

Effects of Age on Immune Cell Modulated Annulus Fibrosus Repair in Mouse Intervertebral Disc Herniation Injury

Timothy D Jacobsen¹, Timothy Hoang¹, Emma Cavendish¹, Damien M Laudier¹, Woojin M Han¹, Alice H Huang², James C Iatridis¹

¹Mount Sinai School of Medicine, New York, NY. ²Columbia University, New York, NY.

Timothy.Jacobsen@mssm.edu

Disclosures: The authors have nothing to disclose

Introduction: Back pain and disability are associated with intervertebral disc (IVD) degeneration and herniation; these conditions increase with age, with disability peaking around 50-70 years of age [1]. Aging of the IVD is known to involve changes in cells, extracellular matrix composition, structure, and function [2]. Young neonatal mice IVDs heal from large annulus fibrosus (AF) injuries with functional regeneration, and this regenerative capacity is lost by skeletal maturity [3]. Regenerative responses in neonatal mice tendons are dependent on the presence and involvement of immune cells, in particular macrophages and regulatory T cells [4], suggesting cellular changes are critical to regenerative healing. There are no studies characterizing how aging affects the IVD healing response and immune cell involvement using preclinical models at ages when back pain is high. This study determined effects of age on: (1) immune cell populations in naïve IVDs; (2) responses of IVD immune cells to injury; and (3) AF reparative responses. A large AF injury with herniation, in a mouse model, was created and evaluated 14 days post-injury (dpi) for active immune-modulated healing responses.

Methods: Mice were used at three ages, Neonatal (14 days; regenerative), Adult (4 months; skeletal maturity), and Aged (1 year; equivalent to human age with peak back pain disability). AF-herniation injury was created in mouse coccygeal IVDs using 26- or 30-gauge needle to produce an injury ~80% of IVD height and 50% of IVD depth; adjacent IVDs were uninjured controls. *Single-Cell RNA Sequencing (scRNA-Seq):* Cells from 6 mice were isolated from whole naïve coccygeal IVDs and pooled, processed using the 10X Genomics Chromium 3' Kit, and sequenced using an Illumina S1 NovaSeq chip. Cell Ranger software mapped reads to mouse mm10-2020-A reference genome. Data processing via Seurat included quality control filtering, normalization, and unsupervised clustering. Cell clusters were visualized with uniform manifold approximation and projection (UMAP). Differential gene expression analysis identified gene markers for individual clusters and canonical markers facilitated annotation of clusters. *Immunohistochemistry (IHC):* IVD segments were collected 14dpi, fixed, decalcified, paraffin embedded, and sectioned. IHC for CD68 assessed macrophage presence. Sections were stained for collagen and glycosaminoglycan (GAG) content using Picrosirius Red-Alcanin Blue (PR-AB); and investigated for collagen alignment with polarized light.

Results: Single-cell RNA sequencing revealed distinct cell clusters at each age including expected native IVD cells: AF, nucleus pulpous (NP), and notochordal clusters (Fig. 1). Distinct immune cell populations were also observed in naïve discs at all ages and included macrophage markers: *Cd68*, *Lyz2*, *Mpeg1*. Macrophages (investigated using CD68 IHC) were present in endplates and outer AF of control (un-punctured) neonate IVDs, with macrophage presence diminishing with age (Fig. 2A). Control un-injured IVDs had decreased NP cellularity as well as widening and buckling of AF lamellae with age (Fig. 2B,C). Following injury there was a noticeable macrophage immune response localized to the outer AF region (Fig. 3A). Adult and Aged IVDs had a cellular/fibrotic cap surrounding the injury site which was less robust in Aged than Adult mice and absent in Neonates. Neonates exhibited a regenerative response characterized by infiltration of cellularized repair tissue within the AF puncture tract, retained organization of AF layers adjacent to the injury site, and better retention of GAG staining within the NP compared to adult or aged mice (Fig. 3B,C).

