Resident Intervertebral Disc Neutrophils are Lost with Age and Implicated in Neonatal Regeneration in Mice

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Introduction: Back pain and disability are associated with intervertebral disc (IVD) degeneration and herniation, and all these conditions increase with age [1]. IVD aging involves changes in cells, extracellular matrix composition, structure, and function [2]. Aging from neonatal growth to skeletal maturity also changes IVD healing responses from functional regeneration to fibrosis [3]. Last year we determined distinct macrophage responses are involved in the age-related shift from regenerative annulus fibrosus (AF) healing in neonatal mice to robust and limited fibrotic healing in adult and aged mice, respectively [4]. Regenerative tendon healing similarly occurs in neonatal mice and is immune cell-dependent with T-cells implicated [5]. Immune cell responses are therefore implicated in regenerative and fibrotic healing to motivate a deeper investigation of immune cells in IVD aging and injury. This study determined effects of age on: (1) immune cell populations in Naïve IVDs; (2) immune cell changes in Injured IVD using an *in vivo* mouse model. We hypothesized IVD immune cell populations change with age and injury, and distinct neutrophil phenotypes are responsible for the shift from regenerative to fibrotic AF healing.

Methods: Naïve and Injured mouse coccygeal IVDs were evaluated using single cell RNA sequencing (scRNA-seq) and immunohistochemistry for 4 age groups: 0.5 Month (Neonatal mice capable of regenerative healing), 4 Month (Skeletal maturity), 12 Month (equivalent peak human back pain disability) and 24 Month (equivalent peak human back pain prevalence). AF-herniation injury (~80% IVD height & 50% IVD depth) was created using 26- or 30-gauge needle. AF injury samples were evaluated 14 days post-injury (dpi) while immune-modulated healing responses remain prominent. For scRNA-Seq, cells from 6 mice (Naïve or Injured @ 14dpi) were isolated from whole coccygeal IVDs (6 IVDs per mouse) and pooled, processed with 10X Genomics Chromium 3' Kit, and sequenced with 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, integration, and unsupervised clustering. Data from all ages in Naïve and Injured IVDs were integrated, clustered and visualized using uniform manifold approximation and projection (UMAP). Canonical markers facilitated annotation of clusters. A distinct immune cell subset was identified, and re-clustered to further characterize immune cell phenotypes. Populations of Neutrophils, Macrophages, T-cells, and B-cells were identified (Neutrophils: *S100A8, Ly6g*; Macrophages: *Cd68, Mpeg1*; T-Cells: *Cd3d, Trbc2*; B Cells: *Ighm, Ptprcap*). Injured and control IVDs were also evaluated histologically and with immunohistochemistry using neutrophil marker Ly6G (n=4 mice per age).

Results: Cell clusters identified included native IVD cells (AF, nucleus pulposus, and notochordal cells), chondrocytes, immune cells, erythrocytes and endothelial cells (Fig. 1A). The immune cell distribution (Fig. 1B) in Naïve IVDs changed with aging showing many neutrophils in 0.5 Month mice (capable of regeneratively healing) and few neutrophils in 4, 12, or 24 Month mice (capable of fibrotic healing) (Fig. 1C). Immunohistochemistry for neutrophil marker Ly6G similarly decreased with age (data not shown), supporting scRNA-seq results. Injury altered IVD immune cell populations across the lifespan; most notably neutrophils decreased in 0.5 Month Injured and increased in 12 Month Injured (Fig. 2A). Neutrophils in 0.5 Month Naïve IVDs had distinct neutrophil phenotypic markers (Fig. 2B) from 12 Month Injured IVDs while neutrophils in 12 Month Injured IVD were more pro-inflammatory (Fig. 2C).

Discussion: scRNA-seq in mice IVDs showed immune cells are resident in Naïve IVDs across the lifespan (0.5– 24 Months), providing more evidence to refute the long-held belief that Naïve IVDs are immune privileged [6]. Immune cell sub-clustering analysis demonstrated the novel finding that IVD immune cells change with age and injury. Interestingly regeneratively healing neonatal (0.5 Month) mice IVDs had a large population of resident neutrophils in Naïve IVDs that was notably absent at all other later ages, suggesting this neutrophil population could play a role in the robust neonatal mouse IVD healing responses. We further determined that AF injury depleted this resident neutrophil population in 0.5 Month mice which may be due to the loss of neutrophils or infiltration of other immune cells. Interestingly, a population of recruited neutrophils appeared in 12 Month Injured IVDs as infiltrating immune cells. The resident neutrophil population in 0.5 Month Naïve was phenotypically distinct from the recruited population in 12 Month Injured expressing a less mature neutrophil phenotype (i.e., higher *Rpl12* expression) and less pro-inflammatory (i.e., less *Il1b* & *Tnf* expression) state. Future studies will block these neutrophils to confirm their mechanistic role. Further, the notably small numbers of neutrophils at 4 and 24 Months in Naïve and Injured IVDs contrasts their presence in 0.5 and 12 Months suggesting additional inflammatory cell changes occur with aging that warrant further investigation. We conclude that IVD immune cell populations change with age and injury, and that neutrophils may play a role in the shift from IVD regenerative healing in neonates to fibrotic healing in aged mice.

