Imaging mass cytometry reveals distinct cellular phenotypes in CD14 deficient mouse synovium.

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INTRODUCTION: Growing evidence has revealed that inflammation is a major driver of osteoarthritis (OA). However, previous consideration of OA as a noninflammatory disease placed early focus on mechanical and structural characterization. As a consequence, there is a knowledge gap with respect to the full description of the inflammatory state across tissues within the knee joint (synovium, meniscus, cruciate ligaments, etc.) during OA progression. Of these tissues, the synovium has been identified as a reservoir of not only inflammatory mediators but also innate (monocyte/macrophages) and adaptive (T- and B-cells) immune cells.[1] Both the diverse cell populations and unique structure of the synovium, including the lining and sublining layers, undergo unique inflammatory-mediated degenerative changes. CD14, a co-receptor to inflammatory toll-like receptor (TLR) signaling and subsequent macrophage activation, has also been identified as being upregulated in OA synovium and, in our prior work, we showed that global genetic CD14 deficiency in mice is protective against PTOA related bone-remodeling and mobility dysfunction.[2,3] *Imaging mass cytometry (IMC)* is an emerging technology that allows for the *spatial localization* of molecular species across tissue samples, facilitating investigation of cellular subtypes throughout diverse tissue structures, such as the synovium, as they change with disease. Utilizing this technology, ***we hypothesized that CD14 deficiency would modulate the innate immune cell profiles within the synovium during OA progression.***

METHODS: *CD14 knockout (CD14-KO) mice:* Global CD14 deficient mice of C57BL/6 background were obtained from Jackson Laboratories (#003726).[4] *OA model (n=5)*: Destabilization of the medial meniscus (DMM) surgery was performed to induce OA in skeletally mature (10-12 wk old) CD14-KO or C57BL/6 (WT) mice.[5] *Flow cytometry analysis (n=5):* Synovial and fat-pad tissue from 4 knees were pooled for each biological replicate, collected at 0-(preop), 4-, 8- or 16-wks post-surgery, and cells were isolated enzymatically. Cell suspensions were split in half and stained with antibodies for monocyte (CD45, Ly6C), and macrophage (CD45, CD64) cell markers or T cell (CD45, CD3) and T-helper cell (CD45, CD3, CD4) markers. Multicolor flow cytometry was performed (BD LSR II), and data was analyzed with FlowJo software (Version 10). Monocyte/macrophage populations were expressed as percent of the CD45+ population, T cell populations were expressed as percent of the CD45+ or CD3+ populations. *IMC (n=3, 4wks-post DMM)*: Whole knee joints were fixed, decalcified, paraffin embedded, and sectioned. Sagittal sections underwent heat-mediated antigen retrieval, and overnight incubation with a 22-marker multiplex panel of metal-conjugated antibodies, followed by incubation with Intercalator-Ir nuclear stain, and imaging using a Hyperion Imaging System (Standard Biotools). *Spatial protein expression and cellular phenotype analysis (n=3):* Single cell masks were created using the nuclear stain (deepcell.org). IMACytE software was used to create t-distributed stochastic neighbor embedding (t-SNE) dimensionality reduction analysis with arcsin transformation to produce data normalization and cluster analysis.[6] Cell counts per cluster were exported for comparison between experimental groups. *Statistical analysis:* Student's t-test or two-way ANOVA (indicated in figure legends), with $p<0.05$ considered significant.

RESULTS: Initial analysis of immune cell populations via flow cytometry revealed general leukocyte (Ly6C-CD64-), monocyte (Ly6C+CD64-), and macrophage (Ly6C+CD64+) populations to be significantly increased compared to baseline following DMM in both WT and CD14-KO synovium (**Fig. 1A**). Comparing strains, the macrophage (Ly6C-CD64+) cell population was significantly decreased in CD14-KO mice compared to WT at 8-wks post DMM (**Fig. 1A**). Further evaluations revealed T-helper cells (CD3+CD4+CD8-) to be increased in both WT and CD14-KO mice at 4wks post DMM (**Fig. 1B**), however at 8wks post DMM the T-helper cell population in CD14-KO mice was significantly lower than in WT synovium (**Fig. 1B**). IMC spatial protein analysis of synovial sections at 4-wks post DMM revealed notable differences in monocyte/macrophage marker expression (Ly6C, F480) within the synovial lining and sublining layers between WT and CD14-KO groups (**Fig. 2C,D**). Dimensionality reduction analysis (t-SNE) revealed 12 unique cell populations across combined experimental synovial regions, with clustering by differential expression of vasculature (CD31), nerve (PGP9.5), monocyte/macrophage (Ly6C, F4/80, CD64, MHC-II, CX3CR1), T-cell (CD3), fibroblast, and other immune cell markers (**Fig. 3A,B**). The identified clusters could be localized throughout synovial lining and sublining layers (**Fig. 3C**), and evaluation of cells within unique phenotype clusters revealed significant decreases in Cluster 2 (p=0.021) and Cluster 8 (p=0.033), and an increase in cluster 5 (p=0.026) in CD14-KO synovium compared to WT at 4wks post DMM (**Fig. 3D**).

