Macrophage depletion attenuates pain-like behaviors and alters DRG neuron molecular signaling in osteoarthritic mice of both sexes

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INTRODUCTION: Osteoarthritis (OA) is one of the leading causes of chronic pain and disability. Yet, management of OA pain remains poor, and often relies on analgesics with limited efficacy. Recent literature points to the emerging role of innate immunity in mediating OA pain. The knee joint is innervated by sensory neurons whose cell bodies reside in the lumbar level dorsal root ganglia (DRG). We previously found increased levels of F4/80+ macrophages in the knee-innervating DRG, 8 weeks after OA was surgically induced in the mouse knee, coinciding with onset of behaviors indicative of persistent pain[1]. In addition, we identified gene clusters via single cell RNA-sequencing (scRNAseq) that suggested the presence of a variety of immune cell types, including macrophages, in the DRGs of naïve mice. Therefore, the objectives of this study were to determine the effect of macrophage depletion on pain-like behaviors, joint damage, and DRG molecular changes in both male and female mice with OA.

METHODS: All animal experiments were approved by our Institutional Animal Care and Use Committee. We performed destabilization of the medial meniscus (DMM) on male or partial meniscectomy (PMX) surgery on female mice, age 12 weeks old at time of surgery, in Macrophage Fas-Induced Apoptosis (MaFIA) mice[2]. We evaluated hind paw mechanical allodynia (using von Frey fibers) and knee hyperalgesia using pressure application measurement (PAM) in both males and females, and weight bearing in female mice. We depleted macrophages at 8-weeks or 16-weeks post DMM surgery in male mice and 12-weeks post PMX surgery in female mice using AP20187 (Tocris), which binds to the transgenic CSF1R-eGFP receptor in macrophages and induces apoptosis. For flow cytometry, the ipsilateral L3-L5 DRGs were collected at the time points above (pooled two mice per sample, n=6-13 mice per group; n=3-6 for flow cytometry). DRGs were digested using collagenase IV and DNase I to make a single cell suspension, subsequently cells were counted and stained before running through the LSR Fortessa flow cytometer using antibody panels to detect PE-CD45, AF700-CD3, BV711-CD11b, PE/Cy7-MHCII, PerCP/Cy5.5-Ly6G, APC-F4/80, BV421-CD163, BV605-CCR2 (BioLegend), and Aqua-Live/Dead stain (ThermoFisher), and CSF1R-eGFP was detected endogenously. Analysis was completed using FlowJo software. In a separate cohort, we examined DRG molecular changes post macrophage depletion at 8-weeks post DMM surgery and naive age matched controls treated with Vehicle or AP20187 (n=5/group, 4 groups: DMM Vehicle, DMM AP20187, Naïve Vehicle, & Naïve AP20187) from male MaFIA mice by bulk RNAsequencing. We utilized PantherDB for pathway analysis based on input of genes p<0.05 and BioVenn to look at pathways that overlap per comparison. Statistical analysis was achieved by two-tailed t-tests were done at each time point.

RESULTS: To determine the role of macrophages in mediating OA pain, we performed macrophage depletion using the MaFIA mouse model. Systemic macrophage depletion 8-weeks after DMM resulted in some alleviation of mechanical allodynia and knee hyperalgesia (Fig. 1A+B) with some mice returning to normal thresholds. Depletion at 16-weeks post DMM in males also resulted in attenuation of mechanical sensitization, but to a lesser degree than at 8-weeks post DMM (Fig.1C+D). At 12-weeks post PMX, macrophage depletion in females also resulted in analgesic effect (Fig. 1E+F), with improvement in weight-bearing deficits as well (Fig.1G). Flow cytometry of macrophage-depleted DRGs revealed a significant decrease in CSF1R+ and F4/80+ macrophages, Ly6G+ granulocytes, and MHCII+ macrophages in both males and females at all timepoints evaluated. In addition, we found F4/80+ macrophages were reduced in knee joint synovium but TRAP+ osteoclasts were not reduced in the knee joint after macrophage depletion. In addition, there was no change in CSF1R+ spinal microglia after AP20187 treatment, indicating that the drug did not cross the blood-brain barrier. Knee histology for male DMM+8wks and female PMX+12wks post-macrophage depletion showed no significant difference in cartilage degeneration, osteophyte widths, or synovitis scores in both males and females post macrophage depletion compared to vehicle.

