Single Cell and Spatial Transcriptomics Reveal Immune Dysregulation and Fibrosis as a Cause for Delayed Healing in Recalcitrant Fractures

Matthew Patrick[1], Jaimo Ahn[2], Kurt Hankenson[2], and Ramkumar T. Annamalai[1]*
[1]University of Kentucky, Lexington, KY, [2]University of Michigan, Ann Arbor, MI
*rtiruvan@buffalo.edu

Disclosures: Matthew Patrick (N), Jaimo Ahn (N), Kurt Hankenson (N), Ramkumar T. Annamalai (N)

INTRODUCTION: Musculoskeletal injuries are a major cause of severe disability, with 5-15% of the 7 million fractures each year resulting in delayed or nonunion. Hypertrophic nonunions are the clinically predominant form which is caused due to high interfragmentary movement and tissue strain. Although acute inflammation is crucial for establishing the healing cascade, the increased interfragmentary movement can give rise to aberrant immune cell phenotypes that can cause fibrosis and compromised functional healing. However, the specific cellular pathways and mechanisms leading to disruptions in the healing cascades are poorly understood. Therefore, elucidating the strain-mediated effects on the inflammatory response in a fracture milieu can help develop immunomodulatory therapies to prevent nonunion. We have developed a murine delayed healing model that mimics the etiology of hypertrophic nonunion. Using single-cell RNA sequencing and spatial transcriptomics on callus tissue, we elucidate the dysregulated immune response leading to fibrosis in delayed-healing fractures.

METHODS: We randomly assigned 40 C57BL/6 mice in two groups (low and high strain) and created a transverse femoral fracture through blunt trauma. Then we stabilized the femurs in high and low-strain groups using hyperflexible Ni-Ti intramedullary nails with low (2.7 N/mm) and high stiffness (16.5 N/mm), respectively. We collected samples at different time points and analyzed them using histology, single cell RNA sequencing, and Visium spatial RNA sequencing. Fibrosis of the callus tissues was analyzed through Picro-Sirius red staining of the sections and imaging under polarized light. For single-cell analysis of the callus tissue, we sacrificed mice and isolated a 3 mm section of the fractured diaphysis containing the hematoma and the surrounding muscle tissue. The tissues were digested to make a single-cell suspension. 3' single-cell RNA libraries were prepared using a 10X genomics kit following the manufacturer's instructions. For spatial transcriptomics the femurs were isolated, decalcified, embedded with paraffin and cut into 5 µm sections. Sections were processed using a 10X genomics visium kit. The samples were sequenced on a NextSeq 2000 and ran through the CellRanger pipeline for demultiplexing, barcoding, and alignment to a reference genome. Further downstream analysis was done using the Seurat and R studio.

RESULTS SECTION: We used single-cell RNA sequencing and spatial transcriptomics to investigate the differences in cell populations, phenotypes, and intercellular communications between low- and high-strain fracture calluses over a 3-week period. We found that the resolution of proinflammatory signals and the elevation of pro-regenerative immune cell phenotypes were crucial for bone regeneration. We also discovered that macrophages played a key role in fibrosis and bone healing. High-strain macrophages showed a hyperactive M1-like phenotype with increased expression of genes such as Mrfta, while low-strain macrophages exhibited a downregulated M2-like phenotype with reduced expression of genes under high strain. Moreover, high-strain calluses had more fibroblasts and osteoclasts in the fibrotic core, while low-strain calluses had more osteoblasts near the fracture line. These cell populations and their intercellular communications were revealed by spatial transcriptomics and CellChat analysis. Additionally, we identified the enrichment of Spp1 and Tgfb pathways in high-strain calluses and Tnf pathway in low-strain calluses. These results suggest that macrophage polarization, fibroblast and osteoclast signaling, and pathway activation are differentially regulated by mechanical strain and affect the outcome of fracture healing.

We then investigated the role of macrophages in fibrosis by examining the expression of Lgals3, a marker of fibrotic macrophages, in high and low-strain calluses. We found that Lgals3 expression was higher in the high-strain callus, especially in regions with abundant macrophages and in the fibrotic core of the fracture callus. In contrast, the low-strain callus had similar Lgals3 expression, but it was distributed away from the fracture line. We also observed that high-strain macrophages expressed more Fabp4 and Pdgfrb, two genes associated with fibrosis, than low-strain macrophages. Fabp4 and Pdgfrb expression increased by 1.08-fold ($p < 0.0001$) and 0.368-fold ($p = 0.003$), respectively, in high-strain macrophages. These results indicate that Lgals3+ macrophages and their gene expression are involved in the fibrotic response in the high-strain callus.

