

# Identification of a Novel, MSC-Induced Macrophage Subtype via Single-Cell Sequencing: Implications for Intervertebral Disc Degeneration Therapy

Jinsha Koroth<sup>1</sup>, Arin M. Ellingson<sup>1</sup>, Casey P. Johnson<sup>1</sup>, Elizabeth W. Bradley<sup>1</sup>

<sup>1</sup>University of Minnesota Twin Cities, Minneapolis, MN

ebradl1@umn.edu

**Disclosures:** Jinsha Koroth (NA), Arin M. Ellingson (5-Medtronic), Casey P. Johnson (5-Medtronic), Elizabeth W. Bradley (5-Medtronic, 8-JBMR, BMC MSKD)

**INTRODUCTION:** Low back pain affects millions of people worldwide, with up to 80% of adults experiencing lower back pain at some point in their lives. Low back pain strongly associates with intervertebral disc (IVD) degeneration characterized by loss of cellularity, extracellular matrix degradation, macrophage infiltration, and compromised structural integrity of the IVD. IVD degeneration exhibits a complex etiology, with factors such as age, diet, genetics, loading, and injury contributing to morbidity.

Due to their “signaling cell” nature, mesenchymal signaling cells (MSCs) secrete cytokines and chemokines that exert anti-inflammatory and immunomodulatory properties that make MSCs attractive candidates for the treatment of degenerative conditions such as IVD degeneration. Preclinical studies likewise suggest that MSCs may limit disc degeneration and/or promote regeneration. One study suggested that systemic delivery of MSCs alters immune cell levels within degenerated rat discs, but we do not understand how MSCs delivered directly to the disc affect resident and recruited macrophage numbers and/or phenotypes.

In a therapeutic setting, delivery of MSCs into the avascular IVD imparts a change in oxygen tension; however, the effects of altered oxygen tension on MSC characteristics and their potential paracrine effects are often overlooked. In our current work, we examined how trophic factors produced by MSCs alter macrophage subsets, specifically examining how oxygen tension (e.g., hypoxia versus normoxia) alters the trophic effects of MSCs.

**METHODS:** To gain insight, we investigated how conditioned medium (CM) derived from MSCs cultured in hypoxia altered macrophage subsets. We cultured human, bone marrow-derived Stro3<sup>+</sup> MSCs in hypoxia or normoxia, and collected CM. We developed and validated a novel magnetic assisted cell sorting (MACS) method to isolate human bone marrow-derived macrophages. MACS-isolated macrophages were then treated with IFN- $\gamma$ , followed by either hypoxic or normoxic Stro3<sup>+</sup> MSC CM. We also cultured macrophages in hypoxic MSC CM in the presence of IL-4 and collected cells for scRNA-seq analyses.

**RESULTS:** We confirmed that MACS sorted cells were Cd14/Cd11b double-positive cells via flow cytometry, and show that MACS sorting yielded nearly three times as many Cd14<sup>+</sup>/Cd11b<sup>+</sup> cells (e.g., 77.5% versus 27.7%) as compared to conventional flow cytometry. MACS sorted Cd14<sup>+</sup>/Cd11b<sup>+</sup> exhibited known bifurcation in Cd14 levels, producing both Cd14<sup>low</sup> and Cd14<sup>high</sup> cells as described within the literature; however, only a fraction of Cd14<sup>high</sup> cells were observed via flow cytometry. Using this approach, from a starting volume of 10 ml whole bone marrow aspirate, 5 $\pm$ 0.92% of nucleated cells were Cd14<sup>+</sup>/Cd11b<sup>+</sup> cells. Isolation of double positive cells via MACS sorting depended on sequential isolation of Cd14<sup>+</sup> cells followed by isolation of Cd11b<sup>+</sup> cells. In contrast, sorting for Cd11b<sup>+</sup> cells and subsequent isolation of Cd14<sup>+</sup> cells yielded two populations of cells, Cd11b single positive cells, as well as Cd11b/Cd14 double positive cells.

