

Single-cell RNA-Sequencing of herniated human intervertebral discs reveal small populations of macrophages and fibroblasts as dominant drivers for the pro-inflammatory environment and fibrosis

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INTRODUCTION: Back and neck pain are major global healthcare challenges causing tremendous socioeconomic burden and costing >\$134.5B in annual US health care costs¹. Intervertebral disc (IVD) degeneration (IVDD) and herniation are strongly associated with back and neck disability. Disease modifying treatments are needed to address the extensive cellular and structural changes in the complex IVDD conditions. Improved understanding of cells involved in IVDD from back pain patients can give insights into pathophysiology and inform new treatments. Single-cell RNA-Sequencing (scRNA-Seq) with bioinformatics can decipher the heterogeneous cell populations and their functions. There remains little clarity on the cells responsible for propagating pro-inflammatory and painful processes in human herniated IVDs, even though scRNA-Seq in human IVDs has been performed and identified IVD cells in healthy and degenerated stages²⁻⁴. Furthermore, scRNA-Seq is a new and dynamic field requiring replication, validation, and integration of multiple studies to better understand roles of human IVD cells. This study used scRNA-Seq on human IVDs collected from subjects undergoing anterior disc fusion (ADF) surgery for painful IVDD and herniation and identified cell populations responsible for pain-driving and pro-inflammatory processes.

METHODS: IVD herniation tissue from 3 subjects (1 male, 2 female) from ADF surgery were collected under an IRB-approved protocol and digested for 12 hours (400U/mL collagenase II). Single-cell suspensions were processed using the 10X Genomics Chromium 3' Gene Expression V3 Kit and sequenced using an Illumina S1 NovaSeq chip. Cell Ranger software v7.1 mapped reads to the human GRCh38-2020-A reference genome. Data processing via Seurat v4.3 included quality control filtering, normalization, dataset integration, and unsupervised graph-based clustering. Cell clusters were visualized with uniform manifold approximation and projection (UMAP) and annotated with UniCell, a semi-automated deep learning annotation model comprising 28M annotated cells from 898 studies⁵. Gene set enrichment analysis (GSEA) through EnrichR identified functions of clusters using gene ontology (GO) terms and MSigDB Hallmark 2020 pathways. Cytokines in ADF of 9 subjects (6 male, 3 female) were measured with multiplex assay on conditioned media from herniated IVD tissues⁶.

RESULTS: IVDD tissues from 3 ADF subjects enabled isolation and sequencing of 13,259 cells, which were divided into 14 clusters (Fig. 1A). UniCell annotated specific cell clusters based on their probability of association with previously reported cell types like chondrocytes (Fig. 1B). The close proximity of all IVD populations and overlapping gene signatures suggests limited phenotypic distinction between annulus fibrosus (AF) and nucleus pulposus (NP) cells. Furthermore, markers related to vascularization and sensitization, especially VEGFA and NGF, were expressed broadly in most IVD cells (Fig 1C, D). Cluster 6 showed high expression of pain-related markers BDNF, TAC1 (Substance P) and CALCA (CGRP) that had faint expression by other clusters (Fig 1D). Cluster 11 was a distinct fibroblast population expressing several collagens and TGF- β 2 (Fig. 2A), and GSEA revealed it to have involvement in vascularization and fibrotic remodeling (Fig 2B). Multiplex protein assay of herniated IVD conditioned media identified many pro-inflammatory cytokines and chemokines including IL-6, TNF α and MCPs (Fig. 3A). Importantly, Cluster 13 alone expressed the majority of these cytokines (Fig 3B) and was annotated as macrophages by UniCell (Fig 3C). GSEA showed Cluster 13 was enriched for TNF α signaling and inflammatory responses (Fig 3D), and volcano plot (not shown) confirmed upregulated differentially expressed genes IL1B, CCL3 and CCL4 supporting their role in pro-inflammatory conditions.