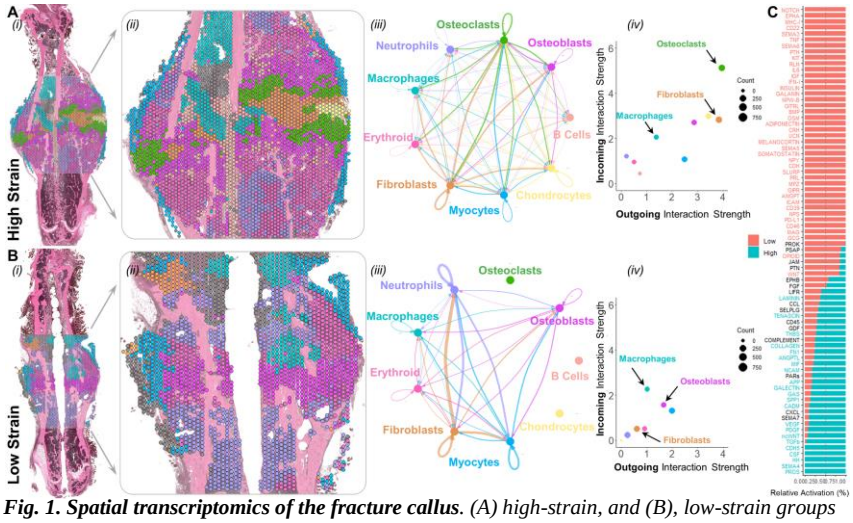

***Fig. 1. Spatial transcriptomics of the fracture callus**. (A) high-strain, and (B), low-strain groups at week 3 post-fracture: (i) H&E-stained femur; (ii) Zoomed-in image showing supervised annotation of cells based on known marker gene expression; (iii) plots displaying putative ligand-receptor interactions, with the width of lines representing the strength of the communication; and (iv) outgoing vs. incoming communications in each cell type. (C) relative enrichment of signaling pathways by information flow across cells in low and high groups.*

DISCUSSION: Our single-cell analysis of high and low-strain groups reveals a disruption in the macrophage inflammatory response at early time points under high-strain conditions. This disruption triggers a cascade of events, leading to aberrant macrophage phenotypes and a healing response that skews toward fibrosis. Spatial data further substantiates this profibrotic response, as evidenced by the increased presence of fibroblasts and osteoclasts in the high-strain callus. Moreover, our observations indicate a differential enrichment of signaling pathways in high and low strain conditions. Specifically, the SPP1 and Tgfb pathways, known to promote fibroblast recruitment and differentiation, were predominant in the high-strain callus. In contrast, the low-strain callus was enriched with the Tnf pathway, which is associated with a reduction in the fibroblast population. In our in-depth exploration of the role of macrophages in fibrosis, we discerned a marked accumulation of Lgals3+ macrophages within the central region of the high-strain callus. Notably, these macrophages were predominantly located in close proximity to fibrotic areas. This observation aligns with previous findings highlighting the critical role of $Lgals3^+$ macrophage populations and their interactions with fibroblasts in the fibrotic response, as evidenced in conditions such as muscular dystrophy. Interestingly, we noted upregulation of fibrosis-associated markers, including Fabp4, Pdgfrb, and Spp1, within these macrophages. This upregulation appears to stimulate the recruitment and differentiation of fibroblasts, culminating in an excess deposition of matrix tissue. This finding underscores the potential of these markers in modulating fibrotic responses and opens avenues for further investigation into their therapeutic implications.

SIGNIFICANCE/CLINICAL RELEVANCE: Our findings elucidate the mechanisms underlying the strain-induced immune dysregulation observed in persistent fractures. By shedding light on this complex process, we pave the way for the advancement of novel diagnostic tools and therapeutic interventions tailored to manage hypertrophic nonunions. This work underscores the potential of our research to make a significant impact on clinical practice and patient outcomes.