DISCUSSION: Flow cytometry analysis revealed significant changes within the synovium following DMM to innate (monocyte/macrophage) and adaptive (T-cells) immune cell populations that persist until at least 8-wks. In contrast, CD14 deficiency reduced the persistence of post-DMM elevations in CD64+ macrophages and CD3+CD4+ T helper populations by 8-wks (to near baseline), compared to WT controls at 8-wks. IMC further supported these results via spatial visualization of monocyte/macrophage and T cell markers across the two strains post DMM. Further, t-SNE analysis of the 22-marker IMC multiplex identified differences in cell cluster populations within CD14 deficient synovium compared to WT, with decreases in two distinct cell populations containing several immune cell markers (CD45, Ly6C, Ly6G, CD56), and fibrosis markers (vimentin: VIM, tenascin C: TNC), and accompanied by an increased cell cluster expressing lining resident (CX3CR1) and general macrophage (F4/80) markers. As CD14 is commonly studied for TLR4-mediated inflammatory signaling, which can influence monocyte/macrophage phenotypic differentiation, it is possible that a global knockout of CD14 is mitigating this.[2] Future work will further identify cell types within differential clusters, their spatial localization within the membrane, and temporal changes with disease.

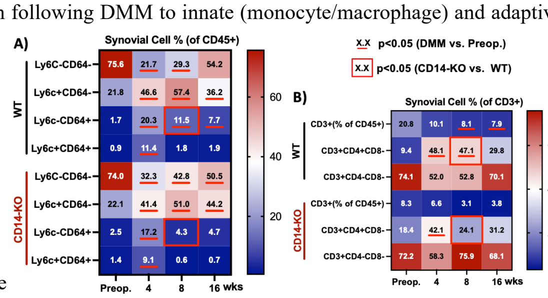

Figure 1: Flow cytometry analysis of synovial immune cell infiltration following DMM. (A) Heatmap of synovial CD45+ leukocyte (Ly6C-CD64-), monocyte (Ly6C+CD64-), and macrophage (Ly6C-CD64+, Ly6C+CD64+) cell populations (% of CD45+ synovial cells) and **(B)** T cell (CD3+, CD3+CD4-CD8-), and helper T Cell (CD3+CD4+CD8-) populations (% of CD3+ synovial cells). p<0.05 significance by Student's T-test comparing DMM vs. preop and CD14-KO vs. WT by two-way ANOVA.

SIGNIFICANCE: These results reveal that CD14 deficiency produces *distinct* immune cell clusters with distinguishable *spatial organization* within the synovium following injury, providing mechanistic support for how CD14 deficiency may be protective against PTOA-associated pathology and mobility dysfunction.

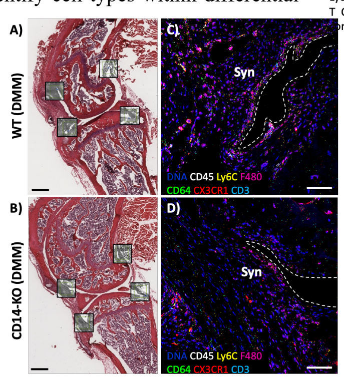

Figure 2: IMC staining of synovium post DMM. (A,B) H&E images of WT and CD14-KO knees **4wks post DMM**, with ROIs indicating IMC spatial protein analysis (grey inset). **(C,D)** Subset of select monocyte/macrophage (Ly6C, F480, CD64, CX3CR1) and T-cell (CD3) marker expression within synovial ROIs. Synovial lining = dashed white line. Scale bars = 50µm.

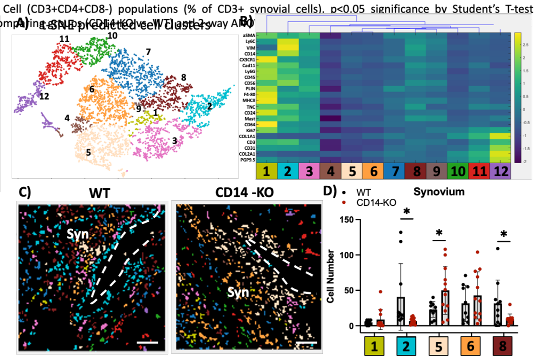

Figure 3: IMC analysis of synovial cell phenotypes post DMM. (A) Unique cell cluster analysis using t-SNE dimensionality reduction with IMC marker expression data. Data points represent individual cell masks from synovial ROIs across experimental groups. **(B)** Marker expression heatmap with cluster labels. **(C)** Single-cell unique phenotype cluster assignment within synovial ROIs. Synovial lining= white-dashed line. Scale bar=50µm. **(D)** Cell number analysis within clusters between WT and CD14-KO DMM synovial ROIs. *p<0.05.

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