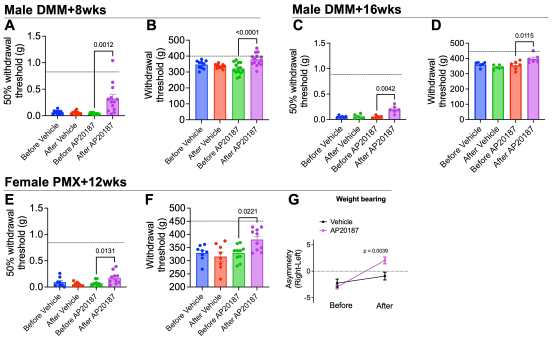

Fig.1. Macrophage depletion attenuates pain-related behaviors in osteoarthritic mice of both sexes. (A) Mechanical allodynia and (B) Knee hyperalgesia of MaFIA male mice 8-weeks post DMM surgery treated with *i.p.* Vehicle (n=10) or AP20187 (n=13). (C) + (D) Same as in (A) and (B) but for male MaFIA mice 16-weeks post DMM surgery treated with *i.p.* Vehicle (n=6) or AP20187 (n=6). (E) and (F) Same as in (A) and (B) but for female MaFIA mice 12 weeks after PMX surgery treated with *i.p.* Vehicle (n=8) or AP20187 (n=10). (G) Weight bearing of female MaFIA mice treated with *i.p.* Vehicle (n=8) or AP20187 (n=10). Statistical analysis by paired two-tailed t-test. Significant if p< 0.05. P values stated on graphs. Graphs show mean +/- SEM.

Bulk RNA-sequencing revealed interesting molecular changes after AP20187 treatment compared to vehicle in male DMM+8wks DRGs. Pathway analysis on genes comparing DMM AP20187 vs DMM Vehicle yielded several pathways in the DRG implicated after macrophage depletion. These pathway changes were weighed against control comparisons, DMM-AP20187-treated vs. Naïve-AP20187-treated, and Naïve-AP20187-treated vs. Naïve-Vehicle-treated and visualized in a Venn diagram of pathways created in BioVenn (Fig. 2A). We focused on immune- and neuron-related (Fig. 2B) pathways and found higher fold enrichment in pathways such as regulation of axon extension, regulation of calcium ion-dependent exocytosis, and synaptic vesicle exocytosis, to name a few. A closer look at the synaptic vesicle exocytosis pathway revealed that most of the genes in this pathway were downregulated after macrophage depletion (Fig. 2C), suggesting less DRG synaptic communication occurs without macrophages present and may be a potential mechanism accounting for less mechanical hypersensitivity in OA mice following depletion.

DISCUSSION: Here, we showed that macrophage depletion causes a variety of molecular changes within the DRG and leads to attenuation of pain-like behaviors in OA mice of both sexes. DRG macrophages were previously demonstrated to contribute to the initiation and persistence of neuropathic pain,[3,4] and here we show they clearly contribute to OA pain. Interestingly, systemic macrophage depletion did not affect joint damage. Future work will further investigate the functional phenotypes of DRG macrophages and gather insights from RNA-sequencing to develop targets for OA analgesia.

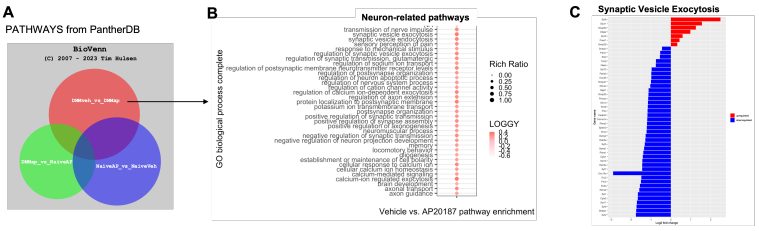

Fig. 2. Gene expression changes in the DRG with macrophage depletion in male mice with OA. (A) Venn diagram generated via BioVenn showing the volume of pathways enriched for three comparisons: DMM AP20187 vs DMM Vehicle (in red), DMM AP20187 vs. Naïve AP20187 (in green), and Naïve AP20187 vs. Naïve Vehicle (in blue). RNA-sequencing from male MaFIA mice 8 weeks post DMM surgery or naïve age-matched controls (n=5). (B) DEGs were input into PantherDB and the output was pathways implicated post macrophage depletion. Neuron-related pathways that were involved post macrophage depletion are shown here. LOGGY is the Log10 of the fold enrichment. The rich ratio is the number of DEGs out of the number of reference genes in the pathway. (C) Showing up (red) or down (blue) regulation of DEGs from DMM AP20187 vs DMM Vehicle group for the pathway, synaptic vesicle exocytosis. Bar graph and Bubble charts generated in R studio.

SIGNIFICANCE/CLINICAL RELEVANCE: This study demonstrates that DRG macrophages play a role in mechanical sensitization in mice with OA. These studies have significant clinical relevance for the development of targeted analgesics for OA pain.

REFERENCES: **1**. Miller RE, et al. PNAS. 2012;109(50): 20602-7. **2.** Burnett SH, et al. J Leukoc Biol. 2004;75(4): 612-23. **3.** Yu X., et al. Nat Commun. 2020;11(1):264. **4.** Raoof R, et al. J Neurosci. 2021;41(39): 8249-8261.

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