Discussion: The scRNA-Seq results on mice IVDs with aging show distinct immune cell populations in naïve IVDs of all ages, contrasting the long-held understanding that naïve IVDs are immune privileged [5]. IHC for CD68 confirmed the presence of macrophages in uninjured IVD that were localized to outer AF layers and endplates, that decreased with aging. The presence of immune cells in the outer AF suggests they may play a role in AF healing response to large herniation-type injury and differences in immune response to AF puncture injury were indeed observed with aging. The Adult and Aged IVDs formed an immune cell rich cellular/fibrotic cap surrounding the injury site which was absent in young Neonates. This fibrotic cap and healing response was similar but less robust in Aged compared to Adult mice indicating a limited response to injury. Increased presence of macrophages in Neonatal IVDs resulted in a more controlled immune response with more organized AF structural healing. Adult and Aged IVDs had fewer immune cells in the naïve condition than Neonatal and also resulted in healing with dysregulated immune cell recruitment, a robust fibrous cap, relatively little AF repair tissue, and a less organized AF structural repair. A controlled type 2 macrophage response was necessary for a regenerative response in neonatal mouse tendons, and this was facilitated by the presence of T regulatory cells [4]. We will therefore investigate T regulatory cells in this model as next steps. We conclude that aging resulted in 3 IVD healing responses to a large AF-herniation injury: Neonates had “regenerative” healing with controlled immune response; Adults had “robust fibrotic” healing with dysregulated immune response; Aged had a “limited fibrotic” healing with dysregulated immune response.

Significance: scRNA-Seq analyses identified immune cell populations in naïve uninjured IVDs, challenging the assumption that IVDs are immune privileged tissues. Aging significantly affected the immune cell modulated IVD healing response, and improved understanding of these immune responses to injury may inform future therapies that better promote a more “regenerative” IVD repair responses as seen in neonatal mice in this study.

References: [1]Hartvigsen+,Lancet,2018. [2]Silwal+,Biomolecules,2023. [3]Torre+,FASEB J,2018. [4]Arvind+,bioRxiv,2021. [5]Sun+,Int J Med Sci,2020.

Acknowledgements: Supported by NIH R01AR080096.

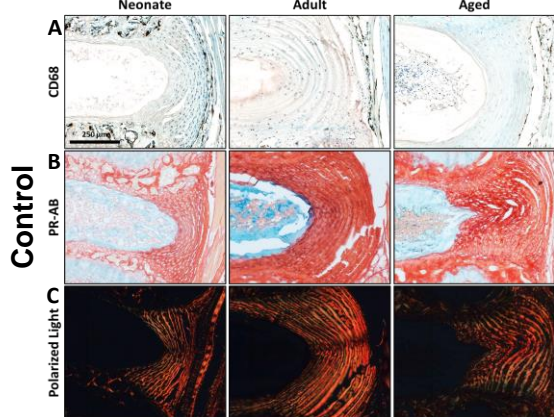
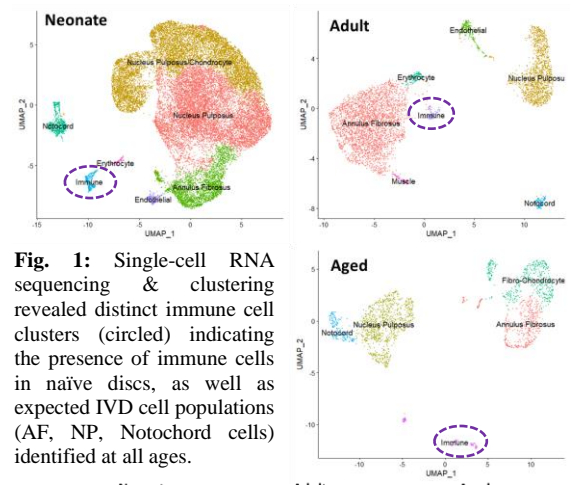


Fig. 2: CD68 macrophage staining (A), PR-AB staining for collagen and GAG (B) and polarized light imaging for collagen organization (C) of uninjured control discs at three ages.

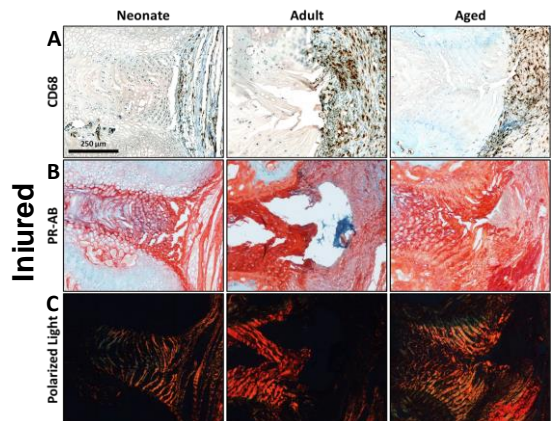


Fig. 3: CD68 macrophage staining (A), PR-AB staining for collagen and GAG (B) and polarized light imaging for collagen organization (C) 14 days post disc injury at three ages.