Δ Age-Related Changes in Macrophage Polarization Affect Osteogenesis

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Purpose: Aging affects many cellular functions of macrophages, and macrophages exhibit various phenotypes depending on the inflammatory environment in which they are located. After injury macrophages exhibit a proinflammatory phenotype, termed M1, and then the macrophages polarize to an anti-inflammatory phenotype (M2). The M2 phenotype is associated with stimulation of healing. We hypothesized that the phenotype and function of macrophages changes with age, and these changes would affect osteogenesis. Therefore, we developed a macrophage/preosteoblast coculture system to assess the interreaction between these two cell types as a function of age.

Methods: Bone marrow monocytes were isolated from mice that were either 10 weeks old or 18 months old. Cells were differentiated into macrophages (bone marrow–derived macrophages [BMM]) in vitro. 10 ng/mL interferon gamma (IFN-γ) combined with 10 ng/mL lipopolysaccharide (LPS) were introduced to the media to activate BMM to a proinflammatory phenotype (M1) and 1 ng/mL interleukin (IL)-4 was used to drive macrophages toward an anti-inflammatory phenotype (M2). Proinflammatory cytokines and anti-inflammatory cytokines were tested by enzyme-linked immunosorbent assay (ELISA) from R&D Systems. M1 marker nitric oxide (NO) was assayed using the Griess reagent system and M2 marker levels (YM1 and FIZZ1) were quantified by quantitative polymerase chain reaction (qPCR). Macrophages of different phenotypes were then cocultured with preosteoblast cell lines (Cab2t3) in 3 configurations: (1) standard coculture, (2) macrophage-conditioned media and Cab2t3 cells, and (3) trans-well coculture. We used qPCR to quantify Runx-2, ALP, Col1a, osteocalcin, osteopontin, and osterix expression at different time points (D3, D7, D10, D14) during osteoblast differentiation. Alizarin red staining was used to quantify mineralization and alkaline phosphatase was quantified to assess osteogenesis.

Results: *BMM from aged mice secrete higher levels of proinflammatory cytokines*: IL-6 levels in 10 weeks BMM and M2 group were significantly lower than 18 months BMM and M2 group, while IL-10 does not appear to be different. *Polarization of macrophages from mice of different ages*: BMM from 10-week-old mice secrete lower levels of NO and upregulated gene expression of YM1 and FIZZ1 compared with BMM generated from 18-month-old mice. *Effect of different phenotype of macrophages on the bone cells differentiation:* In the 10-week group, osteocalcin, osteopontin, and osterix gene expression were reduced in both M1 and M2 group from 10-week-old mice. In the 18-month group, osteocalcin, osteopontin, and osteoterix in M1 group were not significantly different than controls, but M2 macrophages stimulated higher expression levels of these genes. *Effect of different phenotype of macrophages on the bone cells osteogenesis:* In standard coculture system, osteogenesis of Cab2T3 cells did not show any difference between different age groups. In condition media and trans-well coculture system, Cab2t3 cells have more osteogenic nodules in the M2 group than M1 group from 10-week mice. However, for BMM from 18-months mice, more nodules were detected in the M1 group than the M2 group.

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Conclusion: We demonstrate that macrophages from aged mice exhibit a more proinflammatory phenotype compared with macrophages from young adult animals. Our results suggest that osteogenesis may be impaired in inflammatory environments that do not resolve, while M2 macrophages are beneficial for osteogenesis. This is consistent with other conclusions that M2 cells are good for tissue repair and regeneration. These data support our hypothesis and suggest that regulating macrophage polarity may be important for bone repair in aged patients.

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T-Lymphocyte Immune Modulation in Fracture Healing: The Role of IL-17F in a Novel GSK3/β-Catenin Independent Pathway

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Purpose: Previous work established the importance of interleukin-17F (IL-17F) in T-lymphocyte mediated osteoblast maturation during the early inflammatory phase of fracture repair. We hypothesize that IL-17F regulation of osteogenesis occurs through known signaling pathways of bone healing, specifically Runt-related transcription factor 2/core-binding factor subunit alpha-1 (Runx2/cbfa1), a key transcription factor in osteoblast development and the Wnt/glycogen synthase kinase 3β (GSK3β)/β-catenin signaling pathway.

