

# Aging Intervertebral Disc Reveals Upregulation in Immune Pathways and Activated Immune Cell Populations

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**Disclosures:** The authors have nothing to disclose.

**INTRODUCTION:** Low back pain (LBP), one of the most disabling conditions worldwide, is associated with intervertebral disc (IVD) degeneration (IDD). IDD is a multifactorial condition that progresses with age. Regardless of the cause, inflammation is an omnipresent contributor to IDD. The IVD is a composite connective tissue structure made up of three interdependent tissues: central nucleus pulposus (NP), encapsulating annulus fibrosus (AF), and cartilage endplate. Murine aging has been used as a model to study age-associated IVD changes, with higher prevalence of morphological IDD observed at  $\geq 14$  months of age<sup>1</sup>. Importantly, age-associated changes in different IVD tissue types, and the relationship between the effects of aging and inflammation on IDD remains unknown. The objective of the current study was to use the next generation sequencing (bulk RNA sequencing, and single cell RNA sequencing) to assess transcriptomic profiles in the IVD tissue types and immune cell population in aging mice.

**METHODS:** Bulk RNA Sequencing: NP and AF tissues from 6 lumbar IVDs were carefully dissected from C57BL/6 mice at 6, 12, 16, and 20+ months-of-age (M= months-of-age; n=8 for 6M, 12M, 16M; n=2 for 20+M). For each animal, IVD tissue types were pooled and total RNA was extracted (average RIN = 8.6). Bulk RNA sequencing and initial data processing (read alignment) were performed by the Genome Center. For each sample, STRPOLYA kit was used for library preparations and sequenced on Illumina NovaSeq 6000. Gene count data were normalized using the variance stabilizing function using DESeq2. Differentially expressed genes (DEGs) were identified based on  $|\log_2\text{FoldChange}| \geq 1.5$  and adjusted p value (padj.)  $< 0.05$ . Analyses were performed in R to identify differences within NP or AF tissues at various age compared to 6M group (control). In order to explore the up- or down-regulated biological processes and pathways between age groups, gene set enrichment analysis (GSEA) was performed using clusterProfiler package and the Molecular Signatures Database (MSigDB). Enriched biological processes were evaluated using C5 gene ontology (GO) database and pathway enrichment analysis was performed using C2 pathway database. Single Cell RNA Sequencing (scRNA-Seq): 3 lumbar IVDs (L4-S1) were dissected from C57BL/6 mice at ages listed above (n=2/age). Isolated IVD tissues were sequentially digested with 1 mg/mL of pronase for 30 min and 2 mg/mL of type II collagenase for 1.5 hr. Each sample was FAC sorted for viable cells, and scRNA-Seq was performed using 10x Genomics Chromium at the Columbia Genome Center. Raw sequencing data was aligned and converted to expression counts using Cell Ranger (10X Genomics). For all samples, cells with  $< 15\%$  mitochondrial features and gene counts  $> 200$  were included to be of sufficient quality to include in the analysis. In order to identify cell populations, dimensionality of the normalized data was reduced by principal component analysis, non-linear dimensionality reduction was performed using uniform manifold approximation and projection (UMAP), and cell cluster identification was performed using STRING database. All clustering analysis and data visualization were performed using Seurat package in R.

**RESULTS:** Bulk RNA Sequencing: We identified 630, 2754, 388 DEGs in NP tissues and 220, 712, 231 DEGs in AF tissues at 12M, 16M and 20+M compared to 6M group, respectively. Among them, 104 NP genes and 32 AF genes were differentially expressed in all age groups. In both NP and AF tissues, commonly upregulated genes included immunoglobulin subunits and cell surface proteins associated with B cell activation (*Ifitm1* in NP, *Fcrl5* in AF; **Fig. 1A,B**). Notably, NP cell markers (*Krt19*, *Krt20*, *Gpc3*), survival gene (*Ddit4l*), and Wnt signaling genes (*Wnt5b*, *Wnt9a*, *Sostdc1*) in the NP and matrix related genes (*Col9a1*, *Col10a1*, *Susd5*), survival genes (*Ddit4l*, *C1qmf3*), and Wnt signaling genes (*Frzb*, *Sostdc1*) in the AF showed decreasing expression with age (**Fig. 1A,B**). In both NP and AF, GSEA-GO analysis showed B cell activation and phagocytosis recognition as enriched biological processes in all age groups, suggesting potential role of macrophages and B cells in IVD aging (**Fig. 1A,B**). scRNA-Seq: After applying data filtration, total of 36,480 cells were analyzed in the full aging dataset, with median of 1,746 genes detected per cell. Analysis of IVD single cell transcriptome resolved 9 unique clusters, whose identity (Neutrophil, B Cells 1& 2, Macrophage, Monocyte, T Cell, IVD Cells and Granulocytes) was defined based on transcriptome profile (**Fig. 1E**). Longitudinal assessment of cell count for each cluster showed age-dependent increase in B Cell 1 and macrophage clusters (**Fig. 1F**). Given that B cell activation and phagocytosis were upregulated in bulk RNA-Seq data with age, B Cell 1 and macrophage clusters were analyzed for expression levels of activated B cells and macrophage polarization markers. Sub-clustering analysis revealed that B Cell 1 cluster showed age-dependent increase in the expression of activated mature B cell markers, which peaked at 12M (*Fcmlr*, *Ms4a1*, *Cd83*; **Fig. 1G**). Furthermore, macrophage cluster showed increased expression of M1 macrophage markers (*Cd38*, *Serpine2*, *Tlr4*) at 16M and 20+M, while low expression of M2 markers (*Mrc1*, *Arg1*, *Il10*) were detected in all age groups (**Fig. 1H**).

