Immunomodulatory Strategies to Ameliorate Trauma-Induced Immune Dysregulation

by

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DISSERTATION ABSTRACT

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Doctor of Philosophy in Bioengineering

Title: Immunomodulatory Strategies to Ameliorate Trauma-Induced Immune Dysregulation

Severe musculoskeletal trauma is a highly prevalent condition that frequently results in poor healing outcomes and other complications. Following a fracture, the successful initiation of bone repair relies in part on the immune response to injury, which plays crucial roles such as preventing infection, clearing debris from the injury site, and recruiting and activating progenitor cells. Previous studies have highlighted the functions of specific immune cell populations in these processes, and direct correlations have recently been identified between certain circulating immune cells and bone regeneration in preclinical models of trauma. However, therapeutic strategies leveraging or targeting these immune cell populations to improve bone healing outcomes remain understudied. This work aimed to address this problem by characterizing immune cells and cytokines that are dysregulated after severe injury and by investigating novel methods for modulating immune cells that have been correlated to impaired bone healing.

One key population of immune cells that has been previously implicated in impaired bone regeneration in a rat model of trauma are myeloid-derived suppressor cells (MDSCs). MDSCs represent a heterogeneous population of immature myeloid cells that expand rapidly after injury and are hallmarked by their potent immunosuppressive capacity. In this work, we aimed to target and deplete these cells systemically in a rat model of bone and muscle polytrauma using two different therapeutic strategies. First, we employed Synthetic Nanoparticle Antibodies (SNAbs), a multivalent nanotherapeutic platform that serves as a synthetic alternative to monoclonal antibodies for targeted cell depletion. SNAbs were used to target S100A8/A9, a

protein complex known to be expressed by MDSCs. However, systemic administration of SNAbs only decreased the relative frequency of a sub-population of MDSCs in rats with polytrauma, while another sub-population of MDSCs actually expanded and healing outcomes worsened following SNAb treatment. Therefore, in a subsequent study, we attempted to instead modulate MDSCs using all-trans retinoic acid (ATRA), a vitamin A derivative that has previously been shown to promote differentiation of MDSCs into mature myeloid cell types and reduce their immunosuppressive capacity in models of cancer. ATRA diminished MDSCs *in vitro*, but it only resulted in depletion of a sub-population of MDSCs when administered *in vivo* to rats with polytrauma, and it failed to improve bone regeneration.

To provide further insights for potential future immunomodulatory strategies, we next aimed to expand our characterization of the cellular immune response to trauma to tissues local to the site of injury. We accomplished this by employing spectral flow cytometry to analyze several immune cell populations in the bone defect site, the adjacent muscle, and the adjacent bone marrow in a rat model of femur trauma. This revealed multiple immune cell populations that became altered following injury, mimicking certain patterns of immune dysregulation previously observed in the peripheral blood after injury.

Altogether, this work helped elucidate the development and time course of trauma-induced immune dysregulation, identified novel immunomodulatory strategies and their limitations in therapeutically altering systemic immune cells after injury, and ultimately highlighted the need for additional study of trauma immunology as a potential pathway for improving bone regenerative outcomes.

This dissertation includes previously published and unpublished co-authored material.[179, 269, 270]

PUBLICATIONS

Vantucci, C. E. and Guyer T. *et al.* Systemic Immune Modulation Alters Local Bone Regeneration in a Delayed Treatment Composite Model of Non-Union Extremity Trauma. *Front. Surg*. **9**, 934773 (2022).

Khodabukus, A., Guyer, T., *et al*. Translating Musculoskeletal Bioengineering Into Tissue Regeneration Therapies. *Sci. Transl. Med*. **14**, eabn9074 (2022).

Maniar, D., Keenum, C., Vantucci, C.E., Guyer, T. *et al*. Single-cell transcriptomic analysis identifies systemic immunosuppressive myeloid cells and local monocytes/macrophages as key regulators in polytrauma-induced immune dysregulation. *Bone Res*. **13**, 69 (2025).

Guyer, T., Gill, D., Leguineche, K.E., Link, K., Jones, K.A., Williams, K.E., Strait, L.K., O'Neill, K.L., Hastings, M.A., Lin, A., Maniar, D., Roy, K., & Guldberg, R.E. All-trans Retinoic Acid Modulates Circulating Immune Cells in a Preclinical Model of Musculoskeletal Trauma. *Manuscript in Preparation*.

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Dedicated to Alexander Harteau (2011–2025)

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CHAPTER 1: INTRODUCTION

Severe musculoskeletal trauma is exceedingly common, and despite recent advances in acute trauma care that have increased survival, complication rates remain high. The immune response to trauma has been highlighted as one important factor in bringing about successful bone regeneration after a fracture. In particular, immune cells local to the site of injury play crucial roles in the early stages of repair, helping to prevent infection, clear cellular debris, and activate progenitor cells. Recently, attention has also been given to the systemic immune response to trauma, which includes circulating populations of immune cells and cytokines that are dysregulated after injury and correlate with bone regenerative outcomes. In particular, one population of immune cells that expresses both myeloid (CD11b/c) and granulocyte (HIS48) markers has previously been correlated to impaired bone healing in a rat model of trauma. These cells have been referred to in previous literature as myeloid-derived suppressor cells (MDSCs), a heterogeneous population of immature myeloid cells that expand rapidly after injury and possess potent immunosuppressive functions, and they may have potential as a novel therapeutic target. Still, the roles of specific immune cell populations in musculoskeletal regeneration and the potential benefits that could be achieved by therapeutically modulating them remain largely understudied. As such, there remains a critical need to more broadly characterize the immune response to injury in both systemic and local tissues and to investigate novel therapeutic strategies that target immune cells to improve functional healing.

To address this gap, here we employed a rat model of composite bone and muscle trauma to investigate trauma-induced immune dysregulation. The overall objectives of this thesis were to (i) characterize both the systemic and local immune responses to severe musculoskeletal trauma and their relationship to bone healing, and (ii) investigate immunomodulatory strategies

for altering the systemic immune response to bone trauma and their subsequent effects on bone repair. Our overall hypotheses were that (i) both systemic and local populations of immune mediators including cells and cytokines are dysregulated after composite trauma and correlate with bone regenerative outcomes, and (ii) depletion of MDSCs would ameliorate trauma-induced immune dysregulation and subsequently improve bone healing in a rat model. We tested these hypotheses through the following specific aims:

1.1 Specific Aim 1: Characterize the immune response to severe musculoskeletal trauma, including both systemic and local immune cells and cytokines, and identify markers that are dysregulated after injury or correlate to bone healing outcomes.

It is generally accepted that successful bone regeneration is dependent in part on the initial inflammatory response to injury as well as its timely resolution. Excessive or prolonged immune dysregulation appears to be detrimental to healing, and sustained elevation or depression of certain circulating immune cells has been correlated to impaired healing outcomes in preclinical trauma models. The objective of this aim was to investigate how levels of immune mediators including both cells and cytokines in the blood and in tissues local to the injury site respond to severe musculoskeletal trauma and affect its outcomes. This was accomplished using a preclinical rat model of composite bone and muscle trauma. We hypothesized that various immune cell and cytokine populations would be dysregulated in both systemic and local tissues early after traumatic injury and that these populations would correlate with endpoint bone regeneration. Content related to this aim has been adapted from Guyer T., Gill D., Link K., Strait L.K., Leguineche K.E., O'Neill K.L., and Guldberg R.E. (manuscripmanut in preparation).[270]

1.2 Specific Aim 2: Develop and optimize strategies to modulate MDSCs and evaluate their effects on the immune system and bone regeneration in a rat model of severe musculoskeletal trauma.

MDSCs have been previously shown to correlate to impaired bone regeneration in rat models of musculoskeletal trauma. However, it remains unclear whether this correlation indicates that MDSCs directly impair bone healing or whether targeting and depleting them would have a therapeutic benefit. To assess this, we employed two immunomodulatory strategies that have previously been shown to deplete or alter the function of MDSCs in other models. These strategies included the use of Synthetic Nanoparticle Antibodies (SNAbs), a synthetic nanotherapeutic platform that can target specific cells of interest, and all-trans retinoic acid (ATRA), a vitamin A derivative previously shown to promote differentiation of MDSCs into mature cell types. The objective of this aim was to test these strategies *in vivo* in a preclinical rat model of composite bone and muscle trauma and determine their effects on circulating immune cells and bone regeneration. We hypothesized that systemic administration of these therapeutics would result in targeted depletion of MDSCs and would consequently ameliorate trauma-induced immune dysregulation and improve bone healing. Content related to this aim has been adapted from Vantucci C.E. and Guyer T., Leguineche K., Chatterjee P., Lin A., Nash K.E., Hastings M.A., Fulton T., Smith C.T., Maniar D., Frey Rubio D.A., Peterson K., Harrer J.A., Willett N.J., Roy K., and Guldberg R.E. (2022, Frontiers in Surgery),[179] as well as Guyer T., Gill D., Leguineche K.E., Link K., Jones K.A., Williams K.E., Strait L.K., O'Neill K.L., Hastings M.A., Lin A., Maniar D., Roy K., and Guldberg R.E. (manuscript in preparation).[269]

IMPACT

This work provided new knowledge of the immune response to severe musculoskeletal trauma, its time course, its variation across distinct tissue compartments, its relationship with bone regeneration, and novel strategies for modulating immune cells to combat trauma-induced immune dysregulation.

CHAPTER 2: BACKGROUND

2.1 Pathophysiology of Complex Bone Fractures

Severe trauma is the leading cause of death and disability in people below the age of 45,[1,2] and although recent improvements in acute trauma care have increased rates of survival and limb salvage, this has unfortunately been accompanied by a consequent increase in late emerging complications.[3] Such complications commonly include delayed bone fracture healing or bone nonunions. Although the precise definition of the term is subjective, a nonunion is generally defined as a bone fracture in which the normal biological regenerative processes cease to such an extent that functional healing will not occur without further clinical intervention.[4] Such cases often require repeat surgical interventions and may lead to amputation, imparting significant health and financial burdens on patients. Although the incidence of nonunions has varied considerably in past studies and is highly dependent on patient-specific factors as well as injury type and severity,[5] one large population-based study in Scotland recently reported a nonunion rate of approximately 1.9% among all patients with bone fractures, although higher nonunion rates were reported for fractures of certain bones such as the tibia or clavicle.[6] In the United States, this corresponds to approximately 100,000 total nonunions annually.[7] Furthermore, these injuries result in substantial healthcare expenses. One large-scale study of bone fractures that occurred in the United States during the calendar year 2006 found that the median direct cost for treatment of an open tibial fracture nonunion among patients with medical insurance was $25,556 USD.[8] Additionally, this estimate did not include indirect costs such as loss of productivity or costs to society. In cases where amputation is carried out, the projected total lifetime cost for a single patient has been projected to exceed $500,000 USD.[9] Still, bone nonunions and the repeat surgical procedures required for their treatment impart such significant

health burdens on patients that those whose limbs are salvaged following trauma on average report experiencing lower quality of life than those who undergo amputation.[10,11] These findings taken together suggest a critical need for further innovation in the treatment of severe musculoskeletal injuries.

Numerous patient-dependent factors affecting the risk of developing a nonunion after a fracture have been identified, including age, sex, medical comorbidities, genetic factors, metabolic disease, nutritional deficiency, and the use of certain medications such as non-steroidal anti-inflammatory drugs.[12–18] Delayed or failed fracture healing can also be exacerbated by factors related to the injury, including fracture pattern, location, concomitant soft tissue damage, and the presence of infection.[6,19,20] Further complicating matters, therapeutic interventions to treat failed bone healing are often delayed as a result of the time required to diagnose nonunions. The U.S. Food and Drug Administration defines a nonunion as a fracture that has persisted for at least 9 months and has shown no signs of healing for at least 3 consecutive months,[21] and treatments are often administered only after this diagnosis is established via radiographic evidence. Apart from prolonging patient suffering and disability, this delay may also worsen treatment outcomes, as the earlier stages of bone repair immediately after injury feature enhanced wound and systemic healing environments which may be more conducive to successful bone regeneration.[22–25]

Treatment of severe musculoskeletal injuries presents significant clinical challenges due to the need to simultaneously manage stabilization of complex fractures, soft tissue injuries, and local infection. An illustrative example of the clinical treatment of nonunion is demonstrated in Figure 1, which depicts a case of severe lower limb extremity trauma. This patient initially presented with a Gustilo Type IIIA open tibia fracture including concomitant soft tissue damage.

The treatment course for this patient over the first six weeks alone required initial debridement of the bone and soft tissues, stabilization of the fracture with an external fixator, implantation of antibiotic-loaded poly-methyl-methacrylate (PMMA) beads to address significant contamination at the injury site, subsequent removal of the external fixator and implantation of an internal plate fixator, and autogenous bone grafting to the defect site. Despite these interventions, the patient returned after six months post-injury with broken plates due to a lack of bone regeneration, requiring repeat internal fixation and repeat autogenous bone grafting. This clinical case exemplifies both the myriad challenges associated with the treatment of severe extremity trauma and the prolongation of patient suffering resulting from a lack of means to diagnose or predict complicated healing outcomes earlier in the course of treatment. As such, there remains a need for the development of novel therapeutic strategies to address bone nonunions and the identification of novel biomarkers for identifying cases that are likely to progress to nonunion.

