# Distinct Neutrophil Populations Support Regenerative Healing In Neonatal Mice

Timothy D. Jacobsen[1*], Timothy Hoang[1], James Hong[1], Levon Rodriguez[1], Irina Heggli[1], James C. Iatridis[1]
[1]Ichan School of Medicine at Mount Sinai, New York, NY *Timothy.Jacobsen@mssm.edu

**Disclosures:** The authors have no potential conflicts to this work.

**Introduction:** Back pain and disability are associated with intervertebral disc (IVD) degeneration and herniation, and these conditions increase with age [1]. Aging involves changes to IVD cells, extracellular matrix composition, structure, and function that impact healing [2]. In mice, IVDs with a critically-sized herniation-type injury healed regeneratively in neonates and fibrotically in adults [3]. This transition from regenerative IVD healing to fibrosis occurs between postnatal day p14 and p28, with bulk-RNA-seq analyses implicating changes to the IVD immune cell response [4]. Last year, we determined that resident IVD neutrophils are lost with age and implicated in neonatal mouse IVD regeneration [5]. This year, we test the hypotheses that IVD neutrophil phenotypes shift with aging and injury, and play a mechanistic role in IVD healing. This study aims to: (1) determine effects of age and injury on immune cell sub-populations; (2) investigate changes in neutrophil phenotype between regenerative and nonregenerative ages; (3) determine mechanistic role of neutrophils in regenerative IVD healing by knocking them out prior to injury in neonatal mice.

**Methods:** *single cell RNA sequencing (scRNA-seq):* Naïve and Injured mouse coccygeal IVDs were analyzed at 4 age groups: 0.5 Month (0.5 Mo, neonatal mice capable of regenerative healing), 4 Mo (skeletal maturity, young adult), 12 Mo (adult) and 24 Mo (aged, peak back pain prevalence). Injury involved a critically-sized AF-herniation created using 26- or 30-gauge needle (scaled with age to adjust for IVD size). IVDs were dissected and analyzed at 14 days post-injury (dpi) when immune-modulated healing responses are prominent and healing patterns are predictive of steady-state responses. For each group, cells were isolated and pooled from 6 mice with 6 IVDs per mouse. Data from all samples were integrated, clustered and visualized using uniform manifold approximation and projection (UMAP). A distinct immune cell subset was identified, and re-clustered to further characterize immune cell phenotypes. *Flow Cytometry:* Cells were isolated from naïve coccygeal IVDs of 0.5 Month (regenerative) and 1 Month (non-regenerative) mice and stained for Cd45 (Immune Marker), Ly6G (Neutrophil Marker) and CD11b (Activation Marker). Cells for flow cytometry were gated for immune cells (Cd45+) then for neutrophil maturity (Ly6G Hi/Low) and activation state (CD11b +/-). *Neutrophil Depletion:* Neutrophils were depleted from neonatal mice by intraperitoneal injection of a neutrophil depleting αLy6G/Ly6C antibody (BioXCell), depletion was confirmed using Ly6G staining in mouse spleen. Change in disc height index (DHI) was measured in uninjured and injured coccygeal IVDs 14dpi in both control and neutrophil depleted mice using Faxitron x-ray. Paraffin histology with H&E and PR-AB staining were used to investigate IVD morphology.

**Results:** Annotation of scRNA-seq using canonical markers identified a distinct immune cluster within cell isolated from the IVD, with sub-clusters of neutrophils, macrophages, T Cells and B Cells (Figure 1A). Neutrophils decreased with age from regenerative (0.5 Mo) to non-regenerative ages (4,12,24 Mo), while neutrophil infiltration was observed following injury in older ages (Figure 1B). Four distinct neutrophil sub-clusters were identified and neutrophils from naïve neonatal IVDs were predominantly Neutrophil 1 (Neut1) and Neut2 while aged injured samples were mostly Neut3 and Neut4 was distributed across age and injury state (Figure 1C & 1D). Neutrophil clusters had distinct phenotypic expression with Neut1 and Neut2 being less mature and expressing higher levels of cysteine protease inhibitors (Figure 2A). Flow cytometry confirmed neutrophil populations from regenerative ages (0.5 Mo) were phenotypical distinct from non-regenerative ages (1 Mo) showing higher proportions of immature (Ly6G Lo) activated (Cd11b +) neutrophils with an increase in neutrophil maturity (Figures 2B & 2C). Injury in neutrophil depleted 0.5 Mo neonates caused inferior healing from injury with IVD compaction, decreased cellularity, and greater glycosaminoglycan loss (Figure 3A) as well as significantly decreased disc height index (DHI, Figure 3B).

