

Inflammatory Stimulation of the Intervertebral Disc Promotes Chemokine Production and Macrophage Recruitment

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INTRODUCTION: Low back pain is the leading cause of disability and is often associated with degeneration of the intervertebral disc (IVD), which is characterized by an inflammatory microenvironment including increased pro-inflammatory cytokines and chemokines^{1,2}. The production of chemokines by IVD cells has been shown to promote macrophage infiltration further exacerbating inflammation and degeneration^{3,4}. Previous *in vitro* studies have demonstrated the use of lipopolysaccharide (LPS) to simulate the pro-inflammatory microenvironment leading to increased production of the cytokines, TNF α and IL-1 β ⁵. Similarly, *in vivo* injection of LPS into IVDs also increased tissue levels of cytokines and chemokines leading to macrophage migration into the degenerating IVD^{6,7}. However, the contributions of each anatomical region of the IVD to chemokine production and propensity for macrophage recruitment is unknown. Additionally, the extent to which local intradiscal inflammation manifests systemically requires further investigation. The **objective** of this study was to model chemokine production and macrophage recruitment in the IVD in response to local inflammatory stimulation. The **hypothesis** was that *in vitro* LPS stimulation of IVDs will increase chemokine expression and macrophage recruitment, while *in vivo* injection of LPS will cause migration of macrophages to the IVD, specifically to the endplate/growth plate region, ultimately increasing chemokine levels systemically in the blood.

METHODS: Rat IVD Isolation: Rat IVDs were isolated from the caudal spines of Sprague Dawley rats (n=4) and cast in 2% agarose to prevent tissue swelling⁸. **Explant Culture:** *In vitro* cultures were conducted with IVDs in transwells with 1 μ g/mL LPS stimulation for 2 or 7 days. IVDs treated with LPS for 2 days were cultured for another 2 days without LPS to create IVD+LPS conditioned media (CM). 7-day LPS-treated IVDs were separated into annulus fibrosus (AF) and nucleus pulposus (NP) using a scalpel before being stored in RNAlater for RNA isolation. **Macrophage Migration Assay:** Macrophages were plated on a transwell insert overnight with IVD+LPS CM or control media in the bottom chamber. Migrated cells were fixed and permeabilized. Transwell membranes were removed and stained with DAPI for imaging and cell counting. **LPS-Injection Procedure:** Surgeries were performed with approval from the Institutional Animal Care and Use Committee (IACUC). Male Sprague Dawley rats (n=21) were anesthetized with isoflurane and, using fluoroscopic guidance, 4 caudal (C) motion segments, C5-6 to C8-9, were exposed. A 33-gauge needle was used to inject 2.5 μ L of saline (Sham) or 100 μ g/mL LPS. Blood was collected at baseline and 7, 14, 21 and 28 after injection using venipuncture of the retro-orbital vein. Rats were sacrificed at 14 or 28 days and IVDs were isolated and fixed for immunostaining, or snap frozen. Snap frozen IVDs were separated into AF and NP immediately prior to RNA isolation. **Gene Expression:** AF and NP tissues were pulverized and cells were lysed with TRIzol and chloroform prior to phase separation. A high salt solution with isopropanol was used to precipitate the RNA before purification with the RNeasy Mini Kit according to the manufacturer's protocol. Expression of *Ccl2* for *in vitro* IVDs, and *Inos* and *Arg1* for *in vivo* IVDs was measured using RT-qPCR. **Immunostaining:** Fixed samples were imbedded and prepped for application of the CD68 primary antibody (Abcam, ab31630) and AF 594 secondary antibody (Invitrogen, A11005). DAPI and coverslips were applied before imaging on a Zeiss Axio Observer. Mean fluorescence intensity (MFI) within the NP, AF, endplate/growth plate (EP/GP), and tissue peripheral to the IVD (periphery) was quantified using ImageJ. **Serum Cytokines:** Blood samples were incubated at room temperature for 1 hour and centrifuged for serum isolation. Levels of the chemokines M-CSF and MIP-1 α were measured using a Bio-Plex Pro™ Rat Cytokine 23-Plex Assay according to the manufacturer's protocol. **Statistics:** Significance of *in vitro* IVD gene expression and macrophage migration was determined using Student's t-tests between treatments and regions. Significance of immunostaining, *in vivo* IVD gene expression, and serum cytokines was determined using two-way ANOVAs with a Fisher's LSD post-hoc test.

