MC3T3 Cells Co-Cultured with Macrophages Demonstrate Enhanced Osteogenic Differentiation

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Statement of Purpose: Macrophages play a major role in regulating inflammation by secreting chemokines, cytokines and other soluble factors[1]. However, it is still unclear how cells of the osteoblastic lineage respond to co-culture with pro-inflammatory M1 and anti-inflammatory M2 macrophages, or their conditioned media (CM). This is clinically relevant because wound healing in scenarios such as severe trauma, fractures and nonunions, infections and osteolysis starts with inflammation. This study evaluated how osteogenesis of pre-osteoblastic MC3T3 cells is affected by: 1) CM from polarized macrophages and 2) direct co-culture with polarized macrophages.

Methods: Bone marrow macrophages were isolated from 10-12 week old male C57BL-6J mice. Macrophages were polarized for 24 hours. M0 polarization media consisted of RPMI 1640, 10% fetal bovine serum (FBS), 30% L929 conditioned media, and 1% antibiotic-antimycotic. 100ng/ml lipopolysaccharide or 20ng/ml Interleukin-4 was added to the media to obtain M1 and M2 polarization, respectively. CM was collected after 24 hours of culture. In the CM experiment, MC3T3-E1 subclone 4 cells (from ATCC) were seeded at 10^5 cells/well in 24-well plates and cultured for four weeks in 30% CM and 70% osteogenic media (MEMα, 10% FBS, 1% antibiotic/antimycotic, 50μg/mL L-ascorbic acid, 0.01M β-glycerophosphate, and 10nM dexamethasone). In the co-culture experiment, polarized macrophages and MC3T3 cells were seeded together at 10^4 cells/well each in 24-well plates and co-cultured in osteogenic media for three weeks. TNF-α and IL-1ra levels were determined by ELISA. In the CM experiment, osteogenesis was evaluated with Von Kossa staining. In the co-culture experiment, osteogenesis was analyzed with alkaline phosphatase (ALP) assay, osteopontin (OPN) qPCR, and Alizarin Red S staining. 1-way ANOVA followed by Tukey's test was performed with GraphPad Prism. N = 4-8 repetitions. *p<0.05, **p<0.01, ****p<0.0001.

Results: MC3T3 cells cultured in M1 CM/osteogenic mixture produced greater mineralization compared to M0 and M2 CM/osteogenic mixtures (Figure 1). However, mineralization was reduced compared to MC3T3 cells in osteogenic media alone. TNF-α level and IL-1ra:TNF-α ratio were elevated in M1 and M2 co-cultures, respectively, through at least 72 hours, which is consistent with the macrophages' phenotypes (Figure 2). ALP activity, OPN gene expression, and Alizarin Red staining was increased in all co-cultures compared to the MC3T3 monoculture, though not significantly for OPN expression (Figure 3). ALP activity increased from 9.3±0.8 to 12.8±0.7, 14.5±0.9, and 13.8±1.0 IU/L (μmol/(L·min)) for M0, M1, and M2 co-cultures, respectively. Likewise, OPN expression increased from 4.7±1.2 to 8.1±1.5, 10.6±2.2, and 7.0±1.3 and optical density (OD) values at 562 nm for Alizarin Red increased from 0.377±0.026 to 0.449±0.007, 0.459±0.015, and 0.456±0.009.

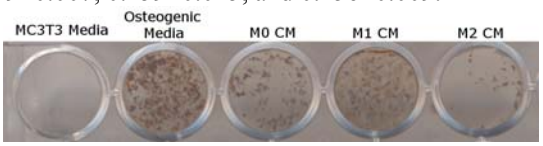

Figure 1. CM experiment: Von Kossa staining at week 4.

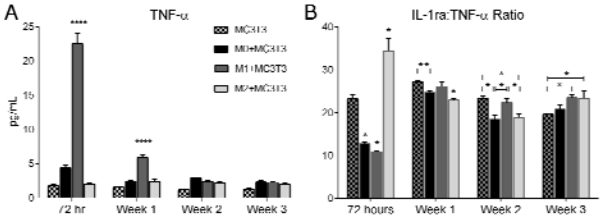

Figure 2. Co-culture experiment: A) TNF-α levels at 72 hours and weeks 1, 2, and 3. B) IL-1ra:TNF-α ratio at the same time points.

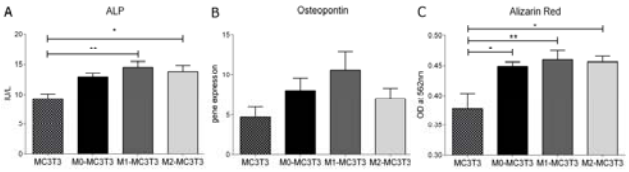

Figure 3. Co-culture experiment: A) ALP activity at week 2, B) OPN expression at week 2 (no statistical significance), and C) OD_{562} readings at week 3.

Conclusions: Compared to the other experimental groups, MC3T3 cells showed increased mineralization in M1 CM/osteogenic mixture, and when co-cultured with M1 macrophages. This suggests that the M1 phenotype and thus, pro-inflammatory signals, are most favorable for osteogenic differentiation. Notably, compared to the control groups, bone formation was reduced when MC3T3 cells were cultured in the CM/osteogenic mixtures but enhanced when they were cultured together with macrophages. This suggests that the presence of macrophages, not just their secreted factors, is necessary to enhance mineralization. This may be due to cross-talk between the two cell types and/or a more consistent supply of chemokines and cytokines in the co-culture system. Together, these findings suggest that direct interaction with M1macrophages is the most beneficial to osteogenesis. A planned transwell co-culture experiment will help to clarify this point. A greater understanding of how macrophages interact with cells of the osteoblast lineage may result in translational therapies to enhance bone healing.

References:

1. Nich C. J Biomed Mater Res A. 2013;101:3033-3045.

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