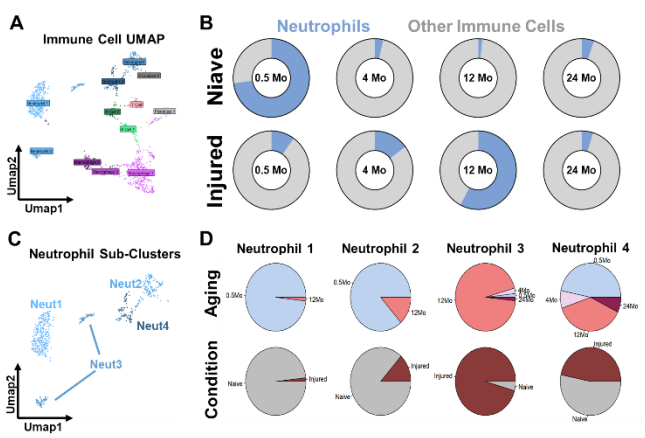


**Figure 1**: Immune cell sub-clustering of scRNA-Seq in mouse coccygeal IVDs identified (A) distinct immune cells populations that were (B) predominantly neutrophils in naïve neonates and increased with injury in adult and aged IVDs. (C) Neutrophil (Neut) sub-clusters on UMAPS showed (D) Neut1 & Neut2 clusters dominant in naïve neonates and Neut3 dominant in injured adult IVDs.

**Discussion:** This study identified distinct neutrophil populations in naïve and injured mouse coccygeal IVD and determined that neutrophil populations shift with agie and injury. Results therefore support and advance recent studies identifying that neutrophils are an important AF cell response to IVD injury [6]. ScRNA-Seq and flow cytometry determined neutrophils present in neonatal (0.5 Mo) IVDs exhibited an immature phenotype compared to neutrophils present in adult and aged IVDs, with a transition to mature neutrophils occurring as young as 1 month. Result point to immature neutrophils playing a mechanistic role in regenerative IVD healing since neutrophil inhibition in neonates worsened IVD healing and since neutrophil populations 1 and 2 were nearly absent at later ages and following injury. In contrast, neutrophil population 3 was dominant in aged and injured IVDs and exhibited the highest neutrophil maturation score and least amount of cysteine protease inhibition. Cysteine proteases include Cathepsins and Caspases which play roles in cell senescence, apoptosis, the complement cascade, and terminal complex formation. As such Cysteine protease inhibition could play a role enhancing neonatal IVD regenerative healing, while its absence may impair adult IVD healing. We conclude that neutrophils are phenotypically distinct in naïve neonatal IVDs and injured mature IVDs, and contribute to neonatal regenerative healing capacity. Future characterizations and gain of function experiments will be important to further elucidate functional roles of the distinct neutrophil populations identified in this study and determine how to translate this knowledge into immune modulating therapies for IVD repair.

**Significance:** A resident neutrophil population was described in neonatal IVDs that is phenotypically distinct differing from recruited neutrophils in injured IVDs at older ages. Neutrophil knockout showed neutrophils contribute to AF regenerative healing implicating suggesting immune modulating therapies is an important area for IVD repair research.

**References:** [1] Hartvigsen+ Lancet, 2018. [2] Silwal+ Biomolecules, 2023. [3] Torre+ FASEB, 2018. [4] D'Erminio+ iScience, 2024. [5] Jacobsen+ Trans ORS, 2025. [6] Clayton+ Exp Cell Res, 2025.

**Acknowledgements:** Supported by NIH R01AR080096.

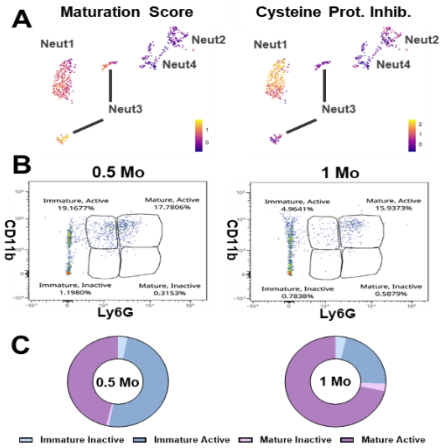


**Figure 2:** Neutrophil sub-clusters Neut1 & Neut2 were less mature and had higher Cysteine protease inhibition by scRNA-Seq. (B) flow cytometry showed (C) neutrophils in naïve IVDs became more mature as early as 1 month of age.

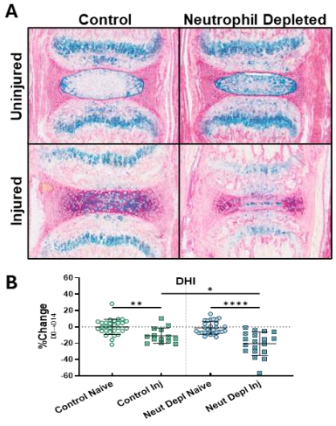


**Figure 3:** Neutrophil-depletion with systemic antibody treatment in neonates impaired IVD healing at 14dpi as shown on (A) H&E and with (B) DHI.