For subsequent experiments, we needed to demonstrate that our MACS isolated cells could functionally respond to pro- and anti-inflammatory cytokines. We first determined if our MACS-sorted cells responded to IL-4, an anti-inflammatory cytokine. IL-4 induced phosphorylation and activation of STAT6, but not STAT1. In contrast, pro-inflammatory IFN- $\gamma$  stimulated STAT1 phosphorylation and activation, but did not alter STAT6 activity. Furthermore, expression of the inflammatory markers IL-6, IL-1 $\beta$ , and TNF- $\alpha$  increased following IFN- $\gamma$  addition, but IFN- $\gamma$ -stimulated cells did not produce changes in levels of the macrophage markers MRC1, PPARG, and IL-1RA. In addition, we also observed enhanced expression of the macrophage marker genes MRC1, PPARG, and IL-1RA following IL-4 addition, whereas IL-4 suppressed expression of the pro-inflammatory markers IL-1 $\beta$  and TNF- $\alpha$  as compared to controls as previously reported. Next, we assessed survival time in hypoxia (i.e. 2% O<sub>2</sub>) of MACS isolated Cd14<sup>+</sup>/Cd11b<sup>+</sup> cells. No significant changes in TUNEL staining were observed following 24 or 48 hours in hypoxic conditions, but an approximate 15% increase in TUNEL positive cells was observed after 72 hours. These data support that MACS isolated macrophages effectively respond to cytokine exposure and demonstrate sufficient survival in hypoxia at 24 or 48 hours.

We then assessed how trophic factors produced by MSCs maintained in normoxic conditions influenced macrophage subsets. Bioinformatic analyses confirmed multiple subpopulations of macrophages within human bone marrow. We first performed comparison analyses between macrophages cultured in hypoxic and normoxic MSC CM. Integration of these two data sets showed large overlap between most macrophage subsets; however, we identified a unique macrophage cluster induced by hypoxic MSC CM. This cluster represented approximately 2-3% of macrophages. Gene Ontogeny (GO) analyses revealed enrichment in several Reactome Pathways, including IL-10, Ccl5/Ccr5, G alpha (i) Signaling, and Rho GTPases within this unique cluster. To determine if factors from MSC CM simulated IL-4, we integrated the data from macrophages cultured in hypoxic MSC CM with and without IL-4 addition. Integration of these two data sets showed considerable overlap, demonstrating that hypoxic MSC CM simulates the effects of IL-4. Interestingly, macrophages cultured in normoxic MSC CM did not significantly contribute to the unique cluster within our comparison analyses and showed differential activation of Tgf- $\beta$  signaling; thus, normoxic conditions did not approximate the effects of IL-4. In addition, neutralization of Tgf- $\beta$  isoforms partially limited the effects of MSC conditioned medium.

**DISCUSSION:** Our data point to a role for Tgf- $\beta$  signaling as an MSC-derived factor impacting macrophage subsets. We first demonstrate that hypoxia induces production of a trophic factor(s) produced by MSCs that mimics the effects of the anti-inflammatory cytokine IL-4; however, this effect was not observed when MSCs were cultured at atmospheric oxygen tension. Comparison analyses between these two datasets implicated altered Tgf- $\beta$  signaling. Likewise, neutralization of Tgf- $\beta$  within the conditioned medium of hypoxic cultured MSCs partially blocked the effects on macrophage responses. Tgf- $\beta$  signaling has a complex role within the intervertebral disc. Tgf- $\beta$  signaling facilitates proper development of the intervertebral disc; thus, it is not surprising that Tgf- $\beta$  likewise has reported roles in promoting intervertebral disc regeneration. In contrast, other studies have demonstrated that aberrant Tgf- $\beta$  signaling can lead to disc degeneration. Similarly, Tgf- $\beta$  limits inflammatory responses of macrophages in other cell contexts. Our study suggests that Tgf- $\beta$  may control macrophage phenotypes; thus, understanding how Tgf- $\beta$  modulates macrophage activities within a model of IVDD is of future interest.

**SIGNIFICANCE/CLINICAL RELEVANCE:** Specific subsets of macrophages may be key to limiting inflammation observed during IVD degeneration, as the effects of broad macrophage depletion in models of IVD degeneration have contrasting results. Given these contrasting effects, defining specific subsets of macrophages that limit local inflammation and/or reduce leukocyte infiltration to the intervertebral disc during degeneration is critically needed. Moreover, understanding how MSCs modulate macrophage subsets may be key to defining mechanisms of action and designing effective modes of therapeutic use.