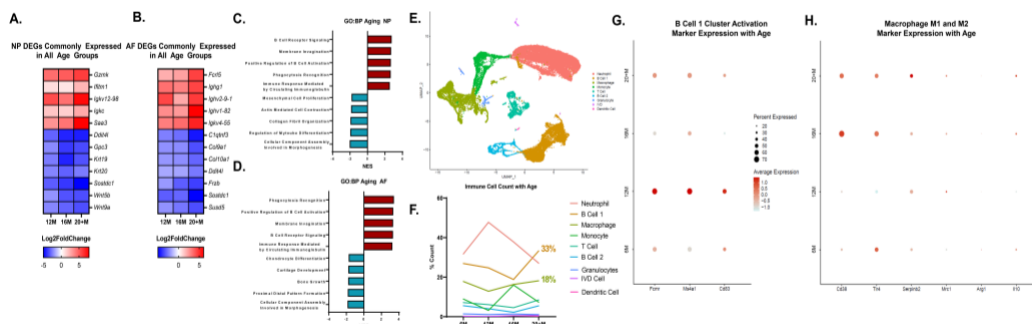
**DISCUSSION:** Decreased expression of notochordal cell markers and Wnt signaling genes in the NP may suggest age-associated decrease in notochordal-like cells or phenotypic shift. Interestingly, a survival gene, *Ddit4l* was commonly downregulated in both NP and AF with age. REDD1 (encoded by *Ddit4l*) is a hypoxia-induced mTOR inhibitor and a positive regulator of autophagy<sup>2</sup>. In human and mouse articular chondrocytes, REDD1 expression decreases with age and osteoarthritis<sup>2</sup>. Decreased REDD1 may suggest increased mTOR activity and autophagy dysregulation contributing to IDD pathophysiology. Notably, both NP and AF cells showed age-dependent increase in genes associated with B cell activation. Longitudinal analysis of B cell cluster showed age-dependent expression and number of activated B cells. Previous scRNA-Seq analysis of caudal IVDs of rat puncture injury model also reported higher percentages of B Cell marker-expressing cells in puncture group, suggesting a potential role of B cells in mediating both injury-induced and age-associated IDD<sup>3</sup>. Another activator of B cells is macrophages. Specifically, phagocytic macrophages serve as antigen presenting cells for B cell activation<sup>4</sup>. Given that our analysis showed increased M1 macrophage marker expression with age, the crosstalk between IVD, M1 macrophages, and B cells may be potential immune pathways contributing to the IDD.

**SIGNIFICANCE/CLINICAL RELEVANCE:** The current study provides a transcriptomic map of age-associated changes in NP and AF, and dynamic profiles of immune cells during IVD aging, which may help identify cellular and molecular targets for potential therapeutic treatments of IDD.

**REFERENCES:** [1] Ohnishi, T., et al., (2018). *JOR*, 36(1), pp.224-232. [2] Alvarez-Garcia, et al., (2016). *Osteoarthr. Cartil.*, 24(9), pp.1639-1647. [3] Rohanifar, M., et al., (2022). *Applied Sciences*, 12(16), p.8244. [4] Harvey, B.P., et al., (2007). *Eur. J. Immunol.*, 37(7), pp.1739-1751.

**ACKNOWLEDGEMENTS:** Studies were funded in part by NIH R01AR069668, R01AR077760, and R21AR080516.

## IMAGES AND TABLES:



**Figure 1.** Heat map of commonly up- and down-regulated genes in NP (A) and AF (B) tissues in all age groups. Top 5 enriched biological pathways of NP (C) and AF (D) at 20+M compared to 6M. (E) UMAP cluster analysis showing 9 distinct cell clusters. (F) Changes in cell count of each cluster with age. (G, H) Dot plot showing marker gene expression for activated B cells (G) and macrophages (H) with age.