Significance: Immune cell populations are present in Naïve IVDs challenging the assumption that uninjured IVDs are immune privileged. The immature resident neutrophils identified in 0.5 Month IVDs could contribute to neonatal regenerative AF healing and contrasts immune cell profiles of mature and aged IVDs that are known to heal poorly, and may point to immunotherapies to enhance future IVD repair strategies.

References: [1] Hartvigsen+, Lancet,2018. [2] Silwal+, Biomolecules,2023. [3] Torre+, FASEB J,2018. [4] Jacobsen+, Trans ORS 2024; [5] Arvind+, bioRxiv,2021. [6] Sun+, Int J Med Sci,2020.

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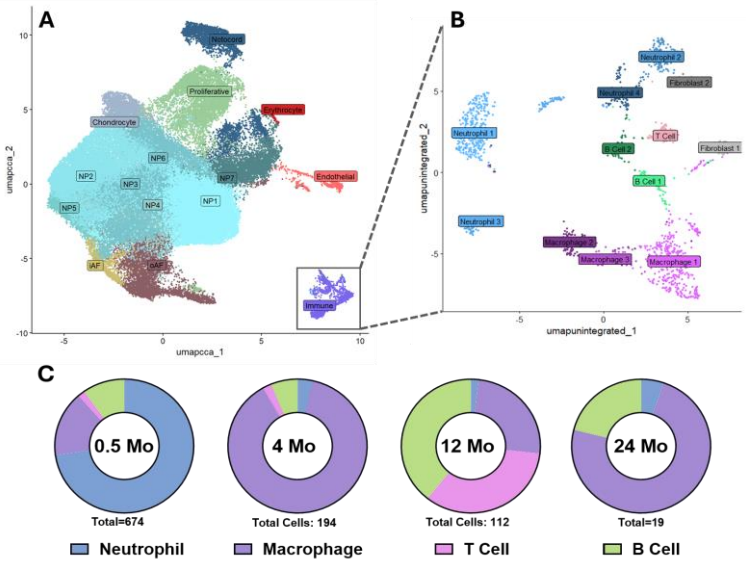

Figure 1 scRNA-Seq of Naïve and Injured IVDs identified (A) immune and IVD cell clusters. (B) Neutrophils, Macrophages, T-Cells and B-Cells were identified in the immune cell subset. (C) Age shifted immune cell distribution in Naïve IVDs with Neutrophils substantially reduced after 0.5Mo

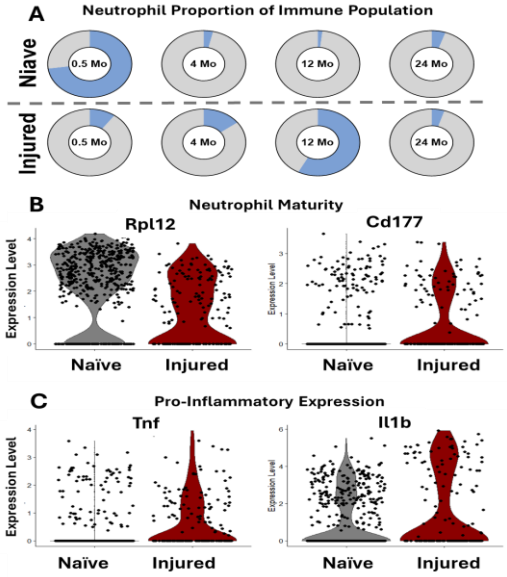

Figure 2 (A) Proportion of Neutrophils from scRNA-seq decrease with Injury in 0.5 Mo mice but increased with injury in 12 Mo mice. Resident neutrophils in 0.5 Mo Naïve are (B) phenotypically immature, and (C) less proinflammatory than recruited neutrophils in 12Mo Injured.