TLR2/4 Inhibitors Rescue the Osteonecrotic Environment Induced Inflammation in PBMC Derived Macrophages

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INTRODUCTION: Legg-Calvé-Perthes disease (LCPD) is a childhood hip disorder caused by the loss of blood supply to the femoral head, leading to bone necrosis, inflammation, and deformity. However, the biological mechanisms that sustain inflammation and delay bone healing remain poorly understood. In many sterile inflammatory conditions, necrotic tissues release intracellular molecules known as damage-associated molecular patterns (DAMPs) that activate immune cells and amplify local inflammation. However, whether similar DAMP-driven mechanisms contribute to the pathogenesis of osteonecrosis has not been clearly defined. A well-established juvenile pig LCPD model was used in this study [1]. Based on evidence that extracellular histones act as potent DAMPs, we postulated that necrotic bone releases histones that stimulate macrophage-driven inflammation through Toll-like receptor (TLR) 2 and TLR4 pathways. The objective of this study was to determine whether necrotic bone derived inflammatory mediators activate macrophage inflammatory responses through TLR2/4 signaling and to evaluate whether pharmacologic inhibition of these receptors can suppress IL-1β–mediated inflammation in osteonecrosis.
METHODS: This study was approved by IACUC. A total of 10 eight-week-old Yorkshire pigs underwent surgically induced ischemic osteonecrosis (ON) at their right femoral heads, while non-operated left femoral heads served as normal controls. Pigs were sacrificed 3–6 weeks after ON induction. Femoral heads from six pigs (five males and one female) were processed for immunohistochemical staining, and those from four pigs were used for scRNA-seq. *Immunohistochemistry (IHC):* Double IHC staining was performed for CD14 and IL-1β using DAB (brown) and Vector® SG (blue-gray) HRP substrates. The number of $CD14^+$ $IL\text{-}1\beta^+$ cells was quantified in ten representative fields per section to compare normal and necrotic bone marrow. *Single-cell RNA sequencing (scRNA-seq):* Normal and necrotic femoral heads from four pigs were enzymatically dissociated for scRNA-seq. Differential gene expression analysis and gene set enrichment analysis (GSEA) were performed, with a false discovery rate (FDR) < 0.05 considered significant. Pseudotime analysis was applied to define differentiation trajectories of macrophage subpopulations and their inflammatory states. *Proteomics:* Three male pigs were surgically induced with bilateral ON and sacrificed two weeks post-surgery for bone marrow extract and true necrotic bone (tNB) collection. Bone extracts were collected as the supernatants from normal (greater trochanter, Con) and necrotic (femoral head, ON) marrow after the centrifugation. Both normal and necrotic extracts were analyzed by proteomics (n = 5 per group). The tNB was prepared by lyophilization of necrotic bone, including both trabecular bone and marrow. *In vitro macrophage assays*: Porcine PBMCs were isolated from whole blood by Ficoll gradient centrifugation. Monocytes were purified using CD14 MicroBeads and cultured in RPMI medium with 10% FBS, 1% Penicillin/Streptomycin, and 50 ng/mL recombinant human M-CSF for 5 days to generate mature macrophages (n = 3). Cells were treated with: Control (Con), TNFα (100 ng/mL), tNB (2 mg/mL), TAK-242 (1 μM, TLR4 inhibitor), TLR2-IN-C29 (50 μM, TLR2 inhibitor), or their combinations. RNA was collected at 6 hours post-treatment for RT-qPCR, and protein lysates and supernatants were collected at 24 hours post-treatment for Western blot and ELISA. *Statistic*s: Paired t-tests were used for *in vivo* comparisons (normal vs necrotic). One-way ANOVA with Tukey's multiple comparisons test was used for cell culture experiments. $p < 0.05$ was considered statistically significant.
