

Mg²⁺-Driven Glycolytic-Mitochondrial Coupling in Macrophage Promotes Periosteal Regeneration

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INTRODUCTION: Delayed bone union remains a significant clinical challenge. Although recent studies have identified dysregulated immunometabolic regulation as a contributing factor, there is a critical gap in accurately characterizing the immunometabolic changes throughout the healing process.

METHODS: Male C57BL/6 mice were used in this study. All surgical procedures were performed accordance with approved protocols from the Animal Experiment Ethics Committee of the Chinese University of Hong Kong (22-283-MIS) and the Hong Kong Polytechnic University (24-25/1137-BME-R-GRF). Mice underwent annular periosteal dissection (PD) at mid-femur. The femur was exposed via the intermuscular septum, and the periosteum, including both the fibrous and cambium layers, was carefully removed using a surgical blade under the guidance of a retractor, creating either a 3 mm- (PD 3 mm) or 6 mm-length (PD 6 mm) annular periosteal defect. Data were analyzed as follows: one-way ANOVA with *Tukey's post hoc* test for comparisons among multiple independent groups; two-way ANOVA with *Tukey's post hoc* test for comparisons among multiple groups with two independent variables; two-tailed *Welch's t-test* for comparisons between two independent groups. Data are presented as mean ± SD. *P* < 0.05 was deemed statistically significant.

RESULTS SECTION: In small-size PD (PD 3 mm), Tb.Th at week 4 group was significantly lower than in week 2 group. In large-size PD (PD 6 mm), both BV/TV and Tb.Th decreased significantly from week 2 to week 4 (Fig. A and B). Bulk RNA sequencing analysis of regenerating tissue confirmed suppression of energy production (Fig. C) and upregulation of macrophage-associated genes in PD 6 mm group at week 4 (Fig. D). Notably, serum Mg²⁺ concentrations were significantly lower in PD 6 mm group compared to both sham and PD 3 mm groups (Fig. E). Besides, identical secondary surgery led to further hypomagnesemia (Fig. F). Implantation of Mg-IMN (Mg/PD 6 mm) significantly promoted osteogenesis, accompanied by the formation of a dense neo-periosteum (Fig. G-I). Single-cell energetic metabolism by profiling translation inhibition (SCENITH) revealed that macrophages relied equally on glycolysis and mitochondrial activity for protein synthesis at week 2 (Fig. J-L). Macrophages from the Mg/PD 6 mm group exhibited expanded endoplasmic reticulum and Golgi compartments (Fig. M-N). Secreted protein array analysis confirmed significantly higher expression of chemokines, notably CCL2, CCL6, CCL12, and M-CSF, in *ex vivo* macrophages sorted from Mg/PD 6 mm (Fig. O). His-tagged recombinant CCL6 (His-tagged CCL6) was detectable at days 0, 3, and 7 during BMDM-induced osteoclast differentiation, showing a concentration-dependent expression pattern (Fig. P). CCL6 promoted BMDM migration in a time- and dose-dependent manner. Furthermore, treatment with 25 ng/mL CCL6 enhanced the differentiation of BMDMs into osteoclasts (Fig. Q).

DISCUSSION: We utilized a large-size PD model to simulate delayed bone union, in which we categorized the bone repair process into two distinct phases. During the early phase at week 2 post-PD surgery, we observed compensatory mechanisms, whereas in the late phase at week 4 post-PD surgery, healing was impaired within a metabolically compromised niche characterized by Mg²⁺ deficiency, elevated bioenergetic demand, and sustained macrophage activation. Mg²⁺ supplementation accelerated bone regeneration by promoting macrophage CCL6 synthesis via the enhancement of glycolytic-mitochondrial coupling. CCL6 facilitated osteoclast-mediated bone turnover by promoting the migration and differentiation of osteoclast precursors.

SIGNIFICANCE/CLINICAL RELEVANCE: The therapeutic strategies hold substantial translational promise, not only by providing a mechanistic foundation for Mg²⁺-based adjunctive therapies to improve clinical outcomes in patients with delayed union, but also positioning Mg²⁺-based interventions as viable platforms for addressing recalcitrant musculoskeletal disorders.

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