

# Immunosuppression and Impaired Fracture Healing in Polytraumatic Injuries

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## INTRODUCTION:

The field of bone regeneration has been primarily focused on treating fractures and nonunions from isolated musculoskeletal injuries. Compared to isolated fractures, which frequently heal well, fractures in over 30% of polytrauma patients exhibit impaired healing. Clinical evidence suggests there may be distinct physiologic, cellular, and molecular fracture healing mechanisms in polytrauma. Previous models have assessed thoracic trauma and associated changes in the volume and mineralization of healing fractures. However, those studies did not investigate the cellular and molecular mechanisms. Hence, although the effects of local inflammation, neutrophil activity, and macrophage polarization on isolated fracture repair have been studied, little is known about how persistence of systemic, polytrauma-induced inflammation affects local bone regeneration. The purpose of this study was to characterize the temporal local and systemic immune responses to polytraumatic injuries in a blunt chest and femur fracture polytrauma murine model.

## METHODS:

Animal surgeries were performed in the Department of Orthopaedic Surgery at UC Davis in compliance with the ARRIVE guidelines and were authorized by Institutional Animal Care and Use Committee (IACUC). A total of 272 C57Bl6J mice were used in this study (8 animals per group per time point). We had four surgery groups including healthy non-injured, isolated blunt femur fracture, isolated blunt chest trauma, and polytrauma (femur fracture + chest trauma). Chest trauma was induced using a drop weight device resulting in bilateral hemopneumothoraces. Femur fractures were created using an Einhorn drop weight device. To study the local and systemic immune response, the cellular phenotype of immune system, and cytokine expression after injury, we collected blood, bone marrow from uninjured limb, femur tissue, and lung tissue after 0 h, 6 h, 12 h, 24 h, 72 h & 3W from all mice. Expression of proinflammatory cytokines were detected in serum using a 10-plex proinflammatory meso scale diagnostics (MSD) V-PLEX panel. Immune cell distribution from isolated bone marrow, femur, or lung tissue were assessed at each timepoint with a BD Fortessa 18-color flow cytometer. Femur tissues were collected after 3 weeks of healing to study fracture healing using micro computed tomography ( $\mu$ CT), histological staining, multiplex opal immunohistochemistry, and light sheet microscopy. Lung tissue was also collected at 3 weeks to assess healing using histological staining, multiplex opal immunohistochemistry, and light sheet microscopy. Two-way ANOVA on ranks was performed when the normality test failed, using the Kruskal–Wallis test for post hoc comparison. Otherwise, regular ANOVA was performed with a Tukey test for post hoc comparison. All analyses were performed in GraphPad Prism 8 (GraphPad Software Company, San Diego, CA). Significant differences were presented as \* $P < 0.05$  and \*\* $P < 0.01$ , \*\*\* $P < 0.001$  and \*\*\*\* $P < 0.0001$ .

**RESULTS SECTION:** Using flow cytometry we studied the cellular phenotype of the immune system in lung, femur, and bone marrow. after inducing injury in our animal models (**Figure 1**). Flow cytometry demonstrated neutrophil and macrophage expression at the fracture site was significantly higher in the polytrauma group compared to the other groups at all time points, with a peak of expression after 72 h. Neutrophils and macrophage levels returned to noninjured group levels after 3 weeks in single trauma animals but remained elevated for the polytrauma group at 3 weeks. Expression of neutrophils in lung tissue was only significantly higher in the polytrauma group at earlier time points with a peak at 12 h, and it reached the range of the healthy group's neutrophil expression after 72h. Macrophage presence was significantly higher for both polytrauma and chest groups in bone marrow during the first 24 hours, indicating the overall high systemic polytrauma response. Expression of adaptive immune cells (B and T cells) was significantly lower in the polytrauma group after 6 hours of injury both locally and systemically and remained low after 3 weeks of healing compared to all groups. Our MSD data supported these observations as there was a significantly higher expression of proinflammatory cytokines at 0 h, 12h and 3 weeks in the polytrauma group. Although the expression of these cytokines was also high for the isolated femur fracture group, the concentration of these cytokines decreased to the level of the noninjured group range after 24h of healing. The expression of IL-10, an anti-inflammatory cytokine, was lower for the polytrauma group compared to other groups with a significant decrease at 72 h compared to the isolated femur fracture group. In addition, our  $\mu$ CT data showed a significant decrease in bone volume/tissue volume and bone mineral density in the polytrauma group after 3 weeks of healing compared to the isolated femur fracture group.

**DISCUSSION:** Our cellular and molecular analysis confirmed that after inducing the polytrauma chest and femur injuries, the expression of acute proinflammatory markers and cells significantly increased. The systemic inflammation response in the isolated femur fracture group decreased to the range of the healthy group's expression after 3 weeks, indicating inflammation resolution and bone healing. However, inflammation remained significantly higher in the polytrauma group at 3 weeks. Expression of B and T cells and related cytokines (interleukin 2 and 4) was lower in the polytrauma group compared to other groups and continued to decrease more over time.  $\mu$ CT data also showed significantly lower amount of bone formation in the polytrauma group, these data could support our hypothesis that the prolonged and hyper-elevated immune system response to polytrauma leads to fracture nonunion. However, the decreased expression of B and T cells demonstrates an exhaustion of the adaptive immune system response that may contribute to fracture nonunion. Our data confirms the early, dysregulated inflammatory state in polytrauma which correlates with decreased bony healing at the fracture site. We will examine the immune exhaustion effects on fracture healing in the polytrauma group after 3 and 6 months in future studies.

**SIGNIFICANCE/CLINICAL RELEVANCE:** There is an increased incidence of fracture nonunions in polytrauma patients resulting in morbidity and economic loss. There are significant gaps in our understanding of how fractures heal in this polytrauma environment. This study elucidates the role of persistent immune and inflammatory dysregulation that may contribute to poor fracture healing in polytrauma.

## REFERENCES:

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**Figure 1.** Flow cytometry results comparing neutrophil and macrophage presence in the femur fracture site (local), bone marrow (systemic), and lung. BM – bone marrow.