RESULTS: *Macrophage activation in necrotic bone*: The number of $CD14^+$ $IL\text{-}1\beta^+$ macrophages in necrotic bone marrow (42 ± 11.8 cells/field) was significantly higher than in normal bone marrow (15.8 ± 6.1 cells/field, $p = 0.0062$) (Fig. 1). This indicates an increased population of IL-1β–expressing macrophages in necrotic femoral heads. *scRNA-seq identifies pro-inflammatory macrophage cluster*: Although total macrophage numbers were not elevated in necrotic bone, scRNA-seq revealed a distinct pro-inflammatory macrophage cluster in necrotic femoral heads with significantly higher *IL1A* and *IL1B* expression. GSEA indicated enrichment of TLR signaling, cytokine-mediated pathways, and NF-κB activation. Pseudotime analysis demonstrated a trajectory shift toward an activated pro-inflammatory state in a macrophage cluster within necrotic femoral heads. *Proteomics detects histone enrichment in necrotic bone extracts:* Proteomic analysis showed a marked increase in histone protein content in necrotic bone extracts (41.75 ± 5.97%) compared with normal extracts (4.3 ± 3.24%, $p = 0.0006$), indicating that necrotic bone releases nuclear components into the extracellular space (Fig. 2). Given the known DAMP role of histones, these findings suggested they may trigger macrophage activation. *tNB induces IL-1β production in macrophages*: *In vitro*, TNFα-primed macrophages cultured with tNB exhibited a significant increase in IL-1β mRNA (13.07 ± 0.61 vs 1.01 ± 0.20, $p < 0.0001$) and secreted IL-1β protein (1322 ± 34.9 pg/mL vs –4.22 ± 6.2 pg/mL, $p < 0.0001$) compared with control. Western blot confirmed increased IL-1β precursor protein levels. *TLR2/4 inhibition reduces necrotic bone–induced inflammation*: Treatment with TAK-242 (TLR4 inhibitor) reduced IL-1β secretion (1059 ± 21 pg/mL, $p < 0.0001$). TLR2-IN-C29 (C29) or combined TLR2/4 blockade (T2/4i) further suppressed IL-1β at both mRNA (C29: 7.23 ± 1.43, $p = 0.0014$; T2/4i: 3.57 ± 0.40, $p < 0.0001$) and protein levels (C29: 391.1 ± 4.1; T2/4i: 313.6 ± 6.3 pg/mL; $p < 0.0001$) (Fig. 3). Dual inhibition resulted in approximately a 76.3% reduction in IL-1β secretion compared with tNB-treated cells, indicating that both TLR2 and TLR4 mediate necrotic bone–induced macrophage activation, with combined blockade producing the strongest anti-inflammatory effect.
DISCUSSION: This study demonstrates that necrotic bone promotes macrophage-driven inflammation through TLR2/4-dependent signaling in a juvenile pig model of ischemic osteonecrosis that mimics LCPD. Increased $CD14^+$ $IL\text{-}1\beta^+$ macrophages and scRNA-seq identification of a pro-inflammatory macrophage cluster indicate that necrotic bone alters macrophage activation states rather than cell number. Proteomic enrichment of histones in necrotic bone extracts suggests that nuclear proteins released during tissue necrosis may act as DAMPs that engage TLR2/4 on macrophages. *In vitro*, necrotic bone induced robust IL-1β expression and secretion, which was significantly attenuated by TLR2 and TLR4 inhibitors, confirming the functional relevance of these pathways. These findings support a DAMP–TLR–IL-1β axis as a key inflammatory mechanism in osteonecrosis. Limitations include the modest animal sample size and the absence of direct testing of purified histones. Future studies will focus on validating histones as macrophage activators and assessing whether TLR blockade can reduce inflammation and enhance bone healing *in vivo*.
SIGNIFICANCE/CLINICAL RELEVANCE: This study identifies a necrotic bone–driven TLR2/4-IL-1β signaling axis as a potential mechanism underlying chronic inflammation in pediatric osteonecrosis. Targeting TLR2/4 pathways may offer a novel therapeutic approach to reduce macrophage-mediated inflammation and preserve femoral head integrity in LCPD patients.
REFERENCES:
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FIGURES: Figure 1: $CD14^+$ $IL\text{-}1\beta^+$ macrophages (Con vs ON). Figure 2: Histone percentages (Con vs ON). Figure 3: IL1β ELISA of different treatments.