Transcriptomic Characterization of Porcine Peripheral Blood Mononuclear Cells Derived Macrophages Treated with Necrotic Bone Fluid

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INTRODUCTION Legg-Calvé-Perthes disease (LCPD) is a juvenile ischemic osteonecrosis (ON) of the femoral head. A disruption of blood supply to the femoral head produces extensive cell death, abundance of necrotic cell debris, and damage-associated molecular patterns [1]. Macrophages are the early and central immune cells recruited to the necrotic bone to orchestrate the ON repair process. Macrophages are known to have a wide phenotypic plasticity, ranging from pro-inflammatory M1 phenotype to anti-inflammatory M2 phenotype, depending on the local microenvironment. However, the role macrophages play in the necrotic femoral head and the repair process following ON is still not elucidated. Since the porcine LCPD model is well established and shows similar histologic and radiographic features as LCPD [2], we isolated and cultured porcine peripheral blood mononuclear cells (PBMCs) to investigate the effects of necrotic bone fluid on macrophage phenotype. The purpose of this study was to obtain transcriptomic characterization of porcine PBMC derived macrophages cultured with NBF. We used artificially created NBF, as described previously [3], and M1 and M2 stimulated macrophages as pro- and anti-inflammatory references.

METHODS 3 Yorkshire male pigs were used in this study as biological repeats (n=3). PBMCs were isolated from the whole blood by collecting the “buffy layer” after Ficoll gradient centrifugation. Monocytes were positively selected by human CD14 MicroBeads. CD14+ monocytes were cultured in RPMI containing 1% commercial porcine serum (cPS) and 1% Penicillin/Streptomycin. cPS-coated Petri dishes were used to facilitate cell attachment. After 4 days of culture, cells were treated with no treatment (Control), M1 stimulants (LPS 100 ng/ml + IFNγ 20 ng/ml), M2 stimulants (IL4 20 ng/ml + IL13 20 ng/ml), or artificial NBF (100 µg/ml). All cells were harvested 24 hours after for flow cytometry (FACS) and RNA isolation. For FACS analysis, cells were stained with anti-CD8086 antibody (M1 marker) conjugated with PE and anti-CD203a antibody (aka SWC9, M2 marker) conjugated with Alexa Fluor® 647. Total RNA was isolated for RT-qPCR and bulk RNA sequencing (RNAseq). For bulk RNAseq, a total of 12 libraries were prepared and sequenced in paired-end mode (150 PE) with the depth of 35-45 M reads per sample. The differentially expressed genes (DEGs) were identified between control vs. M1, control vs. M2, and control vs. NBF. Gene Set Enrichment Analyses (GSEA) were performed. The DEGs and their rank scores (calculated based on the fold change and p-value) were input into the WebGestalt. We further analyzed the top 500 DEGs (based on p-value and p < 0.05) of each treatment using Metascape website, which integrated multiple databases to annotate genes and perform pathway enrichment and network analyses. One-way ANOVA with post-hoc Tukey’s multiple comparison tests was used to compare among four culture conditions. A p-value < 0.05 was considered statistically significant.

RESULTS FACS analysis We compared the expressions of M1 and M2 surface markers, CD8086 and CD203a respectively, among 4 culture conditions. As expected, the percentage of CD8086+ cells in M1 condition was significantly higher than the M2 condition (p = 0.0497), while NBF treatment did not show any significant difference. The relative median fluorescence intensity (MFI) of CD8086 showed dramatic differences among the 4 culture conditions. The MFI of M1 condition was significantly higher than the Control (p = 0.026), M2 (p = 0.0040), and NBF (p = 0.039) conditions. Comparison of MFI of CD203a (SWC9) among 4 conditions revealed significantly lower MFI in the M1 condition compared to the M2 condition (p = 0.0499). RT-qPCR analysis We compared the RNA expression among the 4 conditions. The expression of M1 cytokine TNFα was significantly higher in M1 condition compared to M2 condition (p = 0.013). The expression of IL1β and IL8 was also higher in the M1 condition but there was no significant difference (p = 0.39 and p = 0.077, respectively). We did not observe any significant changes of M2 gene expression (Arg1, CD163, CD206) among the 4 conditions. Bulk RNAseq analysis Total of 16,694 expressed genes were detected. Pairwise comparison between control vs. M1, control vs. M2, and control vs. NBF exposed specific DEGs of each comparison. GO analyses performed using the human database annotated around 90% of unique gene IDs to the selected functional categories, while only 30% of unique gene IDs were annotated using the pig database. Therefore, the human GO database was applied for GO Biological Process enrichment analyses. For M1 stimulated macrophages, most of the top enriched categories were pro-inflammatory related gene sets (Fig.1). For M2 stimulated macrophages, most of the enriched categories were related to down-regulation of inflammation (Fig.2). For the NBF treatment, the top enriched categories were related to down-regulation of protein translation and mitochondrial metabolism (Fig.3), revealing overall down-regulation of cellular activity and energy metabolism of the cells treated with NBF. The further analyses for the top 500 DEGs of each culture condition using Metascape website validated the findings from the GSEA analyses.

DISCUSSION This study represents the first of its kind to determine the transcriptomic profile of porcine macrophages culture using the artificial necrotic bone fluid (NBF) and provides references to known M1 (LPS + IFNγ) and M2 (IL4 + IL13) conditions. Our FACS and RT-qPCR analyses confirmed M1 phenotype of macrophages cultured using M1 stimulants. Bulk RNAseq results were also consistent with M1 phenotype. M2 condition, however, produced mixed results from FACS and RT-qPCR analyses, but bulk RNAseq results revealed anti-inflammatory processes, consistent with M2 phenotype. Surprisingly, the NBF treated macrophages did not show expected M1 phenotype. Instead, there was an overall down-regulation of cellular activity and energy metabolism. This suggests that the macrophage activation and function in the necrotic femoral head may be impaired. Additional studies are planned to confirm macrophage phenotype and activity using the porcine model of ischemic osteonecrosis in vivo.

SIGNIFICANCE/CLINICAL RELEVANCE The findings of this study provide new insight into the role of macrophages in the necrotic bone environment and the repair process. Further studies are needed to confirm macrophage activity in response to the necrotic bone material.

REFERENCES:

FIGURES GSEA results of the top enriched categories in GO biological process: control vs. M1 (Fig 1), control vs. M2 (Fig 2), and control vs. NBF (Fig 3).