Longitudinal Analyses of the Intervertebral Disc Injury Response Reveals Impaired Repair and Divergent Temporal Regulation of Infiltrating Immune Cells Based on Sex

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INTRODUCTION: The intervertebral disc (IVD) is a tissue with limited tissue repair post injury and thus prone to degeneration. Immune cells act as critical mediators of tissue repair by rapidly infiltrating into damaged connective tissues in a temporally regulated fashion to stimulate healing. However, the identities of the infiltrating immune cells, their temporal regulation, and their role following an acute IVD injury remain understudied. There is a critical need to understand crosstalk between the immune system and IVD during injury and how variations arise due to sex, since low back pain due to IVD degeneration is a leading cause of disability with a higher occurrence in female patients. Thus, this study aims to identify the temporal regulation of infiltrating immune cells and measure how immune cell infiltration and IVD repair diverges with sex after an injury. We hypothesize that acute infiltration of immune cells into the IVD post injury is a tightly controlled temporal process that is required for the repair of the IVD post injury.

METHODS: IVD extractions: Five Control (non-injured) and five Injured (bilateral, 30G needle puncture) caudal IVDs were extracted for longitudinal analysis (qPCR) every 2-3 days post injury until 42dpi in 12 wk old female C57/BL6 mice, at key timepoints 3,7,12, 19 and 42 dpi in male mice, and at 3dpi for flow cytometry**(A)**. qPCR: Each sample contains 3 IVDs pooled from Control or Injured discs from the same mouse. Cells were lysed, RNA extracted, and Cp values measured using a QuantStudio3 system. Cp values were obtained and converted to relative fold changes were using ΔΔCt. Gene markers for infiltrating myeloid cells (*Cd11b*), B cells (*Cd19*), T cells (*Cd3g*) were analyzed and HPRT/GAPDH were used as a normalization controls, n=3-5. Statistical Analysis: Line and bar plots were constructed in GraphPad Prism. Gene expression level changes were analyzed using a mixed design two-way ANOVA with or Holm-Sidak post hoc analyses. The main effects of dpi and injury and the interaction: $p < 0.05$. Flow cytometry: 20,000 cells were collected by pooling 15 IVDs for each treatment group from 3 mice at 3dpi. IVDs were rendered into a single cell suspension stained with the following antibodies: 7AAD (live/dead), Cd45, Cd11b, Ly6G/C, Cd4, or Cd8. Immune cells were identified using a BDX-20 analyzer and FloJo 10.0 software for cell population gating. The black numbers are percentages, the red are cell counts. Histology: Sagittal, midline sections from 3,7,12, 19 and 42dpi in Control and Injured samples were stained with Safranin O and degeneration scoring performed on both sexes, n = 1-2. "n" = biological replicates (animals).

RESULTS: Previously, we determined the temporal regulation of immune cell infiltration in 12wk old female injured IVDs between 1 and 42dpi. Data showed that 3, 7, 12, and 19dpi were key infiltration timepoints post injury where 7dpi began the transition from a state of inflammation to proliferation and there was an upregulation of immune cell gene expression at 3dpi (Myeloid, B, and T cells), 12dpi (myeloid cells), and 19dpi (T cells) when compared to controls (dotted line) **(A).** Comparative qPCR analysis of the same infiltrating immune cells genes (*Cd11b*: myeloid, *Cd3g*: T cells, *Cd19*: B cells) in 12week old male mice at the key timepoints show divergent gene regulation of immune cell infiltration over these acute stages of IVD injury when compared to female mice **(B).** Flow cytometry analysis at 3dpi, the peak time of inflammation, in female mice shows a robust increase in myeloid subtypes, Ly6G+ neutrophils, Ly6G+ monocytes, and F4/80+ macrophages, and lymphoid cell types, Cd3+ T cells and C19+ B cells, in injured disc compared to controls **(C)**. Histology of control and injured IVDs of both sexes at 42dpi shows the persistence of the needle puncture wound with impaired healing/ degenerative changes throughout the IVD **(D)**. Degenerative scoring of histology sections from female and male IVDs at 3dpi, n=1, and 42dpi, n=2, show an increase in degenerative changes in both sexes in response to injury that becomes more severe by 42dpi, though males tend to show slightly more severe degenerative changes independent of timepoint **(E)**.

