CCL2 facilitated osteoclastogenesis and osteogenesis in young and aged murine MSC-macrophage co-cultures.

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INTRODUCTION: The aging of the global population has increased the incidence of osteoporosis and associated fragility fractures, affecting quality of life and healthcare costs. Aging is associated with "inflammaging", that is continuous systemic low-level chronic inflammation. Although this prolonged inflammation impairs the initiation of bone regeneration in the elderly, an acute inflammatory reaction is essential for initiating the bone repair process. Thus, appropriate modulation of the acute inflammatory response is a potential therapeutic target for improving bone regeneration in the elderly. C-C motif chemokine ligand 2 (CCL2) is an inflammatory chemokine that is associated with the migration of macrophages and mesenchymal stromal cells (MSCs) during inflammation; CCL2 has been shown to promote osteogenesis by facilitating macrophage migration during acute inflammation in young male murine cells [1]. We hypothesized that CCL2 would also enhance subsequent bone regeneration in aged cells. This study investigated the therapeutic effect of CCL2 on osteogenesis and osteoclastogenesis using young and aged female murine cells.

METHODS: 10-12 week-old and 15-17 month-old BALB/c female mice were used in the following experiments. For osteogenic differentiation assay, bone marrow-derived MSCs and bone marrow-derived naïve primitive macrophages (M0) were isolated from mice as previously described [1]. To investigate the effect of CCL2 on the cell-cell interaction between MSCs and macrophages, direct co-culture of MSCs with M0 (1:1 ratio mix of each type of cells and basal medium) was performed. Cells were seeded and cultured in 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. Alkaline phosphatase (ALP) staining was performed on day 7, and Alizarin Red staining for calcified bone matrix was performed on day 21. For osteoclastogenic differentiation assay, primary bone marrow cells were isolated and cultured in α-MEM supplemented with 10% FBS, 1% A/A, 10 ng/mL macrophage colony-stimulating factor, and 50 ng/mL RANKL. Tartrate-resistant acid phosphatase (TRAP) staining, Catheprin-K assay, and quantitative real-time RT-PCR (for *C-fos*, *Dc-stamp*, *Nfact1*, and *Actin* as a housekeeping gene) was performed on day 7. The gene expression level of each gene was standardized by *Actin*. In both osteogenic and osteoclastogenic assays, cells were cultured with or without recombinant murine CCL2 (recCCL2, 10 ng/mL) for an initial 24 hours. Tukey's multiple comparison test followed by one-way ANOVA or Kruskal-Wallis test with Dunn's multiple comparisons were performed depending on the data were normally distributed. Data were expressed as median with an interquartile range. All analyses were performed using Prism 9. *P* values less than 0.05 were considered significant.

RESULTS SECTION: In the co-cultures of MSCs with macrophages, MSCs from young mice showed higher osteogenic ability than aged mice ($p < 0.01$) (Figure 1). The group treated with recCCL2 showed a greater Alizarin Red-positive area than the non-treated group both in young ($p < 0.05$) and aged ($p < 0.05$) cells (Figure 1). In the osteoclastogenic differentiation assay, the number of TRAP-positive cells was increased in the group treated with recCCL2 both in young ($p < 0.05$) and aged ($p < 0.01$) cells (Figure 2). CCL2 treatment increased the expression level of *C-fos* and *Dc-stamp* both in young ($p < 0.001$, $p < 0.01$) and aged ($p < 0.01$, $p < 0.05$) cells (Figure 3). Young cells showed a higher expression level of *Dc-stamp* than aged cells regardless of recCCL2 treatment ($p < 0.01$) (Figure 3).

DISCUSSION: We demonstrated that CCL2 facilitated osteogenesis in direct co-culture of MSCs with macrophages using young and aged female murine cells. CCL2 also promoted osteoclastogenesis in young and especially aged cells. Osteoclasts play predominant roles in the resorption of old bone prior to subsequent osteogenesis, contributing to the initiation of bone regeneration [2]. We found age-dependent differences in the expression of *Dc-stamp*, the master regulator of cell-cell fusion and osteoclastogenesis. Modulation of CCL2 expression could potentially transform aged cells into younger ones, but further functional investigation is required. In conclusion, CCL2 induced both osteoclastogenesis and osteogenesis not only in young but also in aged cells. Modulation of CCL2 could potentially facilitate the initiation of bone regeneration in impaired aged cells, which might provide a novel anti-aging therapy.

SIGNIFICANCE/CLINICAL RELEVANCE: This study using both young and aged cells suggests that CCL2 could be a potential therapeutic target for anti-aging therapy related to bone remodeling in clinical scenarios such as osteoporosis and associated fragility fractures.

REFERENCES:
[1] Toya M. et al. CCL2 promotes osteogenesis by facilitating macrophage migration during acute inflammation. *Front Cell Dev Biol.* 2023; 11:1213641.
[2] Charles, J.F. et al. Osteoclasts: more than 'bone eaters'. *Trends Mol Med*. 2014; 20(8): 449-59.

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IMAGES AND TABLES:

Figure 1

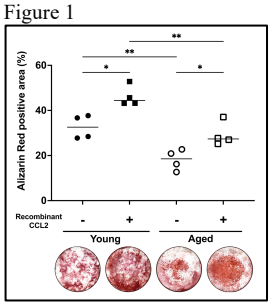

Figure 2

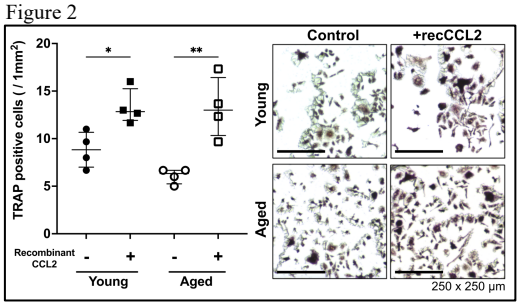

Figure 3