Methods: Preosteoblast mouse cell line MC3T3-E1 and primary bone marrow stromal cells (MSC) differentiated to osteoblasts from 12-week-old wild-type (C57BL/6) mice were cultured and treated with IL-17F. After 3 days incubation, RNA extraction with Trizol and quantitative RT-PCR (real-time polymerase chain reaction) was performed for mature bone formation markers (Collagen 1 [Col1]), osteocalcin, Runx2, and bone sialoprotein [BSP]). For analysis of protein expression, preosteoblast MC3T3-E1 mouse cell line was used and treated with IL-17F or Wnt3a and lysates was obtained after 4 days. Western blot analysis using anti-cbfa1, phospho-GSK3-(Ser9), and (Tyr216) antibodies was performed on cell lysates. Densitometry analysis was used to quantitate the protein expression levels relative to the expression of housekeeping gene, actin.

Results: Preosteoblast cell cultures (MC3T3-E1 and primary bone MSC) treated with IL-17F resulted in a significant increase in expression of markers for osteoblast maturation compared to untreated controls, namely Col1, osteocalcin, Runx2/cbfa1, and BSP ($P < 0.05$). Upregulation of Runx2/cbfa1 expression was confirmed on Western blot analysis showing increased Runx2/cbfa1 levels after IL-17F treatment compared to control with no treatment. In fact, this was similar to expression levels seen with Wnt3 treatment, a known upstream stimulator of Runx2/cbfa1 expression. Interestingly, IL-17F upregulation of the osteoblast transcription factor Runx2/cbfa1 appears independent of the known Wnt-β-catenin pathway. The phosphorylation of the Ser9 site on GSK3β suppresses β-catenin degradation and allows its nuclear translocation to activate downstream targets such as Runx2/cbfa1, to promote bone formation. Western blot analysis showed that IL-17F treatment leads to almost no Ser9 phosphorylation and high levels of Tyr216 phosphorylation, which is consistent with increased GSK3β activity and thus, β-catenin degradation. Yet, despite this, treatment with IL-17F leads to similar increased expression of Runx2/cbfa1 downstream, lending evidence for a GSK3/β-catenin independent manner of IL-17F stimulated osteogenesis.

Conclusion: IL-17F, a pro-inflammatory cytokine secreted by T-lymphocytes, stimulates osteoblast maturation in fracture healing through a novel GSK3-independent pathway.

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Δ Lipopolysaccharide-Induced Systemic Inflammation Affects Bone Healing in a Murine Tibia Fracture Model

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Background/Purpose: The early stages of fracture repair require close coordination between the immune system and skeletal system. Conditions such as diabetes, rheumatoid arthritis, smoking, obesity, and aging adversely affect bone healing. Common to all these conditions is a sustained inflammation. It has been shown that lipopolysaccharide-induced systemic inflammation results in fracture callus of inferior mechanical characteristics in a rat femur fracture model. The aim of this study was to compare fracture healing in a murine tibia fracture model in the setting of lipopolysaccharide (LPS)-induced systemic inflammation to that of control animals.

Methods: Sustained inflammation was created by the injection of lipopolysaccharide (3 μg/animal/day, *E. coli* O55:B5 LPS) intraperitoneally. Control animals received equivalent volume of phosphate-buffered saline vehicle. Concurrently, diaphyseal tibial fractures were created and stabilized with intramedullary pins. Plasma and tibiae were collected on day 1 (D1), D3, D7, D10, D14, and D21 (N = 3 per time course per group). Plasma was isolated from whole blood by centrifugation at 2000*g* for 20 minutes. All plasma samples were immediately stored at –20°C after isolation. Interleukin (IL)-6 levels were detected by ELISA (enzyme-linked immunosorbent assay). Prepared ELISA plates were read as suggested by the manufacturer. Concentrations were expressed in pg/mL. All samples were run in duplicate.

Harvested limbs were fixed in 4% paraformaldehyde and decalcified in EDTA (ethylenediaminetetraacetic acid) for 14 days, after which they were paraffin embedded and sectioned at 5 μm. Every tenth section was stained using a trichrome stain. Adjacent sections were stained using the method of Hall and Bryant. Sections were then examined and tissue types were quantified by stereology. Calculated volumes were expressed in mm^3. Mean volumes were compared using the Student *t* test with a significance level of $P < 0.05$ employed.