DISCUSSION: Comparative qPCR analysis of immune cell infiltration showed stark differences in the regulation of immune cell genes dependent upon sex. Expression of *Cd11b*: myeloid, *Cd3g*: T cells, and *Cd19*: B cells are all down regulated in male mice compared to female mice at 3dpi, which is the peak time of inflammation in female mice, though Cd3 is significantly upregulated at 3dpi in males. Additionally, the peak time of inflammation in male mice has shifted to 7dpi as shown by the robust regulation Cd11b+ cells, which, in females, is where the IVD mciroenviroment switches from proinflammatory to pro-proliferative **(A,B)**. Flow cytometry determined diverse populations of immune cells that either appear, neutrophils and B cells, or robustly increase, monocytes, macrophages and T cells, in response to injury while also showcasing resident immune cells at 3dpi in females. It would be intriguing to determine how these immune cell populations change in both sexes at the peak time of infiltration for females, 3dpi, and males, 7dpi, to begin to unravel which immune cells potentially promote repair differences between the sexes. **(C)**. The severe degenerative changes in both sexes demonstrate an ineffectiveness of innate repair/healing mechanisms independent of sex, but males tend to exhibit higher degeneration scores in control IVDs at 42dpi compared to 3dpi, suggesting a potential off target effect of immune cell infiltration in injured IVDs on neighboring, uninjured IVDs that is sex specific. There also seems to be sex differences in the IVD comparements that show increased degenerative changes in response to injury. Future efforts will increase the biological replicates for histology/ degenerative score, expand flow cytometry analyses to male mice, and include more key time points to determine how the immune cell subtypes identified change over time and with sex.

SIGNIFICANCE: This study will offer a targeted approach to modulate ineffectual IVD repair by elucidating the sex-related differences in IVD- immune system cross talk post injury

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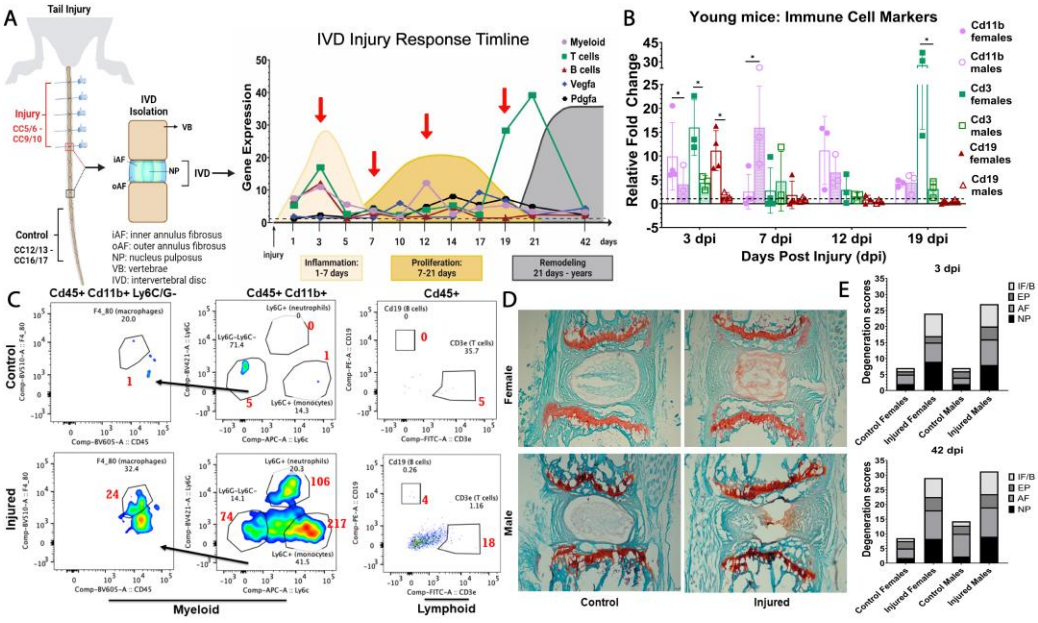

Figure: **(A)** Schematic of Control and Injured IVD tissue used to identify key timepoints, red arrows, of infiltration in 12 wk old female mice n= 3-5. **(B)** Comparative qPCR analysis of immune cell gene expression between sexes, n= 3-4 **(C)** Flow cytometry of infiltrating immune cells in females at 3dpi **(D)** Safranin O/Fast Green stained slides at 42dpi. **(E)** Degeneration scoring of 3 and 42dpi sections. Line colors in A represent the different gene primers from the bar graphs in panel B. Black dashed line represents control values. *= $p < 0.05$