RESULTS: LPS stimulation of IVD explants *in vitro* increased expression of the chemokine, *Ccl2*, in both the NP and AF, with a larger increase observed in the NP (Figure 1a). CM from LPS-stimulated IVDs increased macrophage migration compared to media alone (Figure 1b). *In vivo*, LPS injection caused macrophage migration into the IVD demonstrated by positive staining for the macrophage marker CD68 (Figure 2a). Regionally, significant increases were observed in the NP, EP/GP, and periphery of LPS-injected discs compared to sham 14 days post-injury (Figure 2b). Gene expression indicated that the pro-inflammatory M1 macrophage marker, *Inos*, increased in the AF at Day 14 and 28. Interestingly, expression of the anti-inflammatory M2 macrophage marker, *Arg1*, in the NP significantly decreased 14 days after LPS injection, but at day 28 was increased compared to sham and over time (Figure 3a,b). Injection of LPS led to dynamic changes in chemokine levels in the blood with M-CSF increased at days 7, 21, and 28, and MIP-1 α at days 21 and 28 (Figure 4a,b).

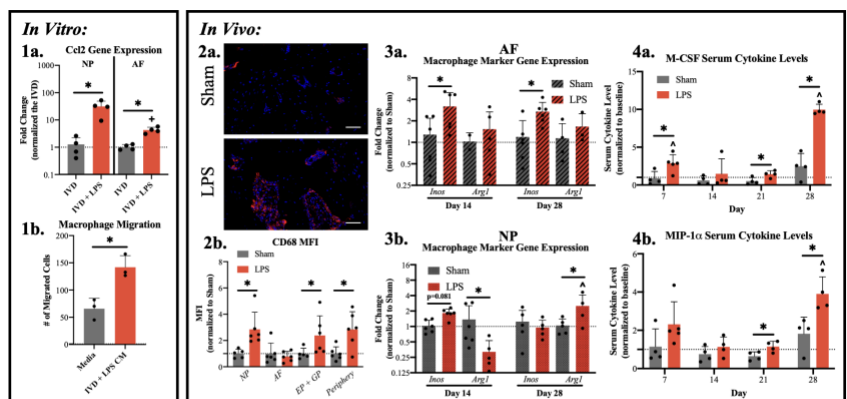
DISCUSSION: The goal of this study was to determine the effects of inflammatory stimulation on regional chemokine production and macrophage recruitment in IVDs. In agreement with our hypothesis, increased gene expression of the chemokine *Ccl2* was observed after 7 days of LPS stimulation causing increased macrophage migration towards CM from LPS-treated IVDs. The larger increase in expression observed in the NP compared to the AF could indicate that NP cells contribute more to chemokine production, and macrophages have a greater propensity to migrate into that region of the IVD (Figure 1a,b). Supporting this theory, staining for the macrophage marker CD68 in IVDs 14 days after LPS injection showed increased migration to the NP, EP/GP, and the periphery, but not the AF (Figure 2a,b). A known mechanism of macrophage recruitment is through ingrowth of blood vessels from adjacent vertebral bodies or through damaged AF⁹. The limited macrophage staining in the AF, but increased staining in the EP/GP region at 14 days could indicate that migration through the endplate may be the primary mechanism of recruitment in this model. Interestingly, gene expression of M1 marker, *Inos*, and M2 marker, *Arg1*, contrasts immunostaining results. *Inos* expression suggests that pro-inflammatory expression is maintained from day 14 to 28 in the AF, while *Arg1* expression in the NP increases over time (Figure 3a,b). Ultimately, local intradiscal injection of LPS led to systemic increases in the chemokines, M-CSF and MIP-1 α , in the blood with the greatest concentrations observed 28 days after LPS injection compared to sham and baseline (Figure 4a,b). Overall, these findings indicate that

SIGNIFICANCE/CLINICAL RELEVANCE:

Demonstration of regional differences in chemokine production and macrophage recruitment provides insight into potential target areas for degeneration prevention. Future studies will use these models to delineate the role of M1 and M2 macrophage subtypes in the progression of degeneration.

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1. a) Gene expression of *Ccl2* and b) Macrophage migration through a transwell towards media or CM from IVDs after treatment with LPS for 2 days. 2. a) Representative images of CD68 staining in IVDs 14 days after *in vivo* saline (sham) or LPS injection (Scale bar = 100 μ m). b) Quantification of MFI in 4 IVD regions 14 days after injection. Gene expression of M1 macrophage marker *Inos* and M2 macrophage marker *Arg1* in the 3a) AF or 3b) NP. Serum cytokine levels of the chemokines 4a) M-CSF and 4b) MIP-1 α . * p<0.05 Between groups indicated. + p<0.05 AF significantly different from NP, ^ p<0.05 LPS significantly different from day 14 (Figure 3) or baseline (Figure 4).