DISCUSSION: IVD tissues from spine surgery subjects with painful IVDD and herniation were collected and analyzed with scRNA-Seq to identify 14 cell populations and distinguish 3 novel cell clusters found to play roles in driving pain and pro-inflammatory responses. The majority of AF and NP clusters had relatively little distinction in expression patterns, and most cells expressed VEGF and NGF, consistent with prior studies showing IVDD cells can promote angiogenesis and neurite growth⁷. Fibroblast Cluster 11 expressed genes implicated in vascularization, matrix remodeling and inflammation, and suggesting a role in fibrotic scarring⁸. Fibroblast Cluster 11 also had close proximity on the UMAP to macrophage Cluster 13 further supporting involvement in scarring. Importantly, macrophage Cluster 13 expressed the majority of pro-inflammatory cytokines found within the herniated tissue, which emphasizes their importance for IVDD progression and provides a potential target for therapies. Macrophages were present in surprisingly small numbers in this study, yet macrophages were shown to interact with NP cells in discectomy NP cells⁹, supporting the concept that a small number of cells can play pivotal roles in orchestrating the responses of much larger cell populations. Cluster 6 expressed highest neuropeptide levels suggesting a role in pain. UniCell identified Cluster 6 as lymphocytic and also identified clusters with characteristics of T cells and fat stem cells that warrant further analysis to elucidate their roles in IVD-specific contexts. Ongoing studies are integrating this dataset with healthy IVD cell data to clarify how IVDD cells differ from healthy IVD cells to inform pathobiology and identify therapeutic targets.

SIGNIFICANCE: Small populations of fibroblasts and macrophages in IVD herniation tissue were identified that may play a role orchestrating painful processes and propagating IVDD.

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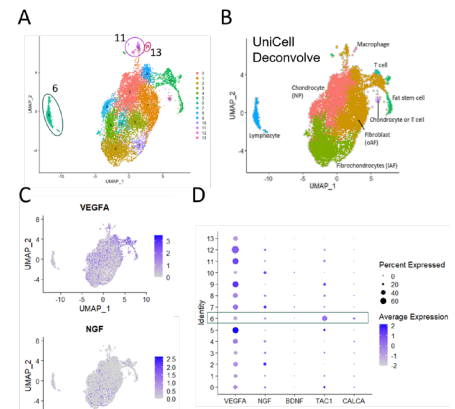


Fig. 1: scRNA-Seq of human IVDD tissue identified (A) 14 clusters (B) annotated using UniCell Deconvolve. (C) VEGF and NGF were expressed throughout all cells, while (D) TAC1 and CALCA were mostly expressed in cluster 6.

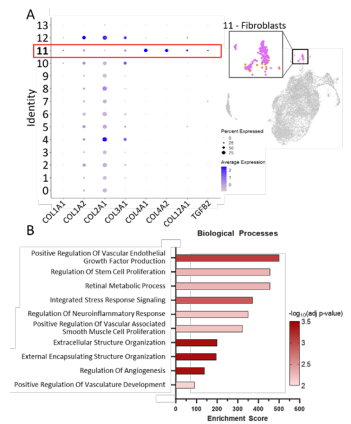


Fig 2: (A) Cluster 11 was identified as Fibroblasts using DotPlot and (B) related to angiogenesis using GO-terms.

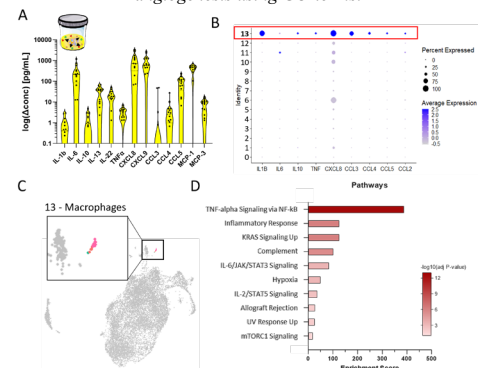


Fig 3: Cluster 13 macrophages were involved in IVD inflammation. (A) Pro-inflammatory cytokines in IVDD conditioned media were (B) expressed in cluster 13, (C) identified to be macrophages. (D) GSEA showed cluster 13 was enriched for TNF α signaling and inflammation.