T-cells and macrophages together further modulate the osteogenesis of mesenchymal stem cells.

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INTRODUCTION: Fragility fractures in the elderly are complicated by the increased prevalence of delayed healings and nonunions, which is a social and economic problem. Open bone harvesting and grafting, the gold standard for the treatment of bone deficiencies, is associated with substantial pain and potential morbidity at the donor site. Therefore, treatment with Bone Marrow Aspirate Concentrate (BMAC) that includes Mesenchymal Stem Cells (MSCs), macrophages, lymphocytes, etc. is minimally invasive, less burdensome to the patient, and therefore attracts attention. However, in the United States, the use of cultured or activated cells, such as MSCs or other immune cells, for treatment is not yet approved by the FDA. Thus, identifying the optimal cell types and proportions for effective bone formation could hold potential for refining treatment strategies. With an increased understanding of osteoimmunology, it has become clear that an early inflammatory phase and a subsequent anti-inflammatory phase are important for bone formation, and that inflammatory macrophages (M1) and subsequently formed anti-inflammatory macrophages (M2) are critical. Recently, it has been reported that the co-culture of MSCs and CD4+ T-cells promotes CD4+ T-cell migration and differentiation, resulting in a proinflammatory phenotype. We hypothesized that direct co-culture of MSCs, CD4+ T-cells, and naïve macrophages (M0) would enhance early inflammation (increase M1) and promote bone formation. In this study, we evaluated the effects of MSCs and non-activated CD4+ T-cells on bone formation and MSCs, macrophages, and CD4+ T-cells on bone formation.

METHODS: Male BALB/c mice aged 10-12 weeks were used in the following experiments. For the osteogenic differentiation assay, primary bone marrow-derived MSCs and M0 were isolated from mice as previously described [1]. CD4+ T-cells were isolated from the spleens using an isolation kit (STEMCELL technology, USA) as previously described [2]. To investigate the effect of CD4+ T-cells, and the interaction between macrophages and CD4+ T-cells on bone formation, direct co-cultures were performed in the proportions specified. The co-cultures were incubated for 3 weeks in a combined medium comprising of 50% osteogenic medium (α-MEM with 10% fetal bovine serum (FBS), 1% antibiotic and antimycotic (A/A), 10 mM β-glycerol phosphate, 50 μM ascorbic acid, and 100 nM dexamethasone) and 50% T-cell or macrophage and T-cell medium. Alkaline phosphatase (ALP) staining was performed on day 7, and Alizarin Red staining for calcified bone matrix on day 21. The data were expressed as median with an interquartile range. Tukey's multiple comparison test followed by one-way ANOVA was performed (Prism 6. $p < 0.05$ was considered significant.)

RESULTS: In the co-cultures of MSCs with non-activated CD4+ T-cells, non-activated CD4+ T-cells showed no significant effect on osteoblastic differentiation by the ALP activity assay (Figure 1A). Similarly, non-activated CD4+ T-cells showed no influence on calcified matrix formation as shown by the Alizarin Red assay (Figure 1B). In a previous study, it was reported that bone formation was favorable with a 1:5 ratio of MSCs to macrophages [3]. Therefore, in our experiments involving MSCs, M0, and CD4+ T cells, we maintained a 1:5 ratio between MSCs and M0, while altering only the proportion of CD4+ T-cells for further investigation. In the co-culture experiments involving MSCs, M0, and CD4+ T-cells, no significant difference was observed in terms of the ALP positive area (Figure 2A). However, in the case of Alizarin Red staining, a significant increase in the Alizarin Red positive area was observed when the proportion of T-cells was at 10, compared to the other groups (Figure 2B).

DISCUSSION: We have demonstrated that co-culturing MSCs with un-activated CD4+ T-cells alone did not enhance bone formation; however, co-culturing MSCs, M0, and un-activated CD4+ T-cells together promoted bone formation. Furthermore, in the tri-cellular co-culture, we observed an increasing trend in bone formation as we increased the proportion of T-cells. This may be attributed to the proinflammatory phenotype shift of M0 to M1 through co-culturing MSCs and CD4+ T-cells, potentially positively impacting bone formation. To further elucidate this, investigating the differentiation ratios of M0 into M1 and M2 during tri-cellular osteogenic differentiation, as well as T-cell differentiation, in future studies will contribute to revealing the optimal cell types and proportions within BMAC.

SIGNIFICANCE/CLINICAL RELEVANCE: Bone marrow aspirate concentrate (BMAC) contains a variable cellular composition from patient to patient. The study's findings offer insights into the role of each cellular component, potentially shaping more effective regenerative therapies for delayed healing and nonunion in fragility fractures.

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IMAGES AND TABLES: