Link N Regulates IL-1β and Inflammasome Activity in the Intervertebral Disc through Interaction with CD14

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INTRODUCTION: Intervertebral disc degeneration disease (IVDD) is a leading cause of chronic back pain due to abnormal sensory input (nociception) by dorsal root ganglia neurons that innervate the disc. Currently, there is a lack of a gold standard to relieve discogenic pain. Recent studies have implicated the NLRP3 inflammasome, a large intracellular protein complex, important for innate immunity and implicated in the activation of caspase-1 and processing of pro-inflammatory cytokines such as IL-1β, in discogenic pain. The NLRP3 inflammasome is primed by Toll-like receptors (TLRs) present on the surface of cells that respond to various stimuli including PAMPs (pathogen-associated molecular patterns) and DAMPS (damage-associated molecular patterns). TLRs typically require cofactors to efficiently respond to ligands including MD2 and CD14. Ligand diversity of TLRs is attributed to CD14. We have demonstrated that LN, a 16-residue peptide derived from link protein, and its active derivative, sLN (an 8-residue peptide) regulates markers of inflammation and pain in IVDs both *in vitro* and *in vivo*, however, the mechanism(s) for this phenomenon remain unclear. In this study, we hypothesize that LN can regulate inflammasome activity through interaction with CD14.

METHODS: **Western blot**: Isolated human NP cells (hNPs) were incubated with LPS [1μg/ml] with and without LN/sLN for up to 72 hours. Lysates were processed for Western blotting to identify changes in Caspase-1, IL-1β, CD14 and P-NFκB. RAW macrophages were cultured as 6 well plates and treated with LPS(1ug/ml) with/without different doses of LN/sLN. **Peptide Docking:** Peptide docking of LN to IL-1β (crystal structure, 9ilb) and CD14 (crystal structure, 1wwl) was determined using the CABS-dock web server. Models were created using PyMOL (Schrodinger, LLC). ***Ca^{2+}-mobilization***-DRG neurons were isolated from lumbar regions L2-5 in 15-week-old C57BL/6 mice and cultured in glass chamber slides for 7 days with IL-1β with or without LN [1 μg/mL]. Cells were loaded with Fura-4, and AM and imaged for changes in intracellular Ca^{2+} either at resting state or following stimulation with capsaicin [100 nM] using a Zeiss LSM800 confocal microscope.

RESULTS: Western Blot demonstrated that LN/sLN inhibited LPS-induced NFκB and caspase-1 activation (n=4, $p<0.01$). Decreases in caspase-1 activation with LN/sLN were accompanied by reduced IL-1β maturation and secretion in NP cells (Figs 1+2; n=4, $p<0.01$). Similar results on inflammasome activity and LN treatment were observed in macrophages. QPCR results demonstrated LN can significantly decrease the expression of inflammasome markers (NLRP3, PYC, IL-1β, IL-6 and NGF; $p < 0.05$, n = 4). *In silico* modelling suggested that LN can interact with CD14 in the LPS-binding pocket. Ca-mobilization is used as a surrogate marker for excitability in DRG neurons. When DRGs were incubated with IL-1β, basal intracellular Ca^{2+} levels were elevated when compared to controls ($p < 0.001$; n = 4) but significantly decreased when LN was present ($p < 0.0001$; n = 4). When DRG neurons were stimulated with capsaicin, IL-1β preconditioned neurons demonstrated a sustained increase in intracellular Ca^{2+}. Co-treatment with LN blunted the sustained Ca^{2+} increase induced by IL-1β.

DISCUSSION: Previous *in vivo* studies have established the repair potential of Link N which has shown to stimulate the proteoglycan content and increase disc height when administered to the degenerative rabbit disc in vivo. In this study, we have determined the effects of LN on inflammasome activity and inflammatory factors in NP cells. CD14 is an important co-factor in the induction of the inflammasome by ligands such as LPS. We postulate that LN may compete with CD14 ligands to mitigate Toll-like receptor activation. In addition to the inflammasome, we demonstrate a direct affect of LN on IL-1β-induced neuronal hypersensitivity. Future studies will need to be addressed to determine the mechanism of LN on inflammasome activation and its effects on inflammasome activity *in vivo*.

SIGNIFICANCE: These results may support the use of LN in the treatment of discogenic pain through the regulation of the inflammasome.

REFERENCES: Mwale et al. (2018) Short Link N promotes disc repair in a rabbit model of disc degeneration. Arthritis Res & Therap v20:201
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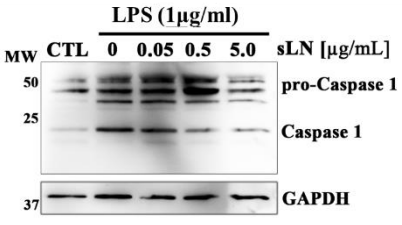

Fig 1. LN abrogates LPS-induced caspase 1 processing in hNP cells. Representative blot showing decreases in LPS-induced caspase 1 maturation in the presence of sLN.

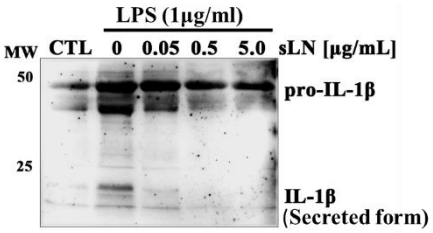

Fig 2. LN decreases LPS-induced IL-1β secretion in hNP cells. Representative blot showing decreases in LPS-induced IL-1β processing in the presence of sLN.