Results: *LPS Injection:* During the 7-day injection period no behavioral differences were noted between treated and control animals. No symptoms of systemic illness were noted in either group. One mortality occurred in the control group. *Plasma IL-6 Levels:* For the LPS group, the IL-6 level rose shapely to concentration of 209.9 pg/mL 24 hours after fracture whereas the maximum IL-6 level reached by controls was 23.5 pg.mL (Figure 1). IL-6 levels decreased in both groups to near-basal levels by postfracture day 3 in controls and day 7 in the LPS-treated animals. *Fracture Healing Under Inflammatory Conditions:* At day 7 the treatment group trended towards a smaller mean callus volume (mean difference 15.5mm^3; $P = 0.21$) (Figure 2). By day 10 and 14 there was no difference in callus size between groups. The difference in callus size was primarily related to an increased volume of cartilage and undifferentiated tissue in control animals (Figure 2). Hall-Bryant quadruple stain confirmed the larger callus in control animals (mean difference 17.4; $P < 0.076$) (Figure 3). The size dif-

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ference noted was present in all tissue types, suggesting an overall smaller callus, rather than a difference in one cell type.

Conclusion: The injection of LPS into the peritoneal cavity of mice concurrent with tibia fracture results in delayed fracture healing based on the results of this study. The injection of LPS is also associated with increased systemic IL-6 production in treatment animals; however, no symptoms of systemic inflammation were noted when compared with control animals. Analysis of fracture site tissue distribution suggests that systemic inflammation causes a smaller fracture callus at day 7. Further work is required to define the individual inflammatory and skeletal cell populations at the fracture site and to determine if the observed histomorphometric differences translate to mechanical inferiority of the callus of treatment animals.

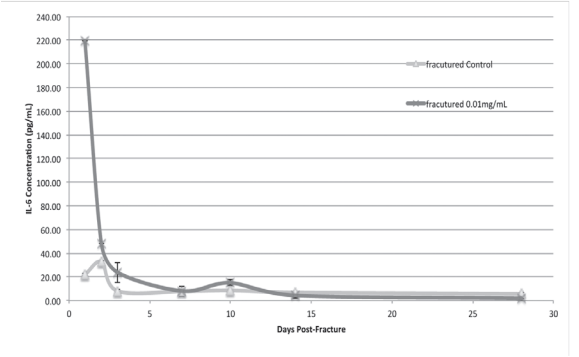

Figure 1: IL-6 plasma concentration after fracture and injection of LPS or control vehicle. Concentrations measured by ELISA.

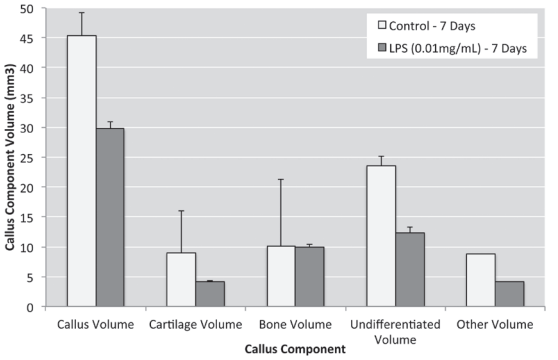

Figure 2: The effect of LPS-induced inflammation on callus size at postfracture day 7 as determined by trichrome staining.

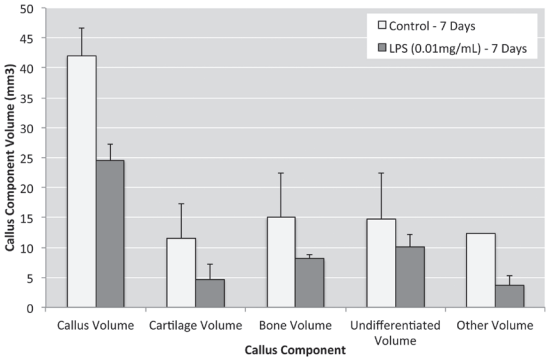

Figure 3: The effect of LPS-induced inflammation on callus size at postfracture day 7 as determined by Hall-Bryant quadruple staining.

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