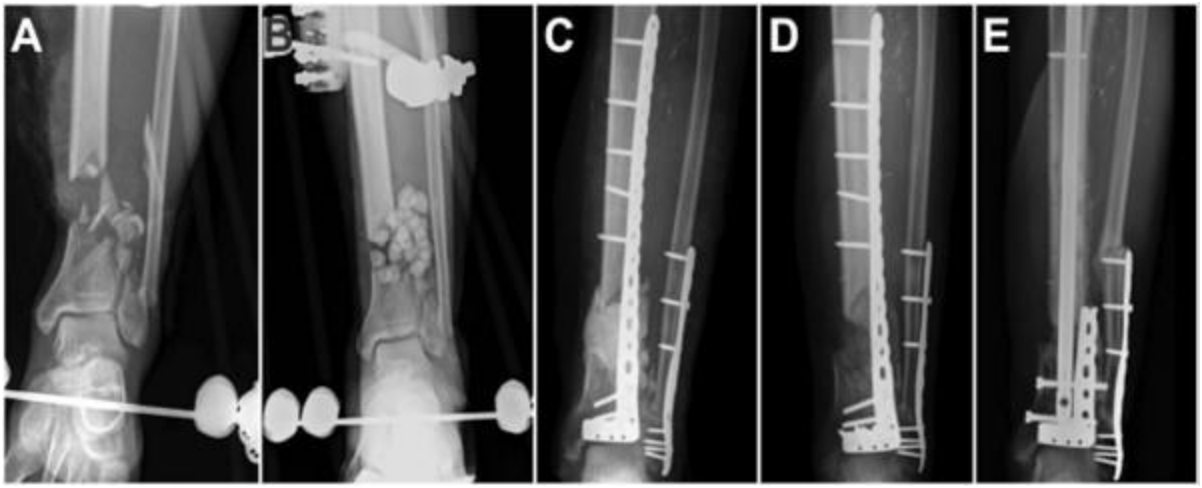


Figure 1. Clinical progression of a typical case of nonunion presented as longitudinal radiographs. (A) 24 year old Male with left Gustilo type IIIA open distal tibia and fibula fracture, initially treated with external fixator, irrigation, and debridement. (B) Due to significant contamination, antibiotic impregnated PMMA beads were implanted during one of the early debridement procedures (day 3 post-injury). (C) 2 weeks after injury, the external fixator was removed, and open reduction internal fixation of tibia and fibula fracture was accomplished with a plate fixator; a PMMA spacer was placed in the bone defect. (D) 6 weeks after PMMA spacer placement, the patient returned to the OR for spacer removal and autogenous bone graft to the defect site. (E) At 6 months, the patient returned with broken plates due to the lack of healing of the bony injury. The patient had revision of internal fixation with an intramedullary nail. Note the small regenerate bone from the bone graft. Courtesy of Dr. Leucht P., NYU Langone

2.1.1 Mechanisms and Stages of Bone Repair

Bone possesses a remarkable capacity for regeneration, even in cases of complex fractures, without the formation of scar tissue. When healing is successful, newly formed bone is indistinguishable from surrounding healthy bone, and this process can therefore be considered a form of tissue regeneration.[26] Depending on the mechanical stabilization of the fracture site, bone repair can occur through one of two mechanisms: primary healing, or secondary healing.[27] Primary healing, also referred to as direct healing, involves the direct union of bone fragments without the formation of an intermediate cartilage callus. This form of bone regeneration occurs primarily via intramembranous ossification, whereby new bone is formed from mesenchymal cells.[28] However, primary healing requires bony fragments to be perfectly reduced, aligned, and

fixed with interfragmentary compression and minimal to no movement at the fracture site.[29] This necessitates specific surgical interventions that are challenging and not suitable for all type of injuries. Secondary or indirect bone healing, which is considerably more common, involves the formation of a callus as an intermediate step in the regeneration of bone. This method of bone regeneration requires a lesser degree of stabilization of the fracture site as compared to primary healing and involves both intramembranous ossification and endochondral ossification, in which bone forms from a cartilage precursor.[28] Secondary bone formation can be divided into four partially overlapping stages: the acute inflammatory response and hematoma formation; formation of a fibrocartilaginous callus; ossification of the fibrocartilaginous network to form a bony callus; and finally remodeling of the bony callus to form compact bone centrally and lamellar bone peripherally.

The inflammatory phase begins immediately after injury and typically lasts approximately 1 week.[30] During this stage, the hematoma coagulates to form a temporary scaffold for later callus formation and acts as a source for inflammatory cells that direct cell recruitment.[31,32] Mesenchymal stem cells (MSCs) migrate to the fracture site from nearby tissues and from circulation.[26,33] These cells possess the capacity to differentiate into a variety of cell types involved in wound healing including adipocytes, myocytes, chondrocytes, and osteoblasts.[34,35] MSCs along with immune cells also release bone morphogenetic proteins (BMPs), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF-α), and various interleukins, all of which stimulate essential cellular biology at the fracture site.[28,36]

The next stage of secondary bone repair is the formation of a fibrocartilaginous callus within the fracture site. During this stage, chondrogenesis occurs, driven by chondroblasts

differentiated from MSCs.[37] Fibroblasts further contribute to this process by secreting collagen and proteins of the extracellular matrix.[38] The resulting soft callus serves dual purposes, both mechanically stabilizing the injury site and providing a scaffold to help enable subsequent deposition of bone tissue.

Over multiple weeks, the soft, fibrocartilaginous callus is gradually replaced with a mineralized bony callus.[39] Osteoblasts differentiate primarily from MSCs in a process guided by BMPs.[40] To help facilitate endochondral ossification, these cells then invade the soft callus and deposit bone matrix which mineralizes to form new bone tissue. The resulting hard callus provides increased structural stability to the fracture site and, in the case of successful healing, effectively bridges the fracture gap.

Finally, bone remodeling occurs over the span of multiple months after hard callus formation. During this step, osteoblasts and osteoclasts respectively deposit and resorb bone tissue, reshaping the hard callus and slowly improving the strength and integrity of the bone.[41] If successful, bone remodeling should effectively restore the original functional and mechanical properties of the bone.

2.1.2 Essential Factors in Bone Repair

Broadly, four essential elements have been recognized as being critical for successful bone regeneration following injury: osteoinductive mediators, osteogenic cells, an osteoconductive matrix, and a pro-regenerative mechanical environment. Together, these factors have often been referred to as the diamond concept,[42] and efforts to provide solutions for impaired fracture healing generally target one or multiple of these factors.

Osteoinductive mediators include growth factors and other proteins involved in bone regeneration. Platelets and immune cells in the early fracture hematoma release various

cytokines that stimulate signaling cascades to initiate healing. Certain interleukins such as IL-1 and IL-6 promote inflammation, TNF-α regulates the inflammatory response and osteogenic protein expression, macrophage colony-stimulating factor influences the recruitment and activity of macrophages which are essential to bone regeneration and remodeling, and osteoprotegerin (OPG) and receptor activator of nuclear factor kappa B ligand (RANKL) regulate osteoclastogenesis, as examples.[43–46] Numerous growth factors play key roles in promoting differentiation of progenitor cells as well, including fibroblast growth factor (FGF), TGF-β, PDGF, and BMPs such as BMP-2 and BMP-7.[47–50] VEGF is another important growth factor which promotes angiogenesis,[51] a crucial early step in bone regeneration. Successful repair relies on the proper coordination of these many osteoinductive mediators.

Osteogenic cells include MSCs and committed osteoprogenitor cells of the periosteum. These cells migrate to the fracture site from adjacent tissues and from circulation, and they play central roles in both fibrocartilaginous callus formation and bony callus formation. MSCs, guided by osteoinductive mediators, differentiate into both chondrocytes and osteoblasts during the regenerative process, secreting cartilage matrix and depositing bone mineral to facilitate endochondral ossification.[52] They also have been shown to influence angiogenesis and help regulate and resolve inflammation.[53,54]

An osteoconductive matrix or scaffold is critical to successful bone regeneration, promoting both recruitment and adhesion of osteogenic cells to the fracture site. Immune cells in the early hematoma have been shown to produce a fibronectin-rich extracellular matrix rapidly after injury, providing an early scaffold that helps conduct infiltration of MSCs to the fracture site.[55] Later, other cells such as fibroblasts and osteoblasts contribute to the osteoconductive

matrix through production of collagenous proteins, proteoglycans, glycoproteins, osteocalcin, and hydroxyapatite.[56]

Lastly, the local mechanical environment affects the course of bone regeneration by multiple mechanisms. The differentiation of progenitor cells into mature cell types crucial for bone repair relies in part on mechanical cues and stability in the physiological environment of the fracture site.[57] Local tension, shear, and compression in conjunction with osteoinductive mediators influence the differentiation trajectories of progenitor cells into fibroblasts, chondroblasts, and osteoblasts.[58] Osteoblasts and osteoclasts are also mechanosensitive, meaning that their activity and resulting bone formation and resorption are dependent on mechanical signals, which therefore play significant roles in both bone regeneration and remodeling.[59] Similarly, cells of the vasculature are mechanosensitive, and therefore angiogenesis after injury is also reliant on mechanical cues.[60,61] For all of these reasons, fixation of the fracture site to ensure mechanical stability is central to the normal clinical treatment of severe bone injuries.[62,63]

2.1.3 Clinical Therapeutic Strategies for Treatment of Severe Bone Injuries

To date, with over a century of successful use, and despite considerable research and development of alternatives, autologous bone grafts remain the clinical gold standard for the treatment of severe bone injuries.[64] The grafting of autologous bone can simultaneously address multiple essential factors in successful bone regeneration. Depending on the type of graft, transplanted bone may provide a porous, osteoconductive, three-dimensional scaffold that enhances the migration of key progenitor cells.[65] Furthermore, autologous bone, and especially cancellous autograft, contains both cells with osteogenic potential and osteoinductive growth factors.[66] Lastly, bone grafts can offer mechanical or structural support to the injury site as well.[64] Still, this gold standard procedure continues to face significant limitations. Most notably,

autologous grafts require a longer and more complex surgery, patients may suffer from donor site morbidity, and the supply of autologous bone that can be harvested is limited.[67] Furthermore, as demonstrated earlier in the clinical case presented in Figure 1, grafts do not always result in robust, successful bone regeneration. Despite these limitations, grafts remain the standard of care for the treatment of large bone defects.

Besides autologous grafts, most alternative therapeutic strategies for severe bone trauma have not seen widespread clinical use. A comprehensive review of the alternative methods for enhancing bone repair that have been studied in clinical settings and in preclinical models is beyond the scope of this work, and this subject has already been reviewed extensively elsewhere.[68,69] However, one notable group of therapeutics that have been approved previously for use in clinics are bone morphogenetic proteins, specifically BMP-2 and BMP-7.[70] These growth factors are powerful osteoinductive mediators capable of robustly promoting bone growth when delivered to the fracture site at sufficient therapeutic doses. Still, the use of BMPs also faces drawbacks including considerable side effects such as postoperative inflammation, ectopic bone formation, osteoclast-mediated bone resorption, and inappropriate adipogenesis.[71] As such, between the limitations faced by both autologous grafts and BMPs, there remains a need for the development of novel therapeutic methods to supplement existing treatment modalities.

2.2 The Immune Response to Musculoskeletal Trauma

The immune response to trauma is crucial to successful wound repair, and therefore strategies aimed at modulating this response are promising for enhancing healing outcomes. As discussed in the previous section, acute inflammation begins rapidly after injury and comprises the first stage of the bone regenerative process. Previous research has demonstrated that this

inflammatory phase is essential to successful bone healing. For example, some studies have found that the use of nonsteroidal anti-inflammatory drugs by patients with long bone fractures significantly increased their risk of suffering complications such as nonunion or infection.[18,72] Similarly, immunodeficient NOD/scid-IL2Rγcnull mice lacking functional innate and adaptive immune cells exhibit delayed endochondral ossification during the later stages of bone repair,[73] and knockout studies targeting various inflammatory cytokines and chemokines in mice have demonstrated delayed fracture healing and poorer functional regeneration.[74,75] On the other hand, excessive or chronic inflammation can also hinder bone repair. Excessive or prolonged local infiltration and activation of neutrophils or pro-inflammatory macrophages has been shown to cause impaired healing, for example.[76,77] Beyond the local fracture site, excessive inflammation can also induce an early catabolic state systemically, resulting in damage to other organs that, in the most extreme cases, may cause multiple organ dysfunction or organ failure.[78]

To balance inflammation and prevent excessive local and systemic catabolism, a simultaneous anti-inflammatory response is also initiated early after injury. Marked by elevation of immunosuppressive mediators such as IL-10 and suppression of adaptive immunity most notably through depletion of T cells, this anti-inflammatory response ideally limits and localizes the immune response to severe trauma.[79] Still, prolonged or exaggerated immunosuppression has also been linked to poor outcomes.[80,81] For example, both acute elevation of IL-10 and prolonged suppression of T cells after injury have been associated with complications in human patients and in animal models.[79,82,83] Altogether, these results appear to indicate that successful bone regeneration requires a properly balanced local and systemic immune response, including both inflammatory and anti-inflammatory signals that are present but not excessively elevated and that resolve in a sufficiently timely manner.

2.2.1 Cellular Mediators of the Immune Response to Trauma

Numerous immune cell populations mediate the immune response to trauma throughout the normal course of fracture healing. These cells and their roles in bone repair have been recently reviewed in detail elsewhere,[84,85] but a brief summary of key findings regarding relevant, critical immune cell populations in trauma will be presented here.

Neutrophils are phagocytic myeloid cells, and they along with other granulocytes are among the first immune cells to infiltrate the fracture hematoma. Within 48 hours of injury, neutrophils make up the majority of cells present at the injury site, and they contribute to fracture healing by synthesizing fibronectin-containing extracellular matrix before stromal cells are significantly recruited to the hematoma.[55] Neutrophils also take part in the removal of cellular and tissue debris and the later dissolution of the hematoma,[86,87] and they are key producers of multiple cytokines that attract monocytes to the fracture site.[88] Still, prolonged local activation of neutrophils has been linked to diminished osteogenic activity, reduced callus mineralization, and poor healing,[76,89] suggesting they may have deleterious effects under excessive or chronic inflammatory conditions. In line with this, studies aimed at depleting neutrophils shortly before or after injury have had mixed results, with some demonstrating improved healing capacity[90,91] and others demonstrating impaired or delayed healing.[92] These discrepancies may have resulted from differences in the timing and methods of neutrophil depletion across studies, or they may be linked to the multiple distinct phenotypes of neutrophils induced after trauma which could have different functions in tissue repair.[93]

Macrophages are also phagocytic cells of myeloid lineage. Macrophages differentiate from monocytes which are recruited to the fracture site from circulation by cytokines including macrophage inflammatory protein-1 (MIP-1) and IL-1β.[94,95] Macrophages are involved in both

the removal of cellular debris and the secretion of key cytokines involved in inflammatory and regenerative signaling cascades.[96,97] Multiple phenotypes of macrophages have been characterized in trauma, and they are generally classified as M0 (naive) macrophages, M1 (pro-inflammatory) macrophages, or M2 (anti-inflammatory or pro-regenerative) macrophages. M1 macrophages have been shown to be important regulators of the early inflammatory response at the fracture site, and they secrete important mediators of angiogenesis such as VEGF and TNF-α.[98] M2 macrophages later help resolve inflammation to yield a local osteoimmune environment conducive to bone regeneration.[99,100] The timely polarization of macrophages from the M1 phenotype to the M2 phenotype is therefore crucial to successful regenerative outcomes.[101] If M1 macrophages remain elevated at the fracture site for a prolonged period, the inflammatory state may fail to resolve in a timely manner, impeding bone regeneration.[102,103]

Lymphocytes, including both T cells and B cells, infiltrate the fracture callus in a two-wave fashion, appearing both during the initial inflammatory phase of healing and later during callus mineralization.[104] T cells and B cells produce both RANKL and OPG, which are involved in regulation of osteoclasts and bone resorption.[105–107] Near the end of the inflammatory phase, B cells appear to be involved in suppression of pro-inflammatory cytokines and the stimulation of T regulatory (Treg) cells.[108] A notable subset of T cells called γδ T cells have also been found to produce IL-17A, which accelerates bone formation by stimulating MSC proliferation and their differentiation into osteoblasts.[74] Tregs, meanwhile, have been shown to suppress osteoclast formation, partially through expression of the cytokines TGF-β, IL-4, and IL-10.[109] Overall, the effects of lymphocytes on bone repair appear numerous and varied.

While most previous studies on the cellular mediators of the immune response to trauma focused on their local effects at the fracture site, more recently, increased attention has been

given to the systemic immune response to trauma as well. Like elevated or prolonged local inflammation, excessive systemic inflammation may also be deleterious to bone repair. For example, inducing heightened systemic inflammation using blunt chest trauma has been shown to impair bone healing following a femur osteotomy,[110] while suppression of systemic inflammation via inhibition of the complement cascade involved in the innate immune system ameliorates this effect.[111] As such, cases of polytrauma in particular, where multiple regions of the body are injured simultaneously, may warrant additional consideration for the treatment of systemic inflammation to improve local bone repair. Recently, our lab has employed a rat model of musculoskeletal trauma to examine correlations between systemic immune cell and cytokine populations in circulation after injury and subsequent bone regeneration. Interestingly, circulating T cells have been found to correlate positively with endpoint bone volume in this model, while circulating monocytes have been found to correlate negatively.[112] However, one especially notable population of cells that have been shown repeatedly to correlate negatively with bone regeneration in rat models are a group of myeloid cells referred to as myeloid-derived suppressor cells (MDSCs), which will be discussed at length in the next section.

2.2.2 Myeloid-Derived Suppressor Cells in Musculoskeletal Trauma

MDSCs are a heterogeneous population of immature myeloid cells that expand in many pathological conditions including trauma and cancer and are hallmarked by their potent immunosuppressive capacity.[113] MDSCs originate from hematopoietic stem cells due to an abnormal form of myelopoiesis.[114] Typically, emergency myelopoiesis occurs as a normal part of the immune response to infection or tissue damage, resulting in an expansion of immature myeloid cells which then differentiate into monocytes and granulocytes. This process is mediated by signals originating from pathogen-associated molecular patterns and damage-associated

molecular patterns, ultimately contributing to acute inflammation.[115] However, certain pathological conditions including chronic inflammation or cancer may result in a form of altered, sustained myelopoiesis which causes immature myeloid cells to deviate from their normal differentiation trajectories.[116] These cells, termed MDSCs, can be distinguished from mature myeloid cells by their morphology, their gene expression patterns, and most importantly, their immunosuppressive functions.

MDSCs mediate immunosuppression of numerous immune cell populations through a variety of mechanisms. MDSCs deplete metabolites, including L-arginine and cysteine, that are critical to T cell functions.[117–119] They produce reactive oxygen species, which are toxic to most cell types including immune cells, and which themselves further hinder MDSC differentiation, creating a positive feedback loop.[120] They produce reactive nitrogen species, which can inhibit IL-2 signaling and T lymphocyte activation.[121,122] Finally, MDSCs participate in the induction, proliferation, and activation of Tregs via cytokines mediators including immunosuppressive IL-10 and TGF-β.[123] As a result of these functions and others, MDSCs ultimately contribute to dysregulation and suppression of the systemic immune response.

The relationship between MDSCs and musculoskeletal trauma remains understudied. Furthermore, early studies on these cells revealed some mixed findings regarding whether they are beneficial or detrimental to trauma outcomes. MDSCs in mouse models of trauma have been shown to cause T cell dysfunction,[113] which could be disruptive to healing given the roles of T cells in bone repair. Furthermore, MDSCs have been associated with a state of chronic immune dysregulation and immunosuppression that often follows sepsis, and their elevated levels have been correlated with adverse sepsis outcomes.[124,125] Elevation of MDSCs after polytrauma has also been associated with an increased susceptibility to infection.[126] On the other hand, these

cells could also be beneficial to healing by acting as counter-inflammatory mediators. One recent study in mice provided evidence that MDSCs contribute to the transition from the early inflammatory phase of bone healing to the later repair phases, in part by supporting osteoblast differentiation and producing osteocalcin.[127] The induction and proliferation of Tregs by MDSCs may also be of some benefit, as Tregs have recently been indicated to play roles in bone anabolism and repair.[128,129] Additionally, while elevated MDSCs have been correlated to worse outcomes in sepsis, depletion of these cells in a mouse model of sepsis actually led to decreased survival.[130] Taken together, these results do not provide a clear consensus on whether MDSCs enhance or hinder trauma outcomes, or whether and how their expansion and function should be manipulated. To further complicate matters, phenotypic markers for MDSCs vary between humans, mice, and rats,[131,132] and the cells also appear to be qualitatively different between pathologic conditions, demonstrating higher MHC class II expression in trauma than in cancer or sepsis for example.[113,133] Nevertheless, our lab has previously demonstrated that circulating levels of MDSCs after injury correlate with impaired bone regeneration and other complications in multiple rat models of bone trauma.[112,134] Systemic MDSCs may therefore represent a novel therapeutic target for ameliorating trauma-induced immune dysregulation and improving healing outcomes.

Previous attempts at modulating MDSCs have primarily been conducted in cancer models, where the cells down-regulate immune surveillance and antitumor immunity.[135] Strategies for targeting MDSCs generally fall into one of three categories: inhibition of MDSC immunosuppressive activity; blockade of MDSC migration and recruitment; and regulation of myelopoiesis or depletion of MDSCs.[136] Modulation of the suppressive activity of the cells has been performed via inhibition of the proteins STAT3 and PDE5. STAT3 is an important

transcription factor involved in MDSC-mediated immunosuppression, and it has been shown to be inhibited by AZD9150 and Napabucasin, for example.[137,138] However, some of the most promising drugs known to inhibit STAT3 have been shown to result in adverse side effects, though this may have been unrelated to STAT3 inhibition itself.[139,140] Inhibition of PDE5 can be conducted using such drugs as tadalafil and sildenafil, and this has been shown to reduce MDSC immunosuppressive activity and increase T cell reactivity via down-regulation of arginase-1 and reactive nitrogen species.[141,142] Other methods for targeting MDSC functions include use of a class I histone deacetylase inhibitor, entinostat,[143] and inhibition of fatty acid oxidation, targeting the cells' metabolic pathways.[144] Meanwhile, methods for inhibiting MDSC migration to the tumor site in cancer models have been reported, primarily by blocking activation of the chemokine receptors CXCR2 and CCR5.[145,146] Finally, mechanisms for depleting MDSCs include strategies that inhibit the conversion of immature myeloid cells into MDSCs, cause apoptosis of the cells, or stimulate differentiation of MDSCs into mature myeloid cells. Multiple tyrosine kinase inhibitors have been shown to limit the differentiation of myeloid cells into MDSCs.[147] Certain chemotherapeutic drugs such as gemcitabine and 5-fluorouracil have been demonstrated to cause selective apoptosis of the cells in the tumor microenvironment.[148,149] Targeting of proinflammatory S100 proteins such as S100A8/A9, which plays roles in MDSC accumulation, has previously appeared to reduce their levels in the blood and secondary lymphoid organs.[150] Finally, all-trans retinoic acid, a vitamin A derivative, notably induces differentiation of MDSCs into terminal myeloid cells such as monocytes and dendritic cells by upregulating synthesis of glutathione, an oxygen scavenger that eliminates reactive oxygen species.[151] Overall, the existing methods for MDSC modulation in the context of cancer are broad and varied. However, it remains to be studied how such methods might affect MDSCs

induced by musculoskeletal trauma or whether successful modulation of the cells would have therapeutic benefits for trauma patients.

2.2.3 Previous Immunomodulatory Strategies for Bone Regeneration

The goal of immunomodulatory therapies for musculoskeletal trauma is to improve functional outcomes either by leveraging immune mediators known to play roles in healing or by ameliorating trauma-induced immune dysregulation to restore a pro-regenerative osteoimmune environment. Many methods of immunomodulation have been investigated. Cell-based strategies comprise a significant category of immunomodulatory therapeutics with great promise. Perhaps most notably, MSCs, in addition to their important proliferative roles during normal bone regeneration, also demonstrate potent immunomodulatory effects including repression of T cell expansion and activation, B cell activation, and dendritic cell maturation and migration.[152–156] They also appear to support the preferential shift of macrophages from a pro-inflammatory M1 phenotype to an anti-inflammatory M2 phenotype.[157] Through such effects, MSCs appear to help regulate and resolve inflammation, and as a result, direct delivery of MSCs systemically or locally to the fracture site and improvement of MSC homing to the fracture site have been explored as immunomodulatory strategies.[158–160] Products of MSCs, such as extracellular vesicles derived from the cells, are also of interest as cell-free alternatives that can be delivered to the injury site to regulate inflammation and promote osteogenesis.[161–163] Apart from MSCs, immune cells could also be delivered locally to enhance healing. One recent study demonstrated that local delivery of Tregs, which also help resolve inflammation, improved tissue healing in bone, muscle, and skin injuries in mice, for example.[164] Similar to MSCs, immune cells are also of interest for products they contain or secrete as well. Exosomes derived from Tregs and macrophages have both been shown to have potential for modulating the immune response and

promoting osteogenesis and fracture repair.[165,166] Finally, immune cells can also be targeted and depleted as a therapeutic strategy. Systemic depletion of myeloid cells in a model of polytrauma with prolonged inflammation was recently found to improve bone healing.[167] Similarly, terminally differentiated $CD8^+$ effector memory T cells have been implicated in impaired bone regeneration, and their systemic depletion appears to improve healing.[168]

Apart from cell-based strategies, various drugs and cytokines have also been investigated for immunomodulatory applications. The effects of corticosteroids and non-steroidal anti-inflammatory drugs, which are both generally immunosuppressive, have been investigated in both animal models of trauma and in trauma patients. However, multiple studies have found that the use of these drugs was actually associated with impaired fracture healing.[169–173] This underscores the importance of inflammation as an early stage of bone repair and suggests that even while timely resolution of inflammation is critical to healing, excessive suppression of the inflammatory response may also be harmful. Therefore, to avoid the detrimental effects of these nonspecific immunosuppressive agents, many more recent strategies have aimed to target portions of the immune response, such as by inhibiting individual cytokines involved in inflammation. Still, these have generally demonstrated limited efficacy in improving outcomes when used clinically. Blockers of the pro-inflammatory TNF-α, for example, have been shown to have no significant effect on bone healing,[174] while also potentially increasing the risk of patients developing infections.[175] Some more promising cytokine-based strategies have directly targeted pathways already known to be involved in bone regeneration. Local delivery of IL-4 in an aged rat model of trauma was recently shown to enhance polarization of M1 macrophages to M2 macrophages at the fracture site and promote bone healing, for instance.[176] Similarly, culturing MSCs in the presence of certain cytokines can alter their secretory profiles and influence their

functions. Preconditioning of MSCs using interferon gamma and TNF-α was shown to increase their anti-inflammatory and osteogenic functions, as one example.[177] Finally, immunomodulatory strategies for enhancing bone repair can also be used in combination with implantable biomaterials to improve their therapeutic effects. One study demonstrated the efficacy of this approach using bone scaffolds which sequentially released first interferon-gamma to promote M1 macrophages early on and then IL-4 to promote M2 macrophages later, and this strategy was found to improve both vascularization of the scaffolds and subsequent healing.[178]

Overall, a wide variety of immunomodulatory strategies have been investigated, with some demonstrating greater potential for enhancing healing outcomes than others. Work remains to be done to successfully translate these therapeutics into the clinic however. Ultimately, the most successful immunomodulatory strategies will likely be both timing-dependent and patient-specific, guided by a deeper understanding of both trauma-induced immune dysregulation and the effects of specific immune mediators on healing outcomes.

CHAPTER 3: SYSTEMIC IMMUNE MODULATION ALTERS LOCAL BONE REGENERATION IN A DELAYED TREATMENT COMPOSITE MODEL OF NONUNION EXTREMITY TRAUMA

Contents of this chapter were adapted from Vantucci C.E. and Guyer T., Leguineche K., Chatterjee P., Lin A., Nash K.E., Hastings M.A., Fulton T., Smith C.T., Maniar D., Frey Rubio D.A., Peterson K., Harrer J.A., Willett N.J., Roy K., and Guldberg R.E. (2022, Frontiers in Surgery).[179]

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3.1 Introduction

Despite the significant clinical challenge posed by fracture nonunions, the mechanisms and biology underlying nonunions are still poorly understood. Nonunions occur in approximately 1.9%–4.9% of fractures worldwide; however, the presence of concomitant soft tissue damage or infection drastically increases this risk.[180,181] Because of this, patients with composite injuries often face multiple interventions, extended rehabilitation time, increased treatment costs, and even amputation or significant long-term disability.[3,182,183] Improved treatment strategies with foundations in the biology and mechanisms of nonunion will be essential to improve patient outcomes, especially in challenging bone healing scenarios like composite tissue injuries.

The advent of osteoimmunology resulted in an increased focus on the local relationship between the immune system and functional bone regeneration.[184] More recently, the role of the systemic immune response has been highlighted as a contributing factor to poor outcomes and increased susceptibility to complications, such as infections, following severe musculoskeletal injury.[185,186] Traumatic injury, especially for open fractures, results in a large increase in damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs).[187] This subsequently sparks a large-scale systemic inflammatory response, often termed the systemic inflammatory response syndrome (SIRS), resulting in an increase of inflammatory cells and mediators (ex: T cells, IL-1, IL-6, TNFa). Left unchecked, this response can result in tissue damage, leading to multiple organ failure or even death. To protect against this, a concurrent, systemic anti-inflammatory response, termed the compensatory anti-inflammatory response syndrome (CARS), results in upregulation of anti-inflammatory cells and mediators (ex: MDSCs, Tregs, IL-10, TGFb). In patients with uncomplicated outcomes, these two systemic responses balance each other out and systemic immune homeostasis is restored.[188,189] However, in patients who experience complications, such as nonunions, the CARS response often overwhelms the SIRS response, leading to systemic immune dysregulation and immunosuppression, marked by mediators such as myeloid-derived suppressor cells (MDSCs) and interleukin-10 (IL-10).[79,190]

MDSCs are a heterogeneous and immature population of immunosuppressive myeloid-lineage cells that dramatically expand in response to traumatic injury during emergency myelopoiesis.[79,191] MDSCs potently suppress immune function, in particular T cells, through factors such as arginase, IL-10, TGFb, and reactive oxygen and nitrogen species (ROS/RNS).[191,192] MDSCs have also been shown to promote expansion and activity of

regulatory T cells, another immunosuppressive cell population, creating a feedforward mechanism to maintain immunosuppression.[193] Recent work in multiple rat trauma models has demonstrated a relationship between systemic levels of MDSCs and bone healing outcomes. For example, in a rat nonunion model, elevated blood levels of MDSCs and IL-10 negatively correlated with endpoint bone regeneration at multiple time points, including as early as one week post-treatment.[112,194] These data were further supported in another study using the same rat nonunion model and a rat composite trauma model in which circulating blood MDSCs negatively correlated with defect bone volumes and the rats with the highest defect bone volumes had significantly lower MDSCs compared to rats with the lowest defect bone volumes.[195] Lastly, in a rat model of infected trauma, infected rats exhibited significantly higher circulating MDSCs, significantly decreased circulating T cells, and a dysregulated cytokine response compared to uncomplicated non-infected fracture.[134] These studies together suggest that systemic immune dysregulation and immunosuppression play an important role in uncomplicated healing outcomes and that MDSCs could be a therapeutic target to restore systemic immune homeostasis, create a permissive and pro-regenerative immune environment, and ultimately improve bone healing outcomes in conjunction with a local treatment strategy.

The Roy lab has previously fabricated bifunctional Janus gold nanoparticles that mimic the structure and function of antibodies, containing an Fc-mimicking peptide on one half of the particle surface and an MDSC-targeting peptide on the other half of the particle surface. These particles were used in a mouse tumor model and shown to systemically deplete MDSCs, resulting in increased T and NK cell infiltration into the tumor environment.[196] The MDSC-targeting peptide was found via phage display screening to target S100A8/A9, a calcium binding protein that has been shown to impair neutrophil and leukocyte function, reduce cytokine

secretion, and inhibit antigen presentation on antigen-presenting cells.[150,197–200] S100A8/A9 is known to be highly present on myeloid-lineage cells following inflammation, such as MDSCs.[201] Based on their efficacy in the cancer model,[196] these MDSC-targeting nanoparticles could be a useful immunomodulatory therapeutic in conjunction with a local treatment strategy to improve healing outcomes following musculoskeletal trauma.

Therefore, the objective of this study was to use a novel, non-surgical nanoparticle approach targeting the systemic immune response to investigate how immunomodulation of MDSCs impacts regenerative outcomes following delayed treatment in a model of composite musculoskeletal trauma containing both a critically sized bone defect and a volumetric muscle injury. While the composite trauma model has been previously established and investigated, here an additional component of delayed treatment was added, representing the clinical delay in treatment of a nonunion defect and the biological changes that occur during that period. Along with a systemic immunomodulatory treatment, we utilized local administration of bone morphogenetic protein-2 (BMP-2), an osteoinductive protein with approval by the U.S. Food and Drug Administration for use in treating spinal defects. We hypothesized that the MDSC-targeting Janus nanoparticles in conjunction with a local BMP-2 treatment strategy would decrease systemic levels of MDSCs, resulting in improved bone regeneration compared to BMP-2 treatment alone. We also longitudinally monitored cytokine and immune cell populations systemically from the blood to identify predictive biomarkers of treatment outcomes and to characterize the immunological factors that contribute to the development of nonunions and poor healing. Single cell RNA sequencing was also conducted to better understand the role of the immunosuppressive MDSC populations in a model of composite trauma. A better understanding of the immunological markers associated with nonunion progression and poor healing could be

essential for facilitating patient-specific immunomodulatory care that optimizes and improves the current standard of care.

3.2 Materials and Methods

3.2.1 Nanofiber Mesh and Alginate BMP-2 Preparation

Nanofiber mesh and alginate hydrogel constructs were prepared as previously described.[202] Briefly, to make the polycaprolactone (PCL) nanofiber meshes, a 90:10 solution of 1,1,1,3,3,3-Hexafluoro-2-propanol, 99+% (HFP; Sigma Aldrich) and N,N-Dimethylformamide, anhydrous, 99.8% (DMF; Sigma Aldrich) was prepared. PCL was added to a concentration of 12% w/v in the 90:10 solution of HFP:DMF and allowed to dissolve overnight. Meshes were then electrospun to an approximate thickness of 500 μM and a laser cutter was used to create 0.7 mm diameter perforations throughout the mesh. UV cure adhesive (DYMAX) was used to glue the meshes to a rolled inner diameter of 4.5 mm and a length of 12 mm. Meshes were then sterilized by Ethylene Oxide and then stored in alpha-MEM at 4°C until use. To make the alginate hydrogel constructs, a 3% w/v alginate solution was created by dissolving RGD-functionalized alginate (FMC Biopolymer) into sterile alpha-MEM (Corning). The solution was then mixed with 0.1% rat serum albumin (RSA) in 4 mM HCl containing 2.5 μg of BMP-2 per defect and cross-linked in an excess of calcium sulfate by thorough mixing. The alginate gels were stored in syringes at 4°C until use.

3.2.2 Fabrication of MDSC-Depleting Nanoparticles

Synthetic nanoparticle antibodies (SNAbs) targeting MDSCs were prepared as previously described using a solid phase synthesis method.[196] Briefly, aminomethyl ChemMatrix resin (SigmaAldrich) was hydrated and then reacted with a disulfide NHS biotin-functionalized

crosslinker (sulfo-NHS-SS-biotin, ThermoFisher Scientific). After washing off unbound crosslinker, 30 nm gold Janus nanoparticles (Nanohybrids) were reacted overnight. Following particle binding, reaction vessels were again washed to remove unbound particles and then reacted with Tris(2-carboxyethyl)phosphine (TCEP, ThermoFisher Scientific) overnight to cleave particles from the resin at the disulfide within the crosslinker. Janus particles were then dialyzed against phosphate buffer to remove excess TCEP. A biotin-functionalized MDSC targeting peptide (G3-Biotin, WGWSLSHGYQVK(K-Biotin)) and an SMCC- functionalized Fc-mimicking peptide (Pep33-SMCC, AQVNSCLLLPNLLGCGDDK-(K-SMCC)) were reacted with the Janus particles sequentially overnight at 4°C with washing in between peptide additions. Final particles were collected via centrifugation, sterile filtered, and resuspended in 1X sterile PBS until injection.

3.2.3 Surgical Procedures

Two surgical models of rat trauma were employed in this study. First, a rat model of trauma with an associated infection was used as a source of MDSCs for *in vitro* experiments. This established, published trauma model has been shown to result in a large, sustained increase in MDSCs that can be isolated repeatedly and easily through blood draws. For these experiments, animal care and experimental procedures were approved by the Veterans Affairs Institutional Animal Care and Use Committee (IACUC) and carried out according to the guidelines. Surgical procedures were conducted as previously described.[134] Briefly, unilateral 2.5 mm segmental defects were created in the mid-diaphysis of the femur using 12-week old female Sprague Dawley rats (Charles River Labs) using a Gigli wire saw (RISystem). Twelve-week-old rats were selected because they are skeletally mature and because approximately 12–15 weeks is when female Sprague Dawley rats reach their maximum growth in weight and femur size. Defects

were then stabilized with an internal modular fixation plate. A collagen sponge inoculated with 107 CFU of Staphylococcal aureus was then placed in the defect site. The 107 CFU was selected based on prior work showing that at this dosage there are sustained, elevated levels of MDSCs and a local infection is maintained with minimal hardware failure or abscess formation through 8 weeks.[134] The incision site was sutured closed with absorbable 4-0 sutures and wound clips. Subcutaneous administration of slow-release buprenorphine (0.03 mg/kg; 1 ml/kg) was used as an analgesic.

Next, for subsequent *in vivo* experiments, a rat model of composite bone and muscle trauma without infection was employed. For these experiments, all animal care and experimental procedures were approved by the University of Oregon IACUC and carried out according to the guidelines. Surgical procedures were conducted as previously described.[195] Briefly, unilateral 8 mm femoral segmental defects with 8 mm diameter, full-thickness quadriceps muscle defects were created in 14-week-old female Sprague Dawley rats (Charles River Laboratories) using an oscillating saw and a biopsy punch. Fourteen-week-old rats were selected because they are skeletally mature and because approximately 12–15 weeks is when female Sprague Dawley rats reach their maximum growth in weight and femur size. Defects were stabilized with a polysulfone, radiotranslucent internal fixation plate and left untreated for 8 weeks at which point animals underwent an additional surgery to treat the bone defect, representing the clinical delay in treatment of a nonunion. The bone defect site was cleared and 2.5 μg of BMP-2 (Pfizer) was added via the nanofiber mesh alginate hybrid delivery system. The muscle defects were left untreated. At 9 weeks post-injury, a subset of animals received no synthetic nanoparticle antibodies (SNAbs) (n = 11), and another subset of animals received arterial injections of a single (n = 5) or double dose (n = 5) (1x SNAb or 2x SNAb) of SNAbs. Animals were euthanized at 20

weeks post-injury. Histology was conducted on a subset of rats (no SNAbs, n = 2; 1x SNAb, n = 1; 2x SNAb, n = 1), and mechanical testing was conducted on the remaining rats (no SNAbs, n = 9; 1x SNAb, n = 6, 2x SNAb, n = 4).

3.2.4 Immune Characterization

Blood was drawn from the tail artery of rats via Vacuette blood collection needles (Greiner Bio-One) at various time points post-injury and treatment including Day 3, Weeks 1, 4, 7, Week 8 Day 5, Week 9 Days 1 and 2, Weeks 10, 12, 16, and 20 to monitor immune cell populations and chemokine and cytokine levels over time. The Day 3 time point was selected to observe the early immune response to injury, whereas the Week 8 Day 5 response was selected to observe immune function prior to SNAb treatment at Week 9. Approximately 300 µL of blood was collected into Microtainer lithium-heparin coated tubes (BD) for FACS analysis and approximately 150 µL of blood was collected into Microvette serum collection tubes (Kent Scientific) for serum analysis. For whole blood analysis, red blood cells were lysed using 1X RBC lysis buffer (eBioscience) according to manufacturer's instructions. Cells were then fixed and resuspended in FACS buffer containing 2% FBS in PBS. Cells were first incubated with anti-rat CD32 to prevent non-specific antibody binding and then stained for various immune cells including T cells (CD3+), cytotoxic T cells (CD3 + CD8+), helper T cells (CD3 + CD4 + FoxP3−), regulatory T cells (Tregs, CD3 + CD4 + FoxP3+), B cells (B220+), monocytes (CD11b + CD68+), and MDSCs (CD11b + His48+). Data were collected using a BD Accuri C6 flow cytometer and analyzed with FlowJo software with gates set based on FMO controls allowing <1% noise. For cytokine and chemokine analysis, serum was collected after allowing blood to clot for approximately 2.5 h at 4°C and then spinning down samples at 1,000 g for 10 min. The supernatant was collected and stored at −20°C until multiplexed chemokine and

cytokine analysis (Luminex) was conducted using Milliplex MAP Rat Cytokine and Chemokine Magnetic kits (Millipore Sigma). Data was collected on the MAGPIX Luminex instrument and analyzed with the median fluorescent intensity values with background subtracted.

3.2.5 Evaluating Bone Regeneration

Longitudinal bone regeneration was qualitatively assessed with radiographic imaging using an UltraFocus digital radiography system (Faxitron), and quantitatively assessed with micro-computed tomography using the vivaCT80 (Scanco Medical) both *in vivo* at six weeks post-BMP-2 treatment and *ex vivo* at twelve weeks post-BMP-2 treatment. The middle region of the defect was analyzed (~6.5 mm) with a 55-kVp voltage and a 145-μA current, and the voxel size was set to 48.5 μm for *in vivo* scans and 24 μm for *ex vivo* scans. Newly regenerated bone was segmented using a threshold corresponding to 50% of the average bone mineral density of native cortical bone. Functional bone regeneration was also assessed quantitatively using biomechanical testing to evaluate torsional stiffness and failure strength. Femurs were excised and potted in Wood's metal and then tested to failure in torsion at a rotation rate of 3 degrees per second using the ELF3200 testing system (TA Instruments). Failure strength was determined as the peak torque within the first 90 degrees of rotation and torsional stiffness was calculated by finding the slope of the linear region before failure in the torque-rotation plot.

3.2.6 In Vitro Co-Culture Assay

SNAbs were tested *in vitro* in a co-culture assay containing MDSCs (target cells) and macrophages (effector cells). The SNAbs require the presence of both target and effector cells, similar to antibodies, in order to elicit specific killing of the target cells (MDSCs). The MDSCs were enriched from the blood of infected trauma rats. Blood was drawn via the rat tail artery and collected into Microtainer lithium-heparin coated tubes (BD). Blood was pooled and then red

blood cells were lysed using 1X RBC lysis buffer (eBioscience). MDSCs were then enriched using magnetic activated cell sorting (Miltenyi) according to the manufacturer's instructions with His48 as the sorting marker. Cells were then washed, counted, and resuspended in RPMI 1,640 media. One week prior to MDSC isolation, macrophages were isolated from the bone marrow of one naïve, healthy female Sprague Dawley rat. The femur and tibia from both legs were excised and a syringe and 20G needle were used to flush the bone marrow cavities with 1X PBS. Cells were then run through a 40 μM cell strainer and red blood cells were lysed using 1X RBC lysis buffer (eBioscience). Cells were then washed, counted, resuspended in RPMI 1,640 media, and plated at 5e5 cells/ml in a petri dish with 20 ng/ml recombinant rat macrophage colony stimulating factor (M-CSF, Peprotech). Cells were cultured for 7 days with media changes on Days 3 and 6 and flow cytometry was used to confirm successful differentiation on Day 7. Cells were plated at a 1:10 ratio of MDSCs to macrophages in a 96 well plate with 500,000 cells per well. In SNAb treated wells, 1e10 SNAbs were added in 50 μL of 1X PBS. 50 μL of 1X PBS was added to untreated, control wells. Following a 24-hour co-culture period, cells were collected and single cell RNA sequencing was conducted on SNAb-treated cells enriched for MDSCs to better understand the impacts of SNAb treatment on rat MDSCs. Untreated, pre-SNAb treated cells were used as a control.

3.2.7 Single Cell RNA Sequencing

Rat PBMCs were isolated from both naïve rats (n = 3) and composite trauma model rats (n = 3) via the tail artery using Vacuette blood collection needles (Greiner Bio-One). Blood was isolated from the composite trauma model rats at Day 5 post-injury and pooled together for subsequent analyses. Cell number and viability were confirmed to achieve the target 5,000 barcoded cells. Single cell RNA sequencing (scRNAseq) was performed using 10X Genomics

Single Cell 3′ Solution (version 3.1), according to the manufacturer's instructions (protocol rev C). Libraries were sequenced on Nextseq500 (Illumina) and data were de-multiplexed, aligned, and counted using Cell Ranger version 3.1.0 (10X Genomics). Data were analyzed using the R package Seurat (https://satijalab.org/seurat/) developed by the Satija Lab and is specifically designed for quality control, analysis, and exploration of single cell RNA sequencing data. The package utilizes Louvain clustering which modularly optimizes large networks to detect communities using an unsupervised algorithm. These clusters can then be visualized using uniform manifold approximation and projection (UMAP) which dimensionally reduces the data into 2 dimensions, UMAP-1 and UMAP-2, or using t-distributed stochastic neighbor embedding (t-SNE) which dimensionally reduced the data into two dimensions, tSNE-1 and tSNE-2. Quality control metrics were utilized and excluded cells with mitochondrial gene percentage greater than 20% or cells with feature counts below 200 or above 2,500.

3.2.8 Statistical Analysis

Statistical significance for quantitative results was determined using appropriate parametric or non-parametric statistical tests and significance was determined by p values less than 0.05. Statistical analyses were performed using GraphPad Prism 9.0 software (La Jolla, CA, USA).

3.3 Results

3.3.1 MDSC Depletion in Vitro from Rat Trauma-Derived Cells

Initial experiments were conducted using MDSCs derived from a rat segmental defect model containing a biomaterial-based Staphylococcus aureus infection. This model was selected due to previous work showing high levels of MDSCs as early as 3 days post-surgery which stay

elevated for several weeks post-injury.[134] The high levels of MDSCs derived from this model allow for *in vitro* evaluation of MDSC depletion using the synthetic nanoparticle antibodies (SNAbs). The SNAbs are 30 nm gold Janus nanoparticles which contain MDSC-targeting peptides conjugated to one domain and Fc-mimicking peptides conjugated to the other domain, allowing the nanoparticles to mimic the structure and function of antibodies, resulting in antibody-mediated killing of the MDSC target cells. Previous work has confirmed the ability of SNAbs to deplete MDSCs systemically in a mouse tumor model;[196] however, *in vivo* reduction of MDSC levels by SNAbs in rats has not previously been evaluated.

SNAbs were tested *in vitro* in a co-culture assay containing MDSCs and macrophages. The MDSCs were enriched from the blood of infected trauma rats using magnetic activated cell sorting and the macrophages were differentiated from the bone marrow of healthy, naïve rats using M-CSF stimulation. Following a 24-hour co-culture period, single cell RNA sequencing was conducted on SNAb-treated cells enriched for MDSCs to better understand the impacts of SNAb treatment on rat MDSCs. Untreated, pre-SNAb treated cells were used as a control. Integration and clustering of both the treated and untreated groups shows the presence of MDSCs, macrophages, and neutrophils (Figure 2A). An integrated overlay of tSNE projections from the SNAb treated and untreated groups shows depletion of the MDSC clusters (Figure 2B), which was also confirmed previously via flow cytometry.[196] In addition, expression of immunosuppressive gene markers known to be highly expressed in MDSCs, including S100a9, IL1b, Arg1 (arginase 1), and Junb (a transcription factor), are all significantly decreased post-SNAb treatment (Figure 2C). The presence of the macrophage clusters resulting from the co-culture assay are confirmed with the expression of macrophage gene markers, including CD68

and Adgre1 (Figure 2D). These results demonstrate that SNAbs are capable of functionally depleting MDSCs, both in cell numbers and via decreased immunosuppressive gene expression.

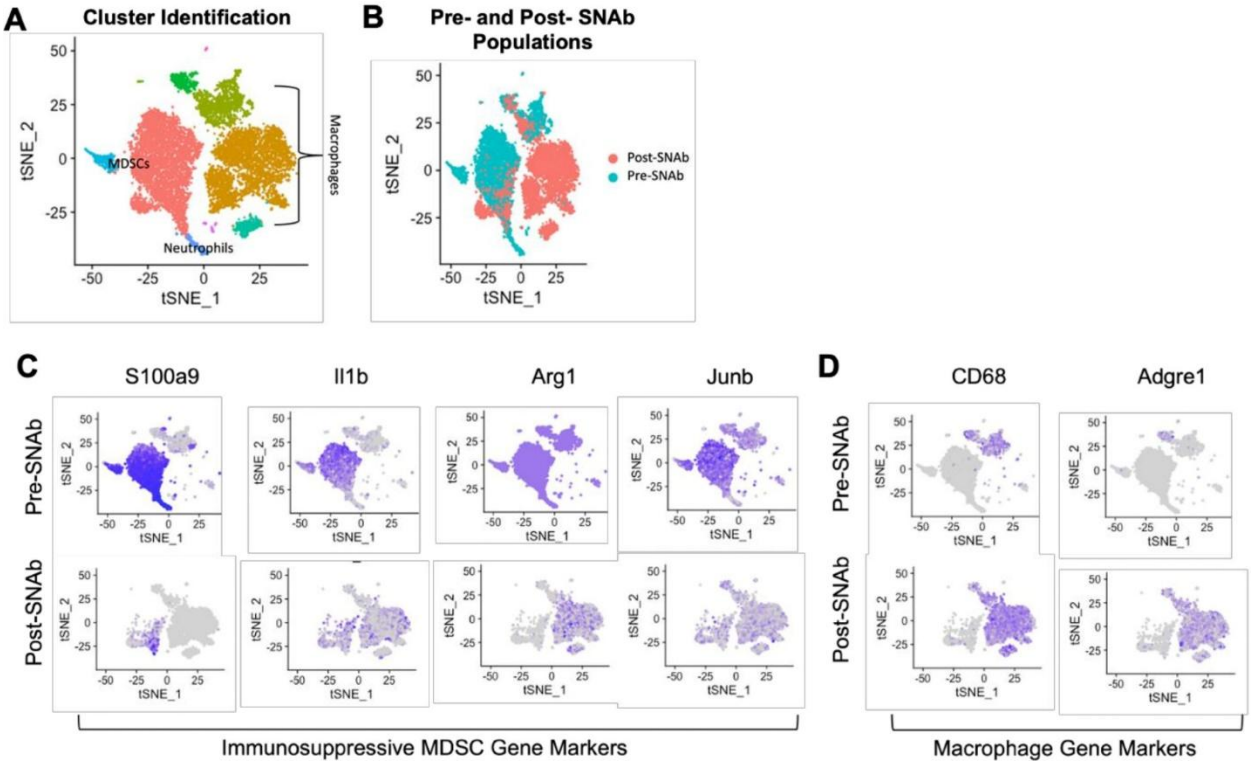


Figure 2. Single cell RNA sequencing following MDSC depletion *in vitro*. (A) Clustering of integrated tSNE plots from the SNAb-treated and untreated cells enriched for MDSCs. (B) Overlay of tSNE plots from the SNAb-treated and untreated groups showing differences in cell populations. (C) Gene expression for immunosuppressive MDSC gene markers (S100a9, ILb, Arg1, and Junb) pre- and post-SNAb treatment. (D) Gene expression for macrophage gene markers (CD68 and Adgre1) pre- and post-SNAb treatment.

3.3.2 Systemic Immune Cells and Cytokines are Altered Following Injury and SNAb Treatment

Blood was collected at the baseline (prior to injury) and at various time points post-injury, post-BMP-2 treatment, and post-SNAb treatment to assess the systemic immune response. At day 3 post-injury, MDSCs and monocytes were significantly elevated while T cells and T helper cells were significantly depressed in the composite defect group compared to naïve controls (Figure 3). T cells and T helper cells were also significantly decreased following BMP-2

treatment at week 8 day 5, although other immune cells did not show significant changes at that time point, in particular MDSCs, in contrast to observations made in previous studies and cohorts.[112,195] In fact, in this study, the MDSC levels were back to pre-trauma levels at the time of SNAb treatment.

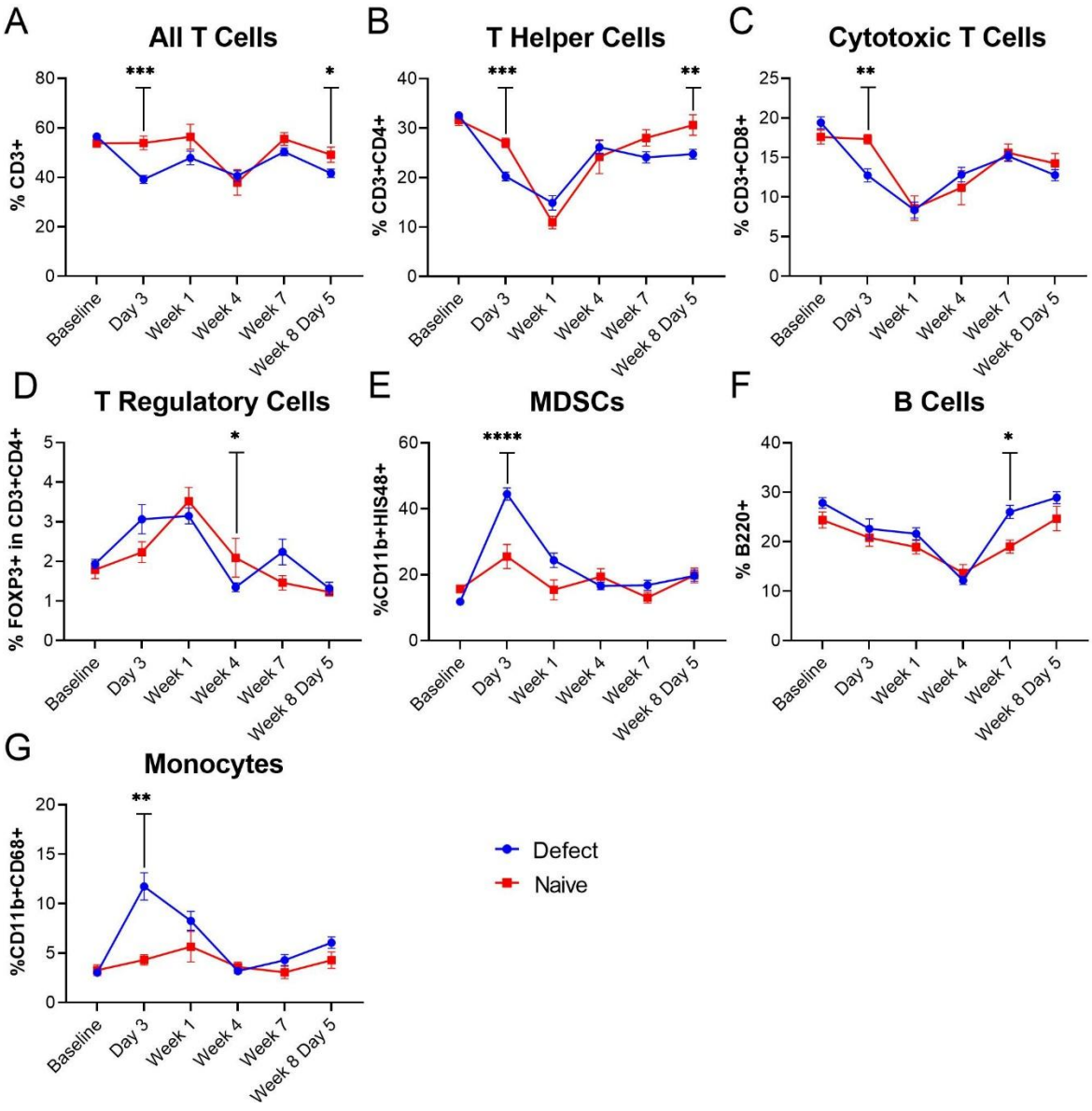


Figure 3. Characterization of circulating immune cells after injury (Day 0) and treatment (Week 8). (A–G) Significant changes in immune cell populations were observed in rats after injury. T cells, T helper cells, and cytotoxic T cells were significantly decreased in injured rats (A–C), while MDSCs and monocytes were significantly elevated (E,G). Data are mean ± SEM, n = 7 to 13 per group. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 as indicated.

At 1 and 2 days post-SNAb treatment (Week 9 Days 1 and 2), both SNAb-treated groups (both the single and double dose groups) showed significantly elevated levels of MDSCs compared to the BMP-2 only and naïve groups (Figure 4). However, looking at the MDSC population via flow cytometry reveals two distinct MDSC sub-populations – a HIS48^{hi} population and a HIS48^{lo} population (Figure 5A). MDSCs are known to have both monocytic and granulocytic subsets in humans and mice,[131] and the observed MDSC sub-populations in this rat data may parallel these known subsets in other species. The two MDSC sub-populations responded differently to SNAb treatment. The HIS48^{hi} MDSCs were significantly decreased at 24 h post-treatment in both SNAb-treated groups, whereas HIS48^{lo} MDSCs were significantly increased at 24 h post-treatment in both SNAb-treated groups (Figure 5C). However, despite a significant decrease in the HIS48^{hi} MDSCs following SNAb treatment, there is still an overall increase in total MDSCs (Figure 5B) due to the larger size of the HIS48^{lo} MDSC sub-population. In addition to changes in the MDSC populations post-SNAb treatment, significant decreases in T cells, T helper cells, and cytotoxic T cells were also observed at Week 9 Days 1 and 2 post-treatment (Figure 4).

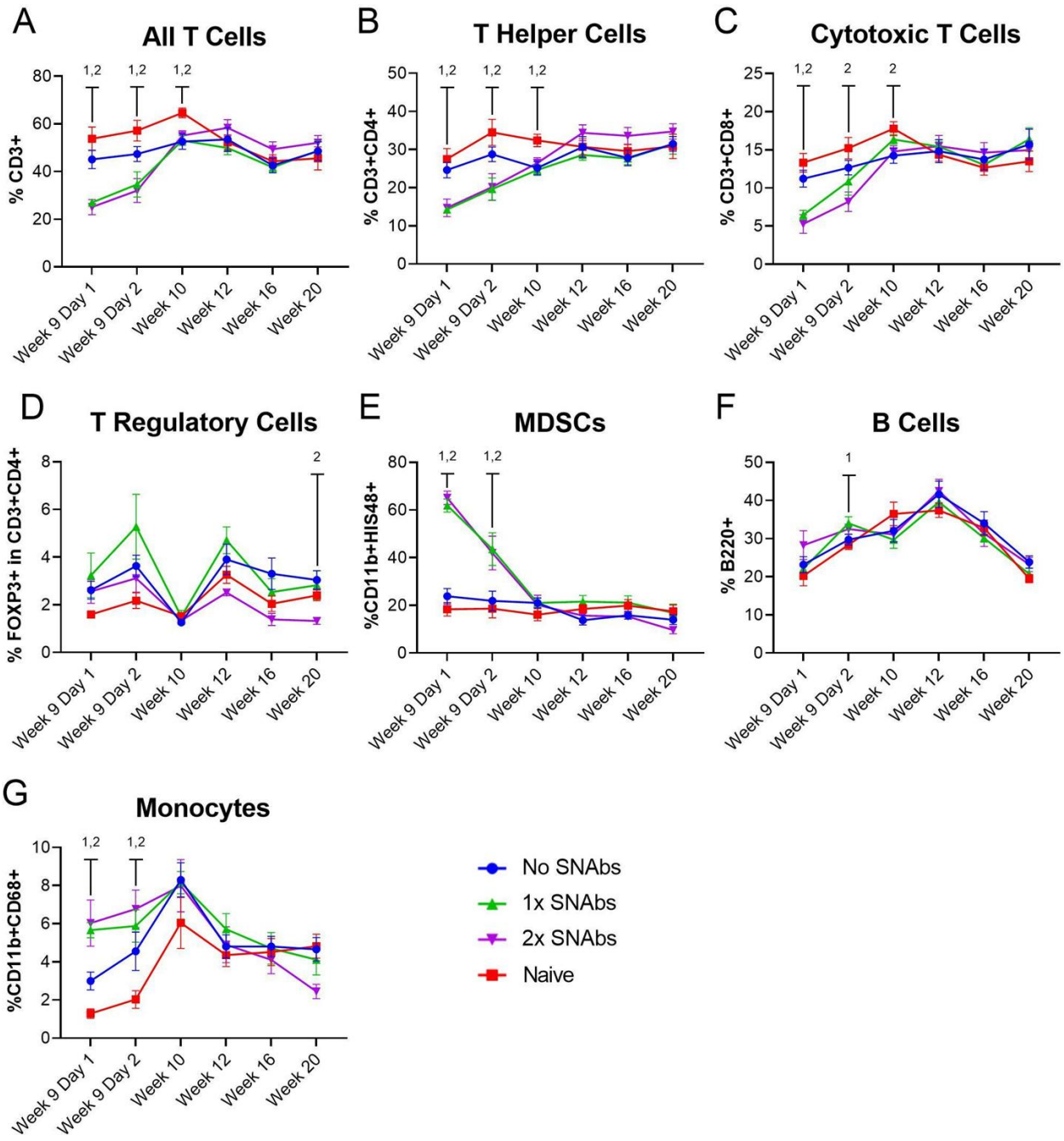


Figure 4. Characterization of circulating immune cells after treatment with synthetic nanoparticle antibodies (SNAbs). (A–G) Significant changes in immune cell populations were observed in rats at multiple time points after SNAb injections. T cells, T helper cells, and cytotoxic T cells were significantly decreased in SNAb-injected rats (A–C), while MDSCs and monocytes were significantly elevated (E,G). Data are mean ± SEM, n = 7 to 13 per group. 1,2 correspond to significant differences between the naive group and the 1x SNAbs group (1) or the 2x SNAbs group (2), with $P < 0.05$.

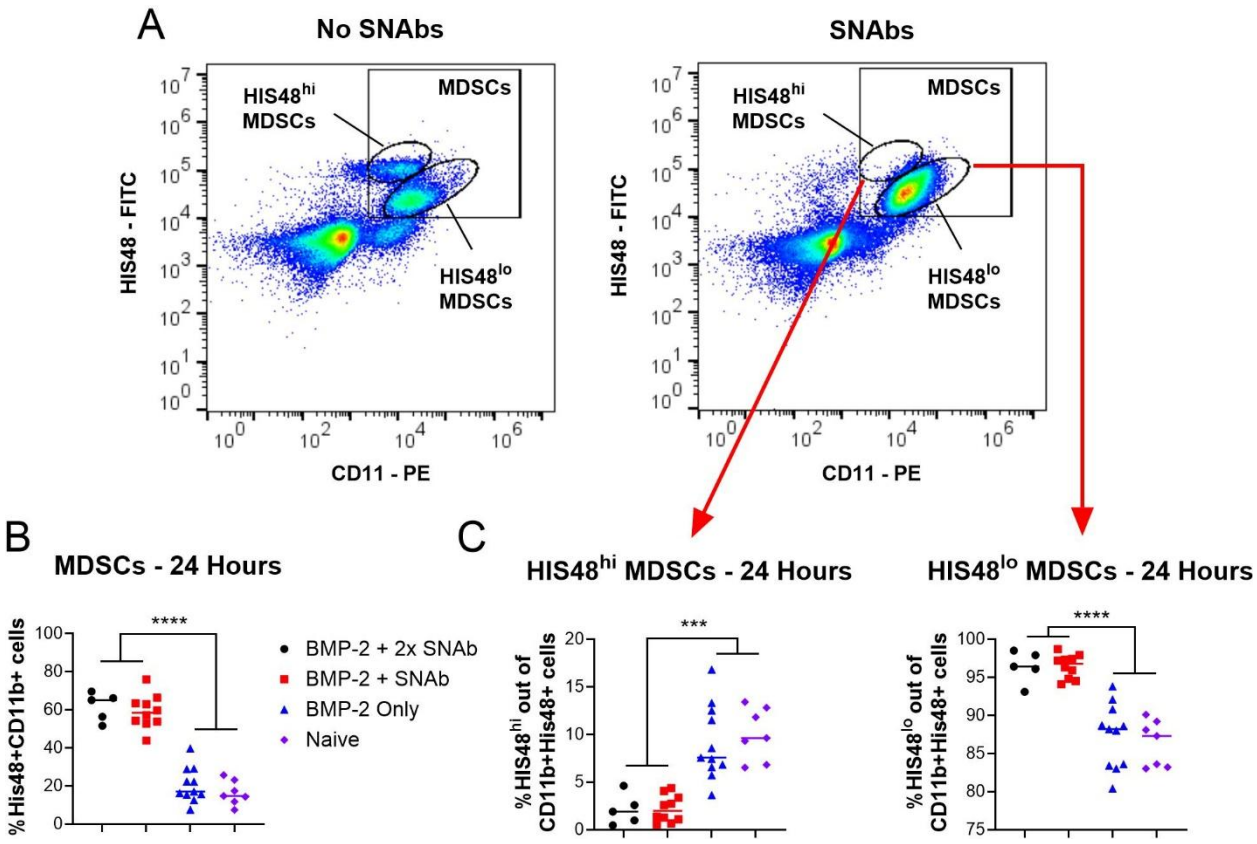


Figure 5. MDSCs at 24 h post-SNAb treatment in the composite trauma model. (A) Flow cytometry dot plots showing two MDSC sub-populations. Systemic levels of total MDSCs (B) and the two MDSC subsets (C) at 24 h post-SNAb treatment in the composite trauma model. Significance was determined using a one-way ANOVA. ***P < 0.001; ****P < 0.0001 as indicated; n = 5 to 11 per group.

Finally, cytokines were also analyzed in the blood at 24 hours and 48 hours post-SNAb treatment. Interestingly, circulating IP-10 was increased and LIX was decreased in SNAb-treated rats as compared to untreated controls at 24 hours post-SNAb treatment (Figure 6, top panel). By 48 hours post-SNAb treatment, numerous cytokines were systemically decreased in the SNAb-treated rats as compared to untreated controls, while IP-10 remained elevated in the SNAb-treated group (Figure 6, bottom panel), demonstrating the wide-ranging effects of SNAbs on the systemic immune response.

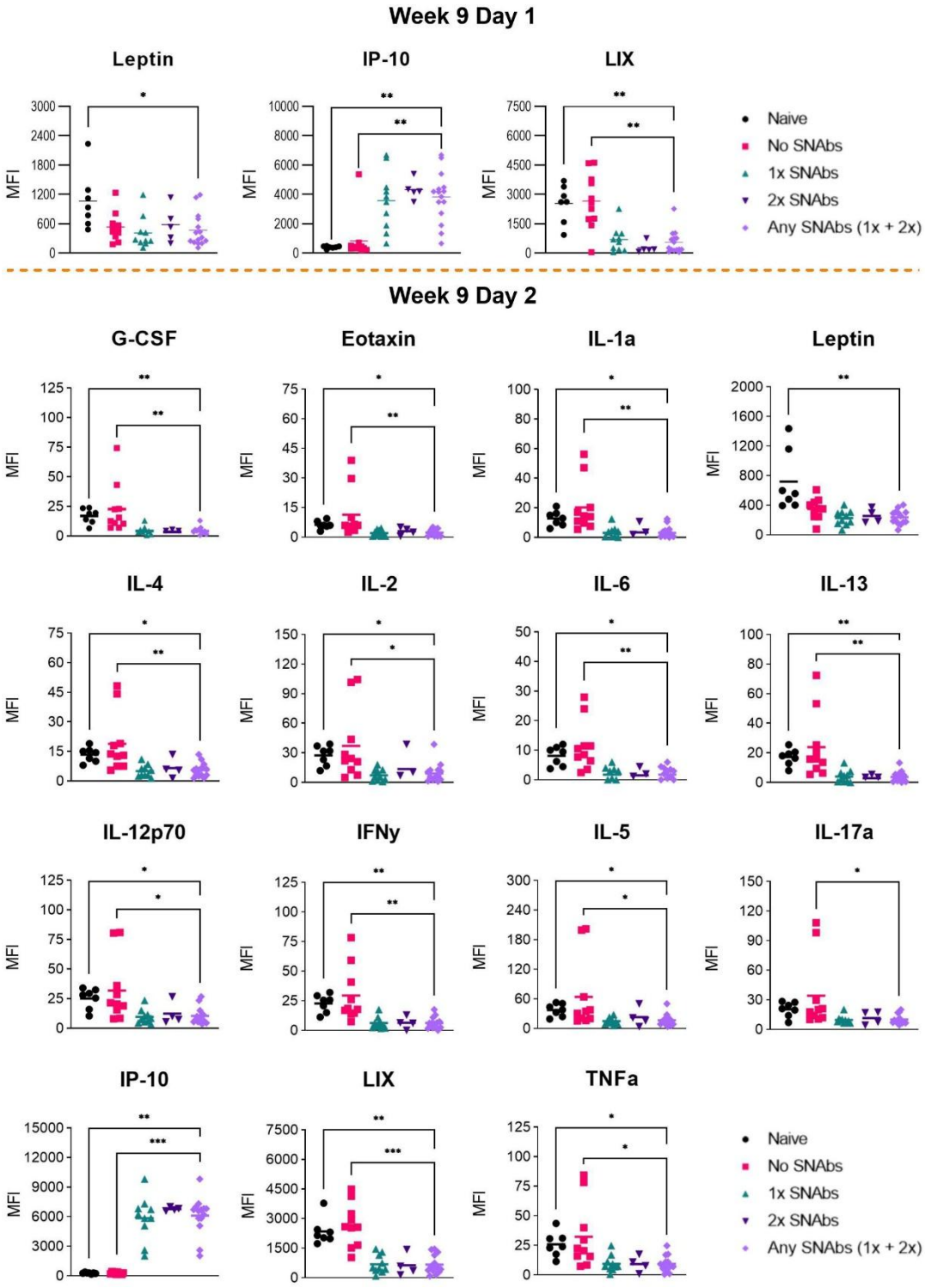


Figure 6. Circulating cytokines are dysregulated post-SNAb treatment. IP-10 was increased and LIX was decreased 24 hours post-SNAb treatment as compared to untreated controls. Numerous cytokines were dysregulated in SNAb-treated rats as compared to untreated controls by 48 hours post-treatment. P-values were obtained using a Kruskal-Wallis test with multiple comparisons. *P < 0.05; **P < 0.01; ***P < 0.001 as indicated.

3.3.3 SNAb Treatment Results in Decreased Functional Bone Regeneration

Functional bone regeneration was assessed by radiographic imaging and micro-computed tomography (uCT) at six weeks (Week 14) and twelve weeks (Week 20) post-BMP-2 treatment as well as biomechanical testing twelve weeks post-BMP-2 treatment. No significant differences were observed in the bone volume or mechanical properties between the BMP-2 only group and either SNAb-treated group individually (Figure 7). However, when comparing the mechanical strength of the femurs treated with BMP-2 only to all SNAb-treated femurs, the BMP-2 only group had significantly increased failure strength compared to the SNAb-treated rats (Figure 5C). Furthermore, the BMP-2 only group (no SNAbs) had higher peak bone volume and higher peak mechanical properties compared to any of the SNAb-treated groups (Figures 3.6A, C, D). Although not statistically significant, the average bone volume, failure strength, and torsional stiffness all decrease with each increasing dose of SNAbs. Further studies with increased power are needed to identify if there is a dose-dependent response to SNAbs that results in decreased functional bone regeneration.

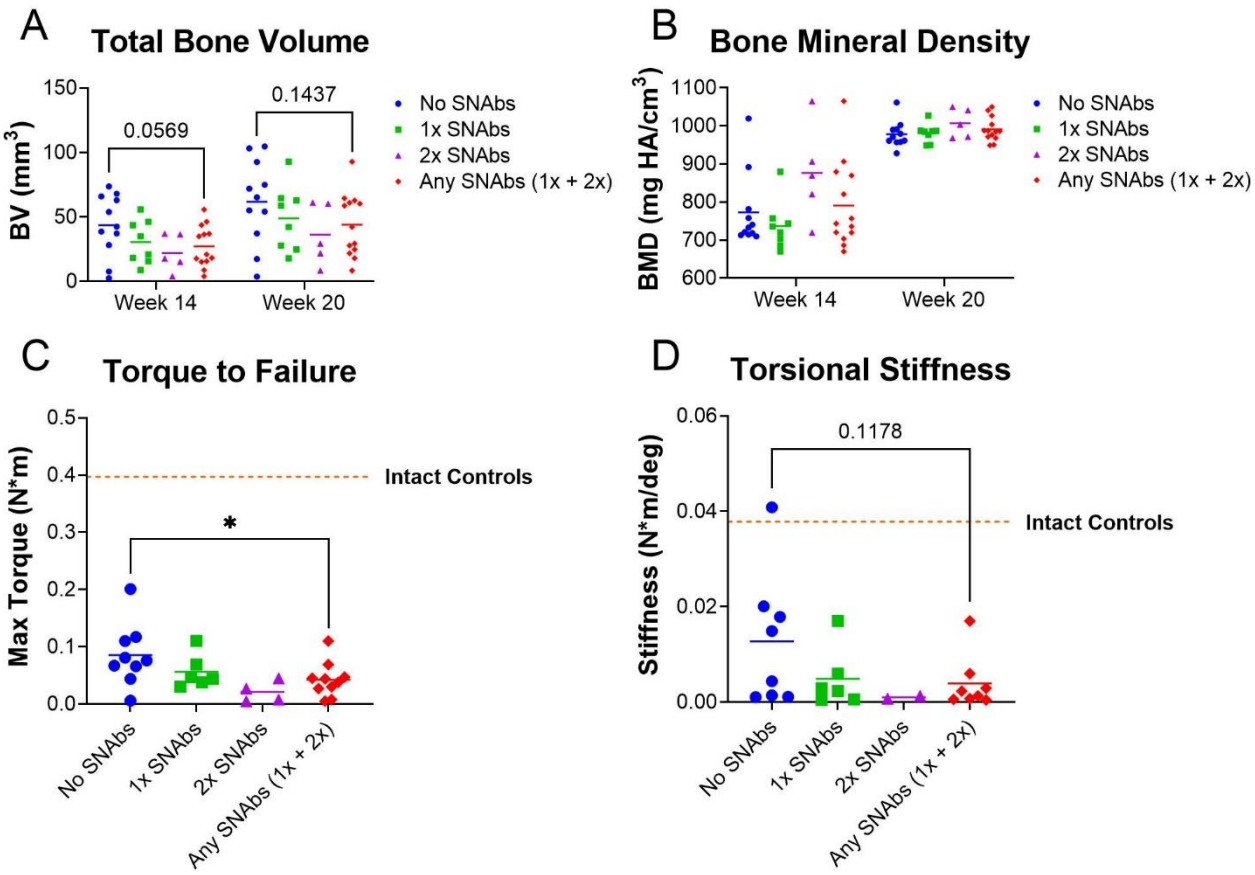


Figure 7. Functional regeneration may be impaired following treatment with synthetic nanoparticle antibodies (SNAbs). (A,B) Total bone volume (A) and bone mineral density (B) of newly formed bone, as quantified by *in vivo* µCT at week 14 and *ex vivo* µCT at week 20. (C,D) Mechanical strength (C) and stiffness (D) of regenerated femurs as quantified by *ex vivo* torsional testing to failure at week 20. The dotted lines indicate mechanical properties of intact bone. *$P < 0.05$ as indicated.

3.3.4 Systemic Immune Cell Populations Correlate with Bone Regeneration

Linear regression analyses revealed correlations between systemic immune cell populations and endpoint bone volume of SNAb-treated rats in the single dose SNAb-treated group (Figure 8). Specifically, T cells, T helper cells, and cytotoxic T cells measured 48 h after SNAb injections (Week 9 Day 2) and at the endpoint (Week 20) were found to correlate positively with healing (Figures 3.7A–C), while MDSCs and monocytes measured 48 h after SNAb injections (Week 9 Day 2) and MDSCs measured at the endpoint (Week 20) were found

to correlate negatively with healing (Figures 3.7D, E). These results generally agree with observations made in previous studies of bone-only injury models, where T cells and T helper cells correlated positively with healing and MDSCs and monocytes correlated negatively.[112]

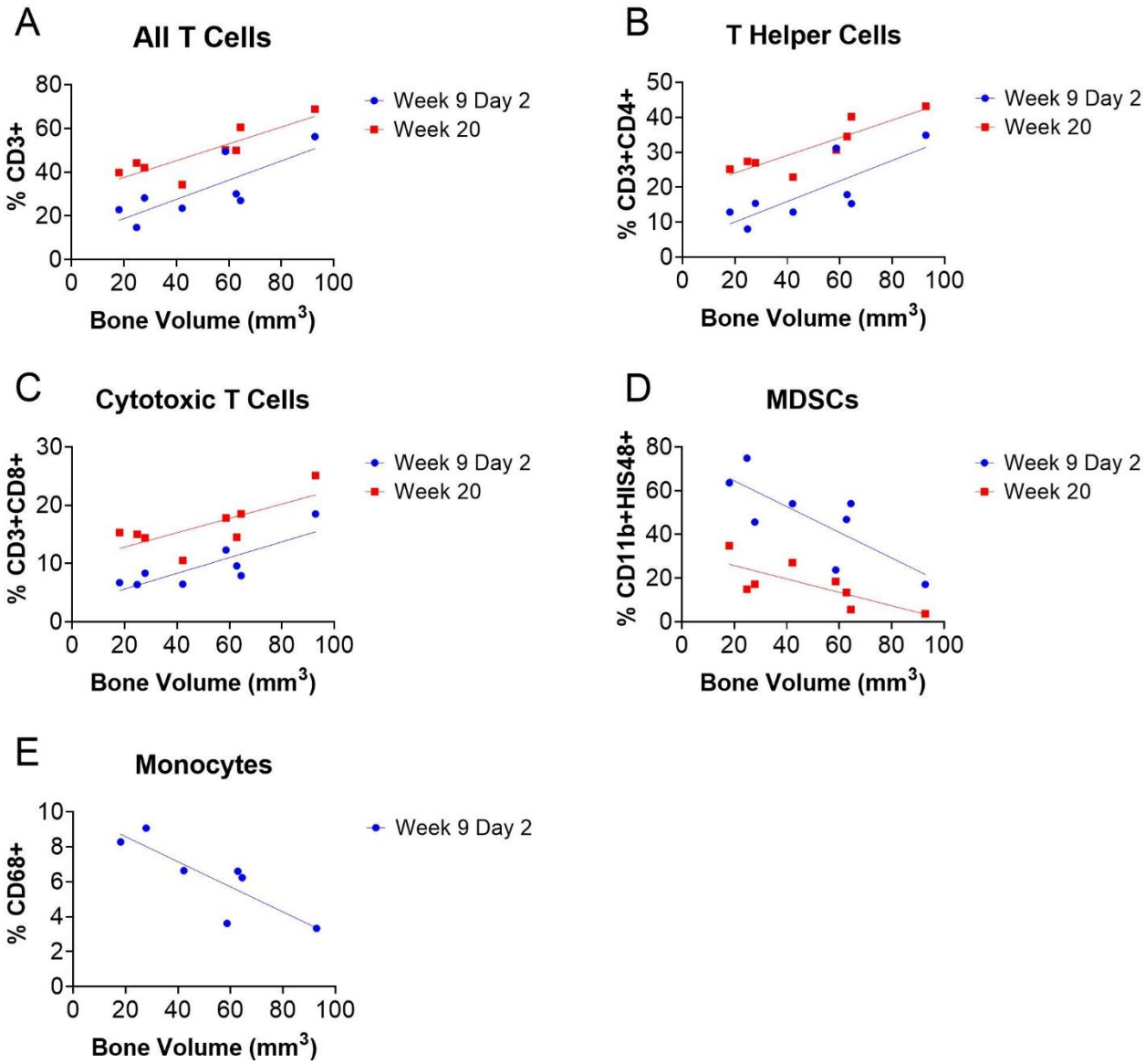


Figure 8. Circulating immune cells correlate with bone regeneration in rats treated with synthetic nanoparticle antibodies (SNAbs). (A–E) Immune cell populations from peripheral blood at multiple timepoints significantly correlate with week 20 bone volumes as measured by *ex vivo* μCT. T cells, T helper cells, and cytotoxic T cells were positively correlated with bone regeneration (A–C), while MDSCs and monocytes were negatively correlated (D,E). Pearson correlation analyses performed, n = 7–8 per time point, slope of linear regression significantly nonzero for all data shown ($P < 0.05$).

3.3.5 Single Cell RNA Sequencing Reveals Differing MDSC Gene Expression Patterns

To better understand why the SNAbs only targeted one subset of MDSCs, we conducted single-cell RNA sequencing (scRNAseq) on PBMCs isolated from the composite trauma model rats at 5 days post-injury. PBMCs from naïve rats were also isolated and data from both the composite trauma and naïve rats were integrated, clustered, and analyzed (Figure 9A). Clusters were identified with marker genes for each immune cell type. Gene expression of MDSC gene markers, including Arg2, Il1b, Pla2g7, Srgn, Entpd1, and Csf3r, was elevated in the two clusters marked MDSCs (Figures 3.8A, B). To further analyze the MDSC populations, these clusters were isolated, re-integrated, and re-clustered, resulting in 3 MDSC sub-clusters (Figure 9C). Cluster 0 accounts for the highest percentage of MDSCs, followed by Cluster 1, and lastly by Cluster 2. Comparing the percentages of MDSCs in the composite trauma rats versus the naïve rats shows an increase in MDSCs in Clusters 0 and 2 only in the composite trauma rats (Figure 9D). Because these two clusters exhibit a marked increase in MDSCs compared to naïve rats, these are likely the two most important clusters to target with the SNAbs. In addition, because Cluster 0 accounts for a much higher percentage of MDSCs than Cluster 2, Cluster 0 may be the most important target in order to achieve a therapeutic effect. The MDSC-depleting SNAbs target S100A8/A9 on the surface of MDSCs, so we next investigated S100A8/A9 expression by looking at the genes for these proteins, S100a8 and S100a9. Please note that S100A8/A9 will be used to refer to the protein, whereas S100a8/a9 will be used to refer to the gene. S100a8 and S100a9 gene expression is highest in Cluster 2, with some expression in Cluster 1, and minimal expression in Cluster 0 (Figure 9E). While gene expression levels are not necessarily equivalent to protein levels, these differences in S100a8 and S100a9 gene expression could account for the MDSC depletion differences observed in the two different MDSC sub-populations via flow

cytometry. Improved markers are needed in order to ensure successful targeting of all MDSC subsets in the composite trauma model.

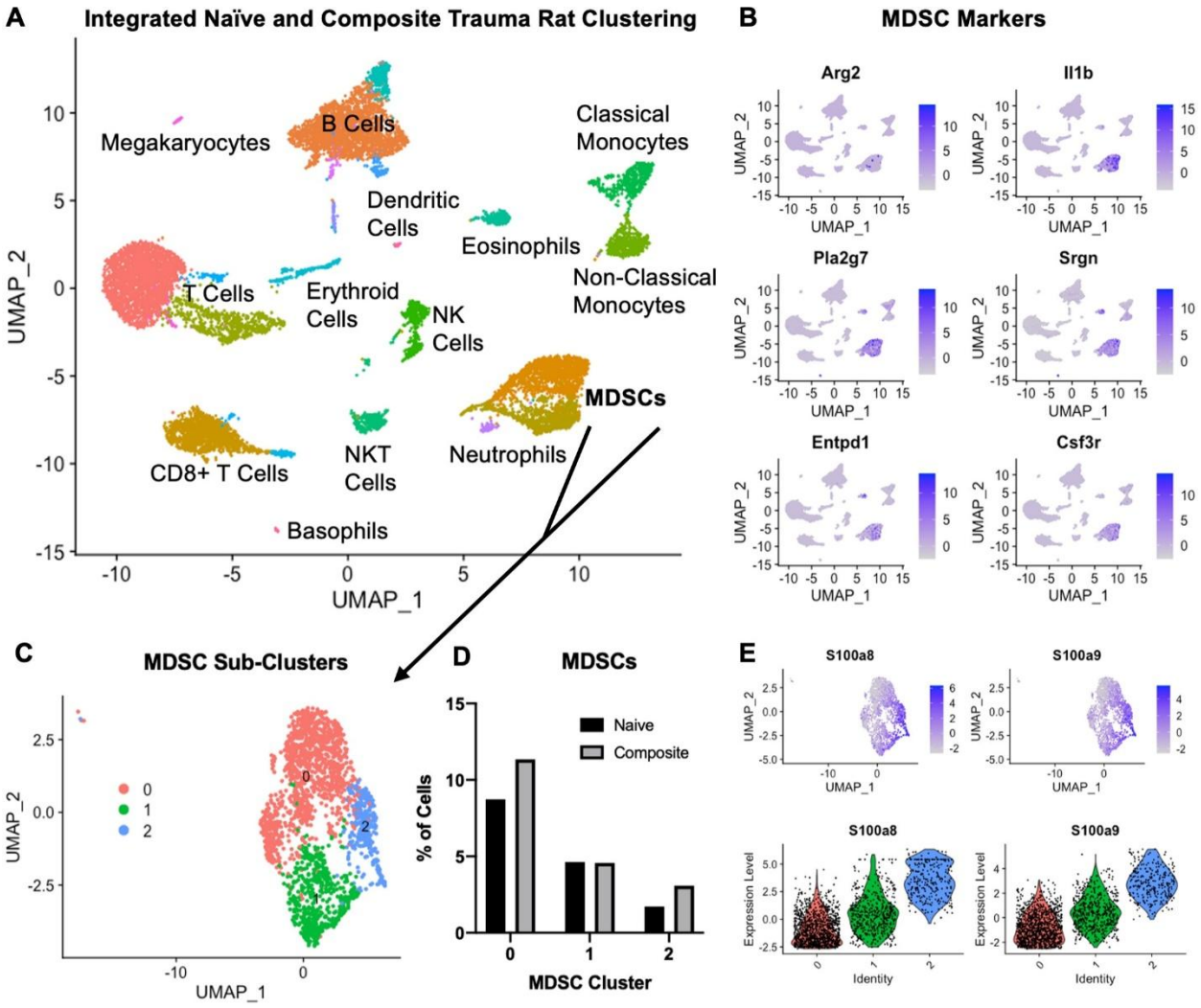


Figure 9. Single cell RNA sequencing in the composite trauma model. (A) Integrated clustering of PBMCs from naïve and composite trauma rats at 5 days post-injury. (B) Gene expression of MDSC gene markers. (C) MDSC clusters identified in (A) were re-integrated and re-clustered for more in-depth analysis. Three subclusters were identified. (D) Comparison of MDSC percentages for clusters 0, 1, and 2 in composite trauma model rats versus naïve rats. (E) Density plots (top) and violin plots (bottom) of S100a8 expression (left) and S100a9 expression (right).

3.4 Discussion

Despite significant advances in trauma care and management, orthopedic surgeons still need improved strategies to address complicated musculoskeletal trauma, especially for more challenging cases, such as nonunions, concomitant volumetric muscle loss, or bone infections. Prior work in our lab has highlighted the role of systemic immune dysregulation and immunosuppression following trauma as a negative predictor of local healing outcomes in both nonunion and composite trauma models.[112,195] Most notably, in the nonunion model, an inverse correlation at 1-week post-treatment was observed between circulating MDSC levels and endpoint bone volume,[112] suggesting that immunomodulatory therapeutics that target and deplete MDSCs could potentially improve healing outcomes. This study seeks to address this question by evaluating the ability of MDSC-targeting synthetic nanoparticle antibodies (SNAbs) to deplete MDSCS, restore systemic immune homeostasis, and alter the local healing environment to improve functional bone regeneration. The relationship between the systemic and the local immune response has been well established, but more recently, there has been more work done investigating the therapeutic relationship between these two environments. For example, one study in cancer immunotherapy demonstrated that systemic immunity is required for successful antitumor immune therapy, and another study showed that systemic immune homeostasis is altered by local biomaterial scaffolds for local tissue regeneration.[203,204] Based on the association between the local and systemic immune environments, we posit that consideration of immune homeostasis and a permissive, pro-healing immune environment at both the local and systemic levels is necessary for regenerative medicine strategies to reach their full potential.

Prior to a full investigation of the impact of SNAb treatment on healing in a composite trauma model, *in vitro* investigations of SNAbs were conducted using MDSCs derived from an

infected trauma model. *In vitro*, SNAbs resulted in a decrease in MDSCs as well as a decrease in immunosuppressive gene expression, including S100a9, Il1b, Arg1 (arginase), and Junb. S100a9 is of particular interest because the S100A9 protein is upregulated on the surface of MDSCs, secreted by MDSCs, and is one of the target proteins of the MDSC-targeting peptide on the SNAbs. Along with arginase (Arg1), S100A9 plays a role in T cell immunosuppression. S100A9 induces upregulation of PD-1/PD-L1, leading to T cell exhaustion and apoptosis.[199] Arginase on the other hand suppresses T cell functions by depleting L- arginine, an essential amino acid for T cell receptor (TCR) signaling.[205] Il-1b (IL1b) is another important MDSC-associated cytokine which recruits and activates MDSCs through the IL-1R/NF-kB pathway, resulting in further accumulation of MDSCs.[206,207] Lastly, Junb (Junb), a transcription factor in the activating protein (AP-1) family, is another marker of aberrant immune cell responses and is involved in the immune cell activation program in MDSCs.[208] Significant reduction in expression of these immunosuppressive and MDSC- associated genes shows promise that SNAbs can be utilized to successfully alter an immunosuppressive environment by targeted killing of MDSCs.

Given these positive preliminary results, the impact of SNAbs on the systemic immune response and functional bone regeneration was investigated in a composite trauma model. This study demonstrated multiple changes to systemic immune cell populations in response to both composite injury and immunomodulation treatment. The elevated levels of MDSCs and monocytes coupled with depressed T cells, T helper cells, and cytotoxic T cells observed after injury indicated the development of trauma-induced systemic immune dysregulation and immunosuppression. Furthermore, depressed levels of T cells and T helper cells were observed following revision surgeries to treat with BMP-2, similarly to the response following the initial injury surgery. These results also agree with observations made in previous studies and models

including a delayed treatment nonunion model and composite trauma model.[112,195] Following the injury surgery, a decrease in T cells and an increase in MDSCs was observed. However, following BMP-treatment (revision) surgery, only a decrease in T cell levels was observed with no increase in MDSCs, which was an unexpected result. MDSCs were only significantly elevated at day 3 post-injury surgery, so a similar spike in MDSCs at day 3 post-revision surgery may have occurred but was not able to be observed at the 5-day post-revision surgery time point used in this study.

Although MDSC depletion was observed *in vitro*, SNAb treatment in the pre-clinical composite trauma model resulted in an overall increase in total MDSCs. Two distinct sub-populations of MDSCs were observed that made up the total number of MDSCs – a $HIS48^{hi}$ population and a $HIS48^{lo}$ population. It was noted that while MDSCs significantly increased in the larger sub-population of $HIS48^{lo}$ MDSCs, MDSCs actually significantly decreased in the much smaller $HIS48^{hi}$ sub-population. These two observed MDSC sub-populations likely mirror monocytic and granulocytic MDSC sub-populations that have been observed in humans and mice.[131] Single cell RNA sequencing analysis revealed differences in S100a8/a9 gene expression, which is the gene responsible for producing the target molecule of the MDSC-targeting peptide used on the SNAbs. S100A8/A9 is a calcium-binding protein that participates in cytoskeleton rearrangement and is released during inflammation to stimulate leukocyte recruitment and induce cytokine secretion.[150,201] Despite its role in inflammation, S100A8/A9 is also known to play a significant immunosuppressive role including by impairing neutrophil infiltration, reducing cytokine secretion, inhibiting antigen presentation on antigen presenting cells, and suppressing leukocyte adhesion and migration.[150,197–200] While S100A8/A9 is known to be highly expressed on myeloid-lineage cells following inflammation, such as MDSCs, it is also

highly expressed in neutrophils, making up approximately 45% of cytoplasmic proteins.[201] Based on the wide and varied roles of S100A8/A9, expression and secretion can change during inflammation and may vary widely in differing causes of inflammation, such as that caused by composite trauma versus infection or cancer. S100a8/a9 gene expression was highest in the smallest MDSC sub-cluster and lowest in the largest MDSC sub-cluster, suggesting that effective targeting may only have occurred for the smallest MDSC sub-cluster. This is supported by flow cytometry data showing successful depletion of the smaller, $HIS48^{hi}$ MDSC sub-population. An improved understanding of S100A8/A9 and how the inflammatory and anti-inflammatory duality impact the systemic immune response over time in different cases of inflammation will be essential for robust and consistent SNAb targeting of immunosuppressive MDSCs. In addition, an improved understanding of MDSC sub-populations and identification of new surface targets that are highly expressed in all MDSC sub-populations could drastically improve MDSC targeting and depletion.

SNAb treatment resulted in a significant increase in MDSCs at Week 9 Days 1 and 2 post-treatment as well as a significant decrease in immune effector cells, including T cells and the T helper cell and cytotoxic T cell subsets. In addition to these cellular changes, multiple significant differences in cytokine levels were observed at both Week 9 Days 1 and 2. These data suggest that SNAbs have the capability to significantly alter multiple aspects of the systemic immune response on both a cellular and protein level. While these cellular and protein differences were not sustained past Week 9 Day 2, we still observed significant correlations between multiple immune cell types at Week 20 and endpoint bone volume in the SNAb treated rats. This suggests that the temporal changes in immune cell populations and cytokine levels at 24- and 48- hours post-SNAb treatment may still have had long-term effects, even 11 weeks

later. Further studies are needed to better understand the mechanisms of SNAb treatment on the systemic immune response and if multiple SNAb treatments are needed in order to achieve more robust responses.

SNAb depletion of only the HIS48^{hi} MDSC subset in the composite trauma model did not improve local healing, likely due to the significant increase in total MDSCs that overshadowed the depletion in the HIS48^{hi} sub-population. SNAb treatment appeared to worsen bone repair; however, because SNAb treatment resulted in an increase in total MDSCs, this is consistent with our previous data showing a negative correlation between MDSCs and healing. When comparing all SNAb treated rats to rats that did not receive SNAbs, the failure strength in torsion was significantly decreased, suggesting poor functional regeneration. Due to aberrant SNAb targeting and the large increase in the HIS48^{lo} MDSCs following SNAb treatment, it is not surprising to see negative effects on bone regeneration. This is further supported by linear regression analyses which demonstrated multiple immune cell populations in SNAb-treated rats that correlated either positively or negatively with healing just 48 h after treatment. These correlations mirror those found in previous studies of segmental defect injuries treated with BMP-2 only,[112] suggesting that the detrimental effects of the SNAbs on healing in this study were likely immune-mediated. Of note, monocytes also correlated negatively with endpoint bone volume, similar to MDSCs. MDSCs and monocytes are both myeloid-lineage cells, and therefore may share similar surface markers, such as the target molecule of the SNAbs. Further work is needed to understand how MDSC-depleting SNAbs interact specifically with monocytes and macrophages, and why monocytes also negatively correlate with endpoint bone volumes. In addition, improved MDSC targeting may be necessary to achieve robust depletion of both subsets of MDSCs to restore immune homeostasis and positively impact regeneration.

It should be addressed that this study included no unmodified particles or scrambled peptide modified particles as controls due to animal limitations and limitations with reagent scale-up. A large sample size was needed to account for the non-responders and responders within each treatment group, and at n = 10 per group, multiple control groups were not physically feasible. However, previous work with SNAbs in a murine cancer model did utilize irrelevant peptide and unmodified particles as controls, and these did not elicit similar responses to the MDSC-targeting SNAbs.[196] It should also be addressed that BMP-2 has been shown to have immunomodulatory effects, including macrophage stimulation and upregulation of cytokines important for cell recruitment and angiogenesis.[209] Because of this, all rats were treated with BMP-2 to eliminate any potential confounding factor due to local immunological changes. It is not expected that systemic MDSC-depleting SNAbs alone can alter local bone healing, and therefore, this necessitates a local treatment strategy, which in this case utilizes BMP-2. We posit that that consideration of immune homeostasis and a permissive, pro-healing environment at both the local and systemic levels is necessary for regenerative medicine strategies to reach their full potential. BMP-2 was selected as the local treatment strategy because it is a potent osteoinductive growth factor with approval by the U.S. Food and Drug Administration in select applications including spinal fusions and some tibia fractures.[210] In addition, pre-clinical and clinical studies over the past decade have shown significant promise for BMP-2 treatment in long bone fractures.[211]

In conclusion, this study examined the effects of a novel, non-surgical nanoparticle approach for modulating MDSCs and the systemic immune response in a composite injury model of trauma. This approach was successful in depleting MDSCs and associated immunosuppressive genes *in vitro* using cells derived from an infected trauma model. Although

total MDSCs were elevated following SNAb treatment in the composite trauma model, closer analysis revealed that a subset of HIS48^{hi} MDSCs were depleted successfully. Single-cell RNA sequencing results suggest that the unexpected effects of SNAbs in the composite trauma model may be related to varied gene expression patterns of S100a8/a9 in MDSC subsets. Elevated levels of systemic MDSCs and monocytes after SNAb treatment inversely correlated to healing while T cells, T helper cells, and cytotoxic T cells positively correlated, even just 48 h post-treatment. These correlations provide insight into why rats treated with SNAbs and BMP-2 may have demonstrated worse healing than rats treated with BMP-2 only. Altogether, these results provide further evidence in support of the role of systemic immune dysregulation and specifically MDSCs in impairing bone repair outcomes and suggest the need for further studies on these cells and their subsets to improve targeted immunomodulatory interventions and enhance healing.

CHAPTER 4: ALL-TRANS RETINOIC ACID MODULATES CIRCULATING IMMUNE CELLS IN A PRECLINICAL MODEL OF MUSCULOSKELETAL TRAUMA

Contents of this chapter were adapted from Guyer T., Gill D., Leguineche K.E., Link K., Jones K.A., Williams K.E., Strait L.K., O'Neill K.L., Hastings M.A., Lin A., Maniar D., Roy K., and Guldberg R.E. (manuscript in preparation).[269]

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4.1 Introduction

Although recent advancements in care have improved survival for patients with severe musculoskeletal trauma, serious complication rates remain high, with nonunion occurring in an estimated 1.9%–4.9% of all bone fractures.[6,180] Additionally, extensive concomitant soft tissue damage can further increase the risk of nonunion. Patients with such complications are frequently faced with costly revision surgeries that prolong pain and may still result in permanent functional impairment.[8,212] As such, there remains a need for the development of novel therapeutic strategies that can reduce nonunion incidence and alleviate the suffering associated with nonunion.

Immune cells are well understood to play critical roles in bone fracture healing. During the early stage of inflammation, immune cells participate in angiogenesis and affect the recruitment, proliferation, differentiation, and activation of mesenchymal and hematopoietic cells

at the injury site.[213] While the research to date has primarily examined the effects of immune cells within the injury site, in recent years, systemic or circulating immune markers have also been implicated as potential factors in healing outcomes.[112,185] Following severe trauma, damage-associated molecular patterns and pathogen-associated molecular patterns are rapidly elevated and subsequently result in an increase in inflammatory cells and mediators.[187] Left unchecked, excessive inflammation may lead to systemic tissue damage, resulting in multiple organ dysfunction syndrome and even death.[78] Consequently, the elevation of inflammatory markers coincides with an increase in anti-inflammatory markers which act to balance systemic inflammation.[214] Ideally, these concurrent inflammatory and anti-inflammatory responses would resolve within days and circulating levels of their corresponding immune mediators would return to baseline values. If this balance is not achieved, however, dysregulated levels of inflammatory and anti-inflammatory mediators can persist for days or weeks, which may contribute to impaired healing and systemic complications.[186,215]

A notable immune cell population that has recently been implicated in poor trauma outcomes such as nonunions is the myeloid-derived suppressor cell (MDSC). MDSCs represent a heterogeneous population of immature myeloid cells which expand rapidly following traumatic injury.[126,216] These cells have potent immunosuppressive functions, including inhibition of immune effector cells such as T cells and natural killer cells and induction or recruitment of other immunosuppressive cell types such as regulatory T cells (Tregs).[119,123,217,218] MDSCs exert these effects through mediators including reactive oxygen and nitrogen species, immunosuppressive cytokines such as interleukin-10 and TGF-beta, and enzymes such as arginase 1.[122,219–222] Various immune effector cell populations including certain subsets of T cells have been shown to be important to the early stages of bone repair,[74] and therefore excessive

immunosuppression could be detrimental to healing. In fact, recent studies have indicated a link between systemic levels of MDSCs and poor outcomes following bone trauma. In multiple rat models of musculoskeletal trauma, circulating MDSCs have been found to negatively correlate with bone regeneration.[112,195] In another study that aimed to target and deplete MDSCs in a rat trauma model using a nanoparticle-based immunomodulatory therapeutic, one sub-population of MDSCs decreased while another sub-population expanded, resulting in an increase in total MDSCs that correlated negatively with healing.[179] Another study using a rat model of trauma associated with infection demonstrated an increase in circulating MDSCs along with decreased circulating T cells and a dysregulated cytokine response in rats with infection as compared to those with non-infected trauma.[134] In humans, the relationship between MDSCs and trauma outcomes remains underexplored. However, in patients who develop sepsis, circulating MDSCs appear to be major actors of sepsis-induced immunosuppression and have been correlated with greater mortality and development of nosocomial infections.[186,223] Taken together, these animal and human studies may indicate potential for MDSCs as a therapeutic target for combating trauma-induced immunosuppression and improving healing outcomes.

Methods for therapeutically modulating MDSCs have been studied extensively in the context of cancer, where the immunosuppressive activity of MDSCs can contribute to tumor growth and lessen the potency of cancer interventions.[224] Numerous therapeutics for diminishing MDSCs have been researched. Notably, all-trans retinoic acid (ATRA), a vitamin A derivative, has been shown to diminish MDSCs by promoting their differentiation into mature myeloid cell types.[225,226] ATRA upregulates the synthesis of glutathione, an oxygen scavenger, which in turn reduces levels of reactive oxygen species.[151] Reactive oxygen species play roles in inhibiting MDSC differentiation,[227] suggesting that reactive oxygen species scavenging compounds may

rescue the differentiation trajectory into mature monocytes, granulocytes and dendritic cells. In certain cancer models, ATRA has been found to decrease the capacity of MDSCs to inhibit T cell function, yielding therapeutic benefits.[228] However, its effects on MDSCs in the context of trauma have not been previously examined.

Here, we examined the effects of ATRA on circulating immune cells and functional bone healing in a rat model of musculoskeletal trauma. We hypothesized that systemic ATRA administration after injury would diminish circulating MDSCs and improve bone regeneration. To investigate this, we employed a previously established rat model of bone and muscle polytrauma involving both a critically-sized bone defect and a volumetric muscle injury.[229] The addition of the muscle injury in this model has been shown to worsen bone regenerative outcomes and exacerbate immune dysfunction, making this an ideal testