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TITLE: **Osteoimmune Mechanisms of Segmental Bone Fracture Healing and Therapy**

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14. ABSTRACT The proposal addresses a very important area of bone fracture healing that is a major health and financial burden in the United States. Towards developing an effective approach to cure segmental bone fractures, it is essential to understand additional mechanisms that play a crucial role in bone healing through participation of cells other than osteoblasts, osteoclasts and their respective progenitors. Bone fractures heal with overlapping phases of inflammation, cell proliferation, and bone remodeling. Osteogenesis and angiogenesis are known to work in concert to control many stages of this process, but when one is impaired, it leads to failure of fracture healing. During fracture repair, there is an abundant infiltration of immune cells at the fracture site that not only mediate the inflammatory responses, but also exert influence on neovasculature. The proposed studies will characterize the role of IMC in healing segmental bone fracture in tibia using an immunocompetent mouse model. Studies will encompass a detailed analysis of IMC function in bone healing through enhancement of cellular and molecular signals on endothelial cell invasion, migration and proliferation, aiding in proper ossification and healing. Overall, the studies will test the hypothesis that signals provided by IMC play a crucial role in interfacing sequential events of angiogenesis and osteogenesis that are vital for proper bone healing.				
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INTRODUCTION

The incidence of segmental fractures is significantly higher in military personnel compared to civilians. Despite efforts involving allografts, surgery and fixators, intramedullary nailing and invasive plate fixing to heal segmental fractures, unfortunately, lingering effects remain when the fracture involves long segmental bone loss. This necessitates the importance of identifying key molecular signals involved during critical phases of bone fracture healing, and developing novel therapies based on such understanding of molecular and cellular interactions during fracture healing.

Large segmental bone defects are still a very serious problem in clinical practice. Especially when a significant quantity of bone is lost in the periosteum, it severely reduces the source of progenitors for remodeling. Current treatment options, especially for long segmental defects still pose complications of morbidity at the donor sites, devitalization of the transplant due to resorption process, leading to reduced mechanical stability - all contributing to difficulties in clinical management. To overcome these limitations, current efforts are focused on: tissue engineering approaches aimed at developing osteoconductive scaffolds, better quality synthetic bone grafts, and use of autologous mesenchymal stem cell transplantation. Although these are promising, the major focus still remains in enhancing osteogenic effects in the fracture area. In order to better approach the problem, it is apparent that key cellular and molecular signals that determine fracture healing during initial and crucial phases of bone healing need to be elucidated.

Following fracture, there is disruption of normal vasculature that results in the release of growth factors by the damaged matrix and surrounding cells, signaling an influx of immune cells and osteogenic progenitors to remodel the fracture site. The initial injury to the blood vessels is contained to the surrounding area and progresses into a hematoma. Immune cells that infiltrate the hematoma to fight infection also secrete various cytokines and growth factors that are responsible for helping initiate the fracture repair cascade. Growth factors such as vascular endothelial growth factor (VEGF), bone morphogenetic

proteins (BMP), transforming growth factor (TGF)- β , and cytokines such as interleukin (IL)-1 and -6 have been implicated in the migration of mesenchymal stem cells (MSC), and aid in recruitment of inflammatory cells to the fracture site. Once fracture healing is initiated, osteogenesis and angiogenesis work in concert to control many stages of bone remodeling. Fractures that do not heal are not avascular, but nonunion are more likely to occur when blood vessel growth is delayed after fracture. Therefore, understanding how the initial inflammatory reaction of fracture healing is coupled to signaling the fracture repair cascade will uncover mechanisms involved in the fate of bone healing, and will provide new opportunities for therapeutic intervention. To this end, the proposal seeks to characterize the roles of a key immune cell population at various stages of fracture healing in a large segmental bone defect model, and therapeutically use it in healing of nonunion segmental fracture in mice.

SPECIFIC AIMS:

- 1. To determine molecular mechanisms of IMC function in healing of segmental bone fractures.**
- 2. To determine therapeutic effects of IMC adoptive transfer in an immunocompetent mouse tibial segmental bone fracture model.**

BODY

Rationale and Approach: Bone fractures heal with overlapping phases of inflammatory influx of immune cells, cell proliferation, endochondral bone formation, coupled remodeling (osteogenesis and angiogenesis). The inflammatory phase characterized by an influx of immune cells and formation of a hematoma. Therefore we want to understand how the initial inflammatory reaction and the recruitment of immune cells at the site of fracture are linked to the fracture repair process. We hypothesize that identification of immune cell phenotypes and accompanying chemokines in fractured microenvironment promises to yield crucial information for designing strategies to treat non-union bone fractures. For instance we envision that identification of particular immune cell subtype(s) and/or chemokine(s) at site of bone fracture could be used as a “bait” to facilitate homing of other angiogenic and/or immune modulators to facilitate bone repair process. To accomplish this goal, in the first part of our project, we focus our efforts on to conduct an unbiased screen to investigate changes in diverse immune cell subsets in femoral bone marrow isolates obtained at critical time-points following a segmental fracture. We have created segmental fractures recently and analyzed data.

The following sections provide highlights of results obtained:

Segmental defect leads to systemic increase in B220+ cells at Day 5 and 10 in C57BL6 mice: We found that segmental fracture leads to a sharp and systemic (effect observed in both contralateral and fracture bearing femurs) increase in B220+ cells in bone marrow isolates at Day 5 and Day 10 following segmental fractures in immune-competent C57BL6 females (8-10 weeks old). To further identify the type of upregulated B220+ cells we also analyzed Plasmacytoid Dendritic cells and B-cell subpopulations. We found a sharp systemic increase in pDC's cell subpopulations at Day 5 and 10 post fracture, time points during bone healing processes such as intramembranous bone formation and endochondral ossification are occurring (**Fig 1**). Further studies will be conducted to establish relationship between the marked upregulation of B220+ immune cell subpopulation and bone repair.

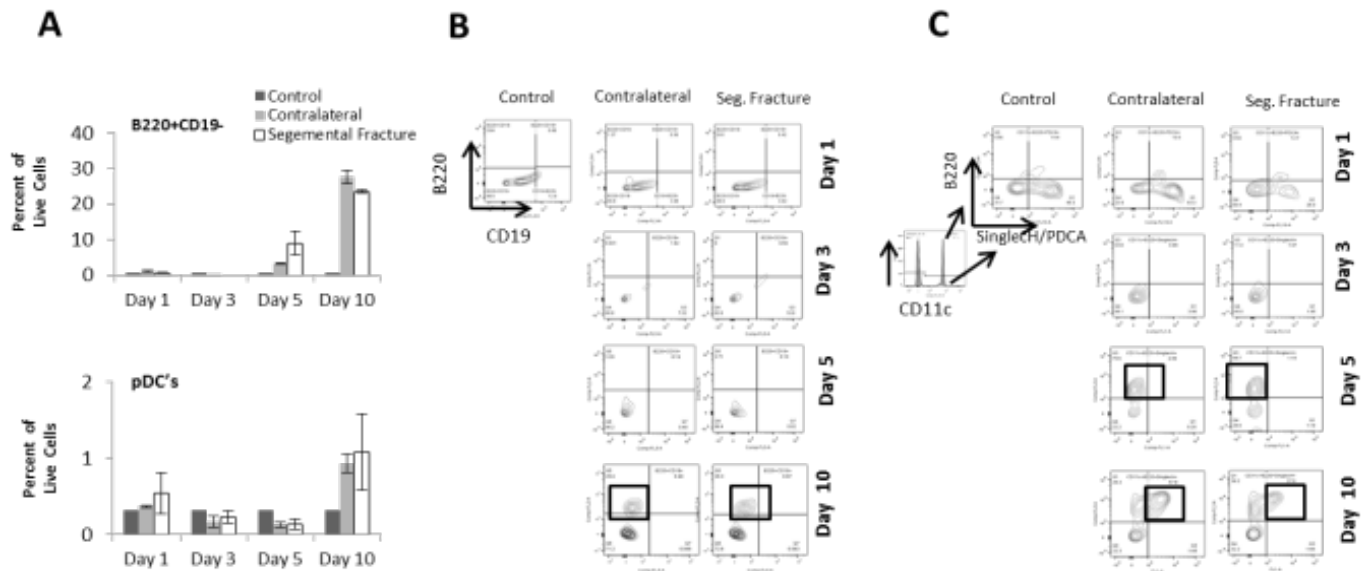


Fig 1: BM isolates from C57BL6 mice bearing segmental fracture were analyzed by flow cytometry. **A**, Quantification of B220 positive and pDC's immune cell subpopulation from control, contralateral and segmental fracture bearing femurs. **B**, **C**. Representative FAC's contour images denoting the gating structure and systemic increase in B cell and pDC subpopulations at Day 5 and 10 (see inset)

Segmental defect leads to a marked systemic increase in Endothelial Progenitor Cells (EPC's) initially, followed by a rapid decline at later time-points: The screen for immune cell subpopulations also revealed a marked and systemic increase in EPC immune cell subpopulation following segmental fracture (**Fig 2**). This finding is particularly encouraging in the light of the observation that bone repair process requires angiogenic mediators. The systemic increase in EPCs as early as Day 1 post fracture may indicate that the BM is primed for angiogenic potential at a very early stage of the bone repair process. Furthermore we found that segmental fracture lead to a modest but a persistent increase in EPC subpopulations in SF femurs

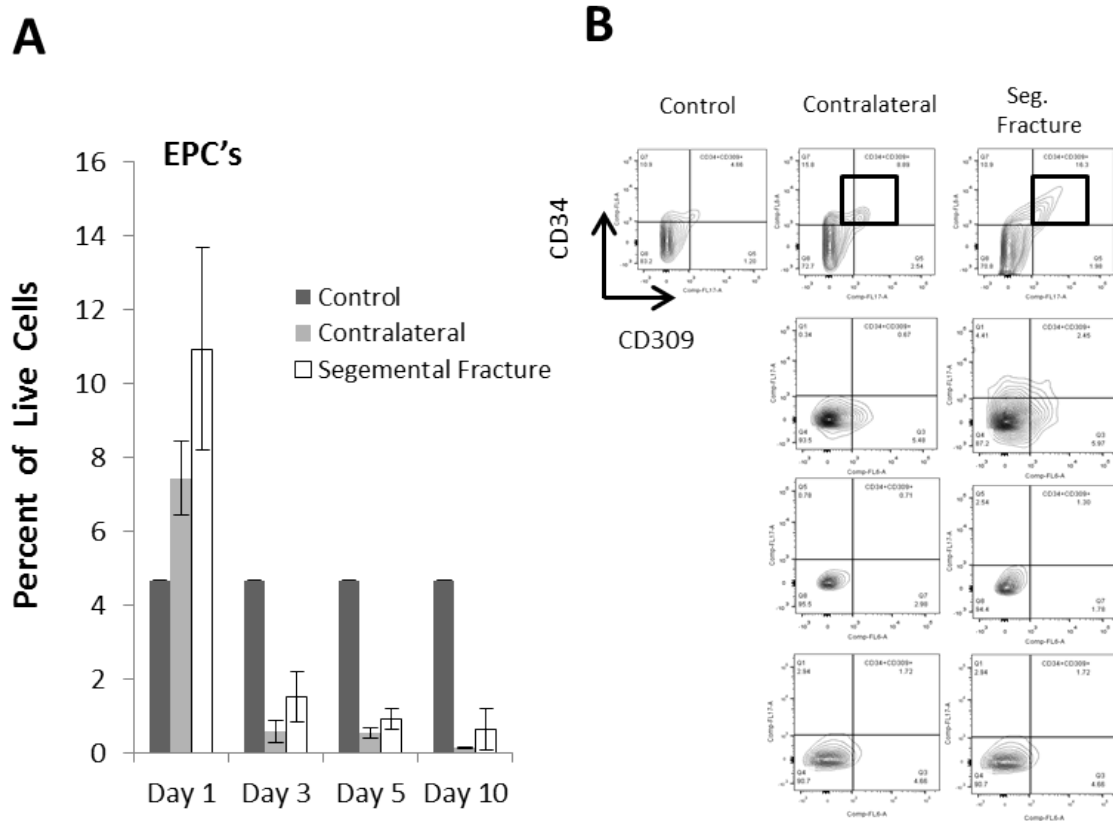


Fig 2: BM isolates from C57BL6 mice bearing segmental fracture were analyzed by Flow Cytometry. **A**, Quantification of EPC's from control, contralateral and segmental fracture bearing femurs. **B**, **C**. Representative FAC's contour images denoting the gating structure and systemic increase in EPC subpopulations at Day 5 and 10 (see inset)

Dendritic cells show a systemic increase on Day 1 post-SF and rapid systemic decline at later time-points: Following critical size segmental defect we observed a sharp increase in Classical Dendritic Cells at very early time points. Interestingly this increase is not sustained at later time-points and we observed a systemic decline in Classical DC's (**Fig 3**). DC's are classical antigen presenting cells that interact with T-cells and B-cells and orchestrate the intensity of adaptive immune response. To assess if the effects in Classical DC's are mirrored in T-cells we next analyzed specific T-cell subpopulations

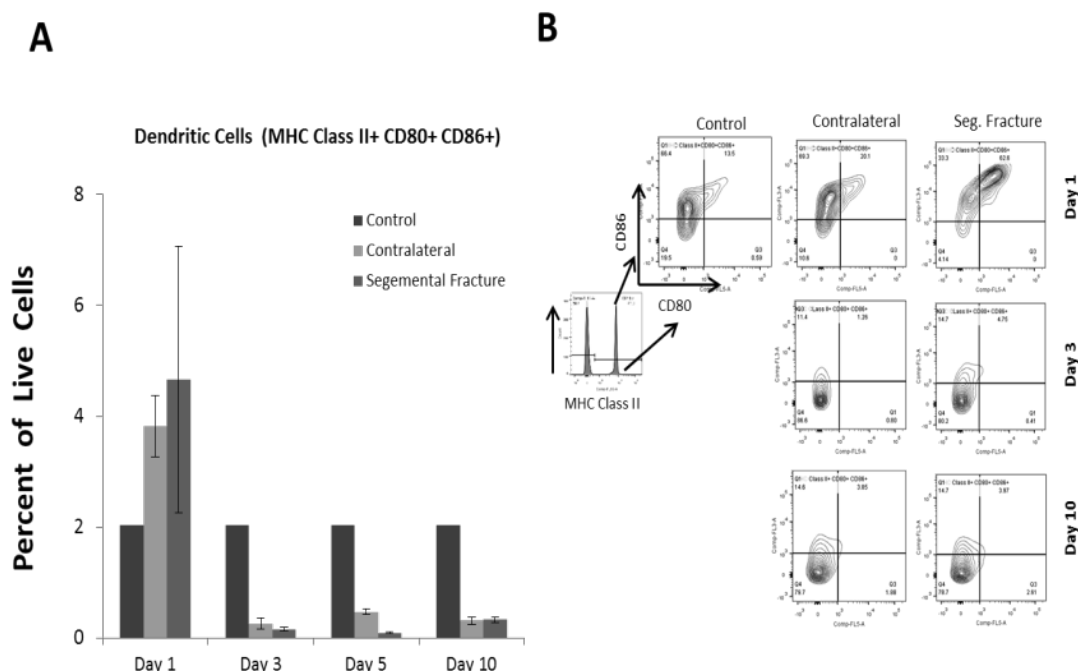


Fig 3: BM isolates from C57BL6 mice bearing segmental fracture were analyzed by Flow Cytometry. **A**, Quantification of classical DC's from control, contralateral and segmental fracture bearing femurs. **B, C**, Representative FAC's contour images denoting the gating structure and systemic increase in EPC subpopulations at Day 5 and 10 (see inset)

Summary of changes in immune cell subtypes following segmental defects:

We analyzed the data obtained in the last 6 months on endothelial cell and immune cell profiling at the fracture site following segmental bone fracture. The alterations observed in specific immune cell subtypes in BM isolates of mice femurs bearing segmental defects are summarized in **Table 1**. The alterations are classified as persistent (observed at all-time points), local vs. systemic (occurring only in segmental defect femurs vs. changes occurring both in contralateral and segmental defects bearing femurs). Future studies will entail using BMD analysis, immunohistochemistry studies, cytokine profiling to correlate the bone healing process timeline with changes associated in immune cell subtypes.

Immune cell subtype	Effect observed
Dendritic, Endothelial Progenitor	Sharp decline after increase on Day 1
B220+, plasmacytoid DCs	Sharp increase on Day 10
Macrophages	Time-dependent decrease
Neutrophils	Time-dependent gradual increase

Table 1: Changes in immune cell subtypes following segmental defects in C57BL6 female mice

Since bone fracture therapy will also require stimulation with bone morphogenetic protein (BMP)-2, our next focus has been to improve stability of this protein and to reduce proteasomal degradation. Towards this major goal, we performed studies to identify domains that are amenable for degradation by proteasomal enzymes. Following are data obtained in this regard.

Proteasomal Blockade Stabilizes Intracellular BMP-2: To assess if proteasomal block causes an increase in BMP-2 precursor, we transfected 293T cells with a construct expressing human BMP-2 precursor under a strong CMV promoter. The cells were treated with two different proteasomal inhibitors post transfection. Intracellular BMP-2 shows dose dependent and time dependent stabilization following proteasomal block, indicating that abrogating conventional routes of intracellular protein degradation can enhance the BMP precursor protein level.

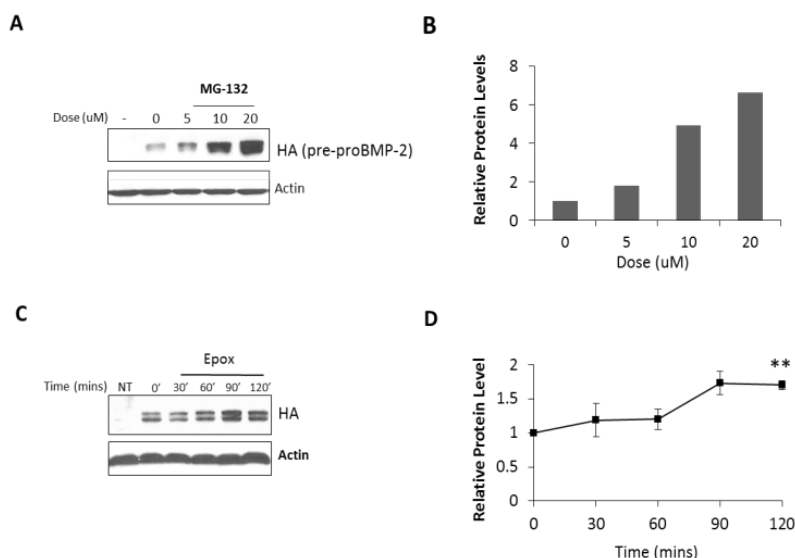


Fig 4: **A, B** 293T cells were transfected with N-terminal HA-tagged pre-pro-BMP2 precursor with increasing doses of MG-132. Protein lysates from harvested cells were immunoblotted with HA antibody. **C, D** 293T cells were transfected with N-terminal HA-tagged pre-pro-BMP2 precursor with Epoximycin for indicated times. Protein lysates from harvested cells were immunoblotted with HA antibody. Pre-pro-BMP-2 exhibits a time-dependent increase in protein levels following proteasomal blockade

Assessment of steady state turnover of BMP-2 precursor: We next assessed the steady state turnover of BMP-2 precursor protein. The cells were treated with Actinomycin D and Cycloheximide to block *de-novo* mRNA and protein synthesis respectively. Following this treatment, short-term degradation kinetics of BMP-2 precursor protein was assessed in presence or absence of proteasomal blockade. We found proteasomal blockade substantially delayed BMP-2 degradation, with dramatic improvement in its intracellular half-life as shown in figures below:

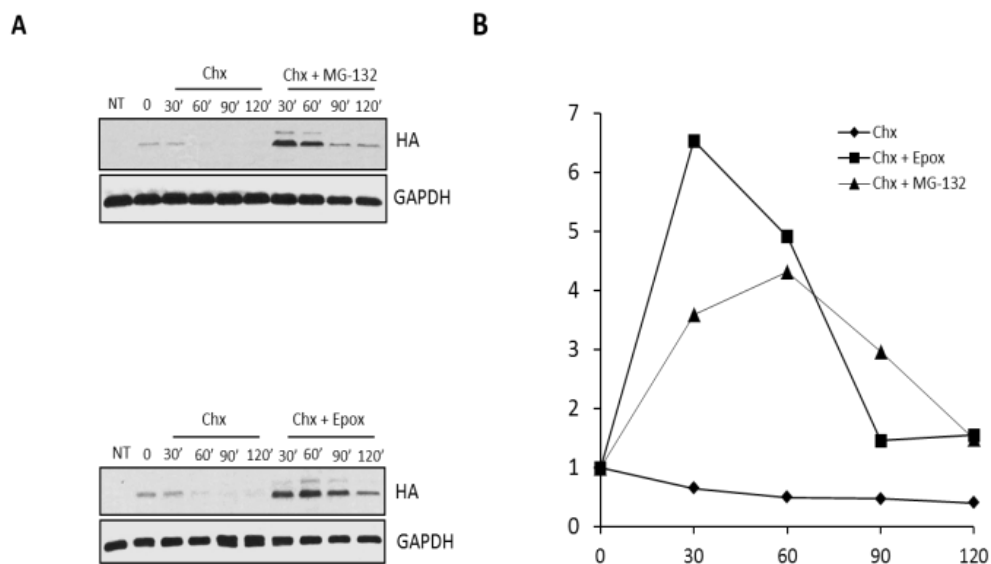


Fig 5: A,B 293T cells were transfected with N-terminal HA-tagged pre-pro-BMP2 precursor and degradation kinetic was followed after blocking de-novo protein synthesis (CHX). **B**, Densitometric quantification of immunoblot shown in Fig 5A. Epoximicin and to lesser extent MG-132 substantially delays BMP-2 degradation.

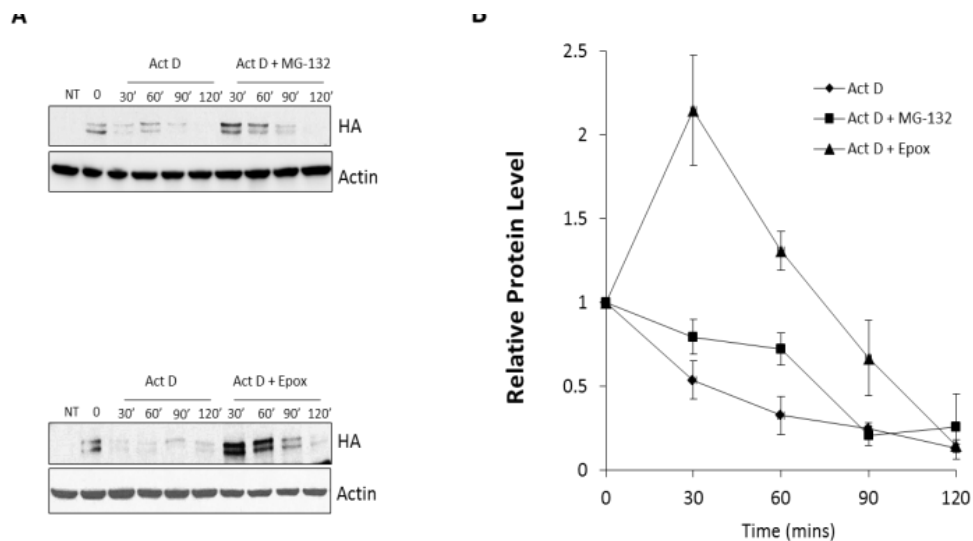


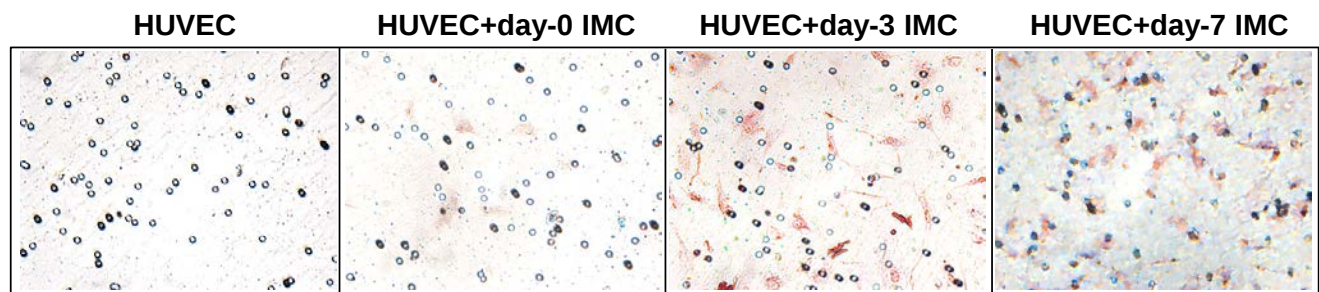
Fig 6: A,B 293T cells were transfected with N-terminal HA-tagged pre-pro-BMP2 precursor and degradation kinetic was followed after blocking de-novo mRNA synthesis (CHX) **B** Densitometric quantification of immunoblot shown in Fig 6A.

Immature myeloid cells increase endothelial cell migration and motility:

It has previously been shown that a subpopulation of differentiated CD34⁺/CD14⁺ monocytes isolated from human peripheral blood will co-express endothelial-specific surface antigens and grow tube-like structures when cultured under angiogenic stimulation. Furthermore, when co-cultured with carcinoma cells, CD11b⁺Gr1⁺ cells have been shown to differentiate into endothelial cells, and incorporate into the tumor vasculature. Considering that angiogenesis and osteogenesis are strongly coupled during bone formation, and that characterization of normal IMC revealed a predominantly monocytic phenotype (Ly6C⁺Ly6G⁻), we sought to determine if IMC in the bone fracture microenvironment displayed an angiogenic phenotype. To this end, IMC infiltrates isolated from the site of broken femur were co-cultured with HUVEC in transwell migration, and motility assays in serum-free media to demonstrate factors from IMC were responsible for any changes in HUVEC phenotype.

Results of this study are shown below:

Figure 7:



IMC isolated from naïve mice or mice after segmental fractures on days 1, 3, and 7 were co-cultured with HUVEC in serum-free medium. IMC were placed in the bottom chamber and HUVEC in the top of the Boyden chamber. HUVEC cells capable of passing through pores were fixed and stained with neutral red after 4 hours.

Figure 7 above demonstrated that IMC, isolated on day-3 and day-7 following bone fracture have the potential to initiate migration of endothelial cells. Quantitative analysis of the motility assay is given in Figure 8. There was a significant difference in the number of HUVEC cells that migrated through the Boyden chamber when co-cultured with IMC isolated on day-3 or day-7 following bone fracture.

Next, a scratch assay was performed. Briefly, HUVEC cells were grown to confluence in 24-well plates and scratched with the narrow end of a sterile p200 pipette tip. Media was changed to remove floating cells and replaced with serum-free endothelial cell basal medium (Lonza). Photomicrographs were taken at 200x magnification immediately after initial wounding, and the scratch area was measured. Transwell inserts were inserted to the wells, and IMC were placed in the upper chamber in the same serum-free endothelial basal media. Cells were then incubated at 37°C with 5% CO₂. After 12 hours, photomicrographs were again taken at 200x magnification and the scratch area was analyzed using ImageJ (NIH). Results are shown in Figures 9 and 10.

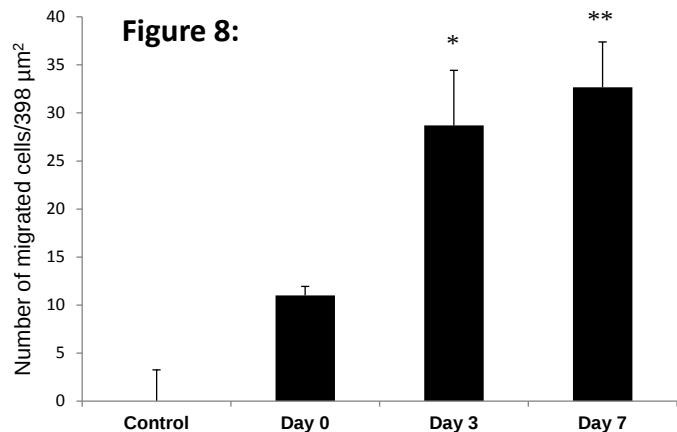


Figure 9:

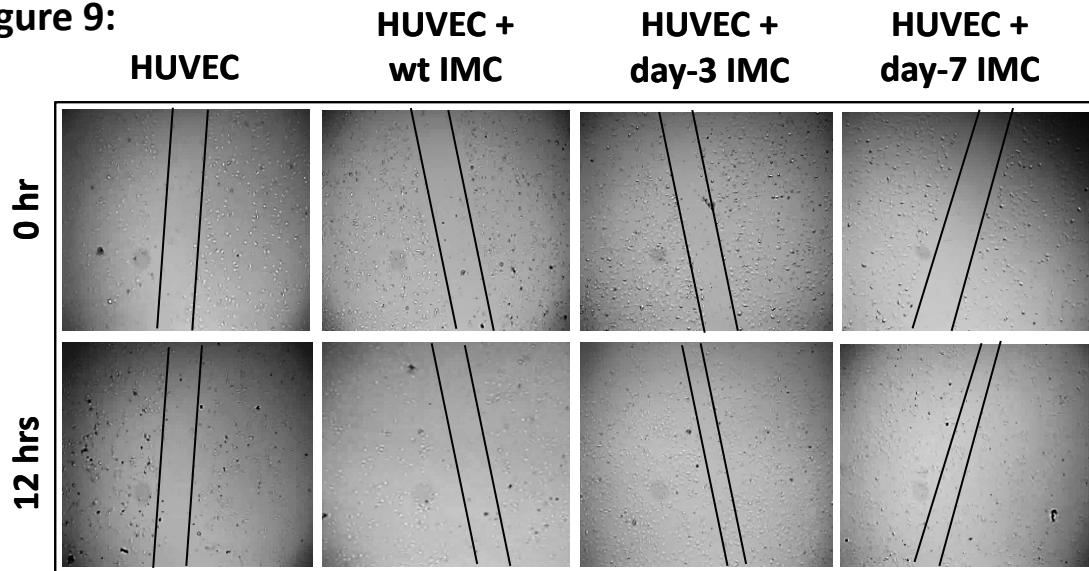
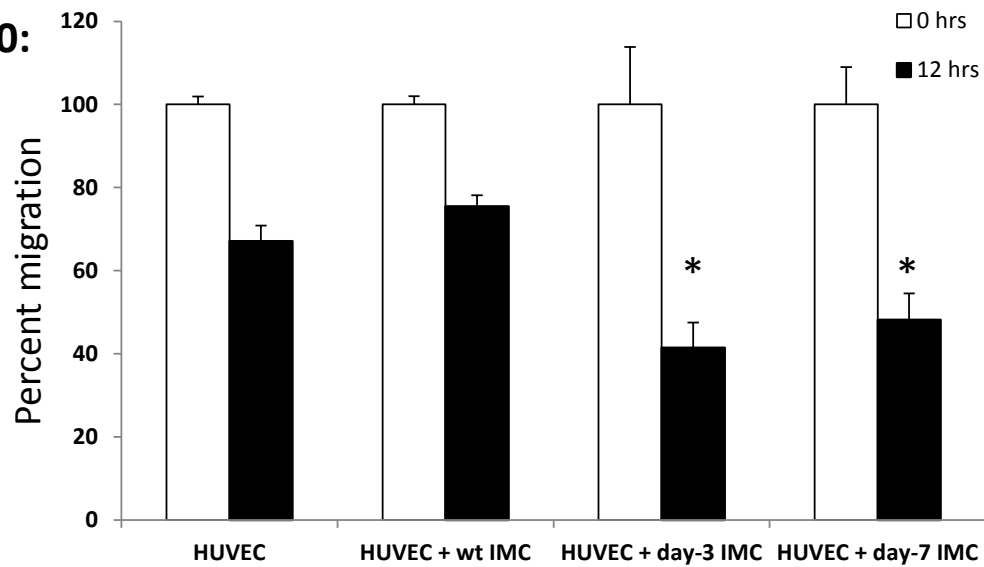


Figure 10:



These data further confirmed that activated IMC caused an increase in cell migration with significant migration observed in HUVEC, co-cultured with IMC isolated on days 3 and 7 after fracture. These results indicate IMC in the post-fracture milieu aid in endothelial cell recruitment during fracture repair.

Immature myeloid cells increase endothelial tube formation *in vitro*. To further elucidate the impact of IMC on angiogenesis in the bone fracture environment, we investigated whether isolated IMC could affect endothelial tube formation. IMC were isolated from wild-type and gemcitabine treated mice, both before and after segmental defect, and co-cultured with HUVEC on reduced growth factor (RGF) Matrigel®. Naïve (control) and activated IMC attached to HUVEC cells and increased the number of branch points and the number of junctions significantly, compared to HUVEC cultured alone (Figures 11-13). This data confirmed IMC provide important paracrine effects on angiogenesis at the fracture site through soluble factors. Briefly, IMC isolated from naïve or 1, 3, or 7 days after segmental defect are co-cultured directly with HUVEC. IMC bind directly to HUVEC. IMC were labeled with CFSE, and HUVEC were labeled with PKH26, 20x magnification (Figure 11). Representative images of HUVEC and IMC co-culture on tube formation assay are given in Figure 12. Quantitative analysis of tube formation using Angiogenesis Analyzer in ImageJ, n = 3 and three 10x images per well is shown in Figure 13 (*P<0.05).

Figure 11:

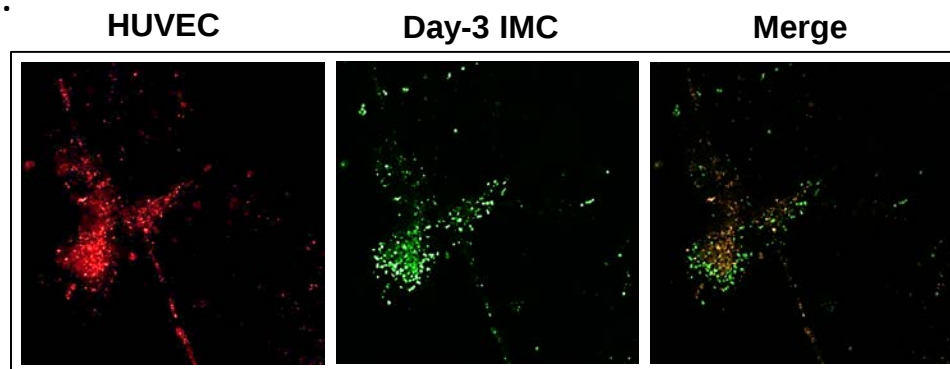


Figure 12:

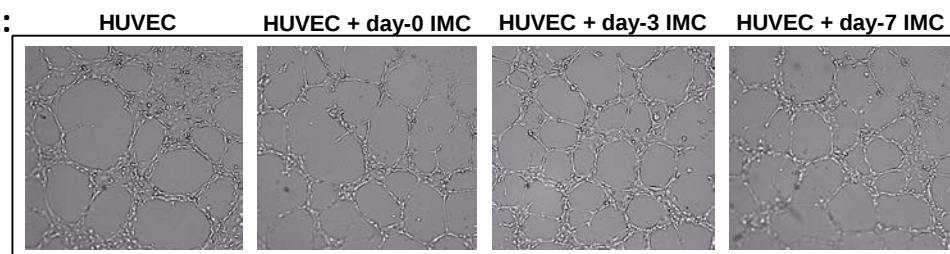
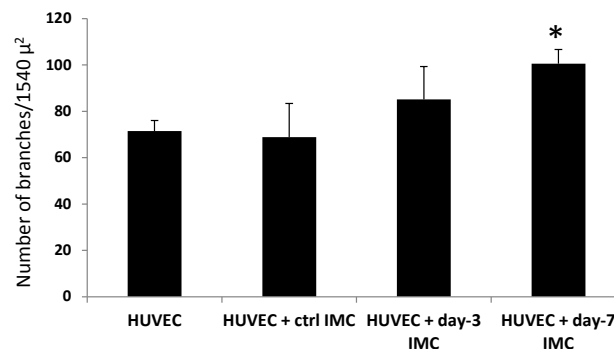


Figure 13:



KEY RESEARCH ACCOMPLISHMENTS:

- Characterization of immune cells following bone fracture was performed by flow cytometry.
- Novel approaches are being undertaken to improve the stability of bone morphogenetic protein (BMP)-2 for therapeutic use in fracture healing.
- Functional assays were performed to characterize immature myeloid cells (IMC) from bone fracture site. The studies were performed to identify properties of IMC in endothelial cell mobilization and vascular remodeling. This is critical since the early phase of fracture healing is greatly influenced by angiogenic cascade.

REPORTABLE OUTCOMES

None

CONCLUSIONS

Immature myeloid cells appear to play a crucial role in promoting angiogenic signaling, which is important for initiating osteogenic response for fracture healing.

PERSONNEL RECEIVING PAY FROM THIS GRANT

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