Intra-articular Depletion of Macrophages following Articular Fracture Results in Bone Resorption and Altered Synovial Macrophage Polarity

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Introduction: Post-traumatic arthritis (PTA) is an accelerated form of arthritis that occurs following joint injury, and it is estimated that nearly 12% of the 21 million Americans with symptomatic osteoarthritis have a post-traumatic etiology. PTA develops most commonly following fracture of the articular surface of a joint. Following articular fracture, C57BL/6 mice develop PTA, whereas MRL/MpJ mice, a superhealer mouse strain, exhibit less severe joint degeneration. Furthermore, the MRL/MpJ mice also demonstrate less synovitis and decreased infiltration of activated macrophages into the synovium, suggesting that inflammation and macrophages within the joint synovium may play a role in the pathogenesis of PTA. The Macrophage Fas-Induced Apoptosis (MAFIA) transgenic mouse strain is on a C57BL/6 background and allows for a specific depletion of macrophages using the non-toxic dimerizing reagent AP20187, which induces macrophage apoptosis via the expression of the inducible Fas-suicide gene. Surprisingly, the MAFIA mice demonstrated an increase in synovial inflammation when macrophages were depleted locally prior to injury. In order to further characterize the role of macrophages in joint injury, a second method of macrophage depletion was investigated in the C57BL/6 mouse strain using a local injection of clodronate liposomes. The goal was to characterize bone morphology and macrophage phenotype following articular fracture and macrophage depletion. We hypothesized that acute intra-articular depletion of macrophages following articular fracture would result in an altered inflammatory response.

Methods: Male MAFIA and C57BL/6 mice were obtained (Jackson Laboratories) and housed until skeletal maturity at 16 weeks, at which point the left hind limb was subjected to a moderate articular fracture as previously reported. Intra-articular depletion of macrophages in the MAFIA mice was accomplished via a single, 6μL intra-articular injection of dimerizing reagent AP20187 (n=6 per time point) at 2 days prior to fracture, immediately following fracture, or 2 days post-fracture. The control group received a single 6μL intra-articular injection of the carrier solution (n=3 per time point). Macrophages in the C57BL/6 mice were depleted via a single 6μL intra-articular injection of clodronate liposomes (n=6 per time point) or control liposomes containing PBS (n=3 per time point) either immediately following fracture or 2 days post-fracture. All mice were sacrificed 7 days post-fracture, the limbs were harvested, and serum and synovial fluid were collected and stored at -80° for future analysis. All hind limbs were formalin fixed and scanned with microCT (SkyScan 1176, Bruker BioSpin). Histological sections of the joint were stained by immunohistochemistry (IHC) with F4/80 (F4/80, Serotec MCA497G) and CD206 (CD206 Mouse anti-Human, Abcam ab64693), to determine synovial
infiltration of mature macrophages, and M2 regulatory macrophages, respectively. Macrophages were quantified by counting F4/80 or CD206 positive cells in the synovial lining and synovial stroma of histological sections. A multi-factorial ANOVA was used to assess bone mineral density of joint by group and time, with limb as a repeated measure. A Student’s t-test was used to compare IHC staining in depleted and non-depleted fractured limbs.

**Results:** Articular fractures were successfully created in all mice. In analyzing bone morphological measures, the tibial metaphyseal region demonstrated significant differences in the fractured limb between depleted and non-depleted mice. MAFIA mice that were depleted 2 days prior to fracture exhibited a significant decrease (p<0.05) in bone mineral density (g/cm^3) in the tibial metaphyseal region (Fig 1A). Macrophage depletion on the day of fracture or 2 days following fracture did not result in significant differences. These variations in bone mineral density were also observed in the bone morphology presented in the microCT images in Fig 1B. However, this decrease in BMD was not observed in the C57BL/6 mice depleted with clodronate. Although macrophage depleted MAFIA mice did not demonstrate a significant change in the quantities of F4/80 general macrophages or CD206 regulatory M2 macrophages in the synovial lining (Fig 2A), MAFIA mice depleted at all time points demonstrated significantly higher levels of F4/80 general macrophages and significantly lower levels of CD206 regulatory M2 macrophages in the synovial stroma as compared to control group at the same time point (Fig 2B, p<0.05). Representative sections stained for F4/80 general macrophages and CD206 regulatory M2 macrophages (brown color for positive staining) are demonstrated in Fig 2C.

**Discussion:** Acute macrophage depletion with articular fracture increased joint inflammation and presented a more inflammatory, erosive condition. With an injury model of an articular fracture, macrophage depletion resulted in a reduction of bone mineral density near the metaphyseal region, an increase in general macrophages (F4/80), and a reduction in regulatory M2 macrophages (CD206). Our data suggest that the intra-articular macrophage population plays an important regulatory role in joint inflammation after injury. Previous studies have reported a rebound of macrophages 2 days following systemic depletion in a skeletal injury model. The observed increase in F4/80 macrophages suggests a similar rebound following local depletion. Intra-articular targeting of macrophages has previously been investigated with OA models, resulting in a reduction of osteophyte formation, fibrosis, growth factors, and synovial matrix metalloproteinases (MMPs); however, we did not observe a reduction of joint inflammation in our fracture model. Interestingly, with the clodronate depletion method, bone morphological changes and degree of macrophage rebound were milder relative to MAFIA mice. The differences between the outcomes in MAFIA and C57BL/6 mice may result from different depletion mechanisms between AP20187 and clodronate. The size of liposomes prevents the clodronate from diffusing through vascular barriers, which restricts the amount of depletion when compared to AP20187 in the genetically modified mouse. Furthermore, it has been shown that clodronate, a bisphosphonate, inhibits bone resorption, providing a potential explanation for the variation in bone morphological alterations in MAFIA and C57BL/6 mice. Our data suggest that though inflammation has degenerative effects on cartilage, macrophages are important in regulating inflammation and bone maintenance after joint injury.

**Significance:** This study provides new insight on the regulatory role of macrophages in joint inflammation and bone resorption and repair after joint injury.
Figure 1: Tibial Metaphysis of macrophage depleted and carrier control mice: (A) Bone Mineral Density (BMD) of control and fractured experimental limb for all treatment groups *p<0.05. (B) MicroCT axial cross-sectional images in fractured experimental joints. Scale bar is 1 mm.
Figure 2: (A) Synovial Lining, (B) Synovial Stroma, and (C) Macrophage IHC Staining (brown color) comparing F4/80 Macrophages (left) and CD206 M2 Macrophages (right) in MAFIA mice depleted 2 days after fracture *p<0.05 (n=3 per group). Scale bar is 200 µm.