations in the progression of OA. Our findings also emphasize the need for a more in-depth understanding of the impact of macrophages on OA progression.

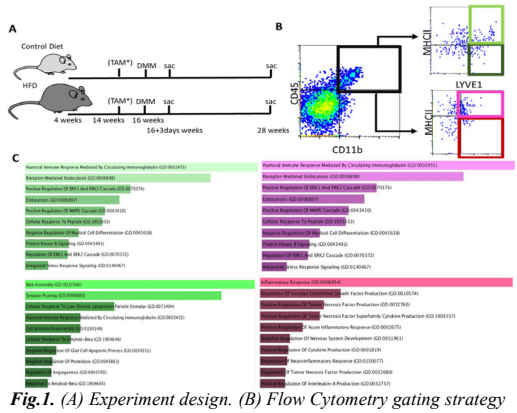

Fig.1. (A) Experiment design. (B) Flow Cytometry gating strategy utilized to distinguish main macrophages populations. (C) Gene ontology analysis utilizing Enrichr package.

SIGNIFICANCE:
The findings of this study further our understanding of the role of different populations of macrophages in synovium and provide potential targets for future OA therapies.

ACKNOWLEDGMENTS:
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REFERENCES: 1. Dick$^+$ *Sci Immunol.* 2022; 7(67):eabf7777, **2.** Culleman$^+$ *Nature* 2019; 572(7771): 670–675.

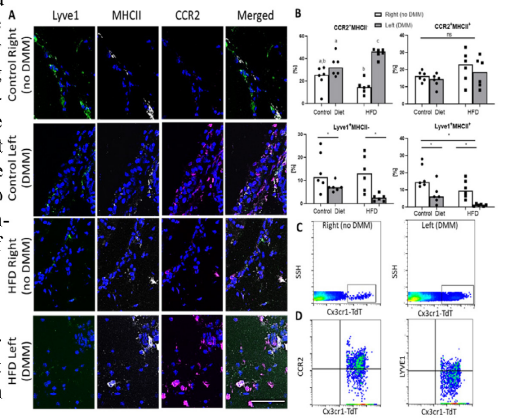

Fig.2 Synovial macrophages at 3 days post-DMM. *(A) Representative immunofluorescent (IF) staining confirmed presence of LYVE1$^+$, MHCII$^+$, and CCR2$^+$ macrophages in the joints. (B) Content analysis of these populations as defined by flow cytometry. (C) Representative flow cytometry analysis of Cx3cr1-TdT cells in Right (no DMM) and Left (DMM) joints. (D) Representative CCR2 and LYVE1 expression in TdT-positive cells. 2-way ANOVA, different letters indicate $p<0.05$ between groups. * $p<0.05$ indicates group effect, n=5-12 per group. Scale bar 50μm.*

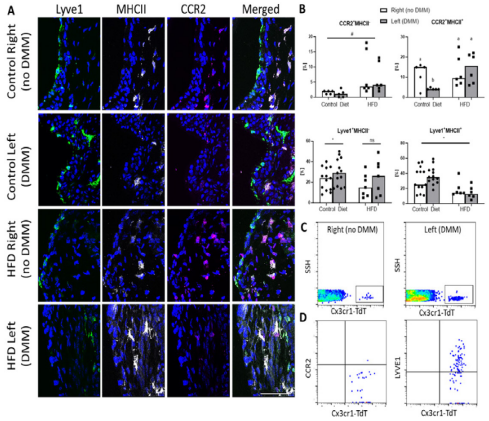

Fig.3 Synovial macrophages at 12 weeks post-DMM. *(A) Representative IF staining confirmed presence of LYVE1$^+$, MHCII$^+$, and CCR2$^+$ macrophages in the joints. (B) Content analysis of these populations as defined by flow cytometry. (C) Representative flow cytometry analysis of Cx3cr1-TdT cells in Right (no DMM) and Left (DMM) joints. (D) Representative CCR2 and LYVE1 expression in TdT-positive cells. 2-way ANOVA, different letters: $p<0.05$ between groups. * $p<0.05$ indicates group effect, n=5-12 per group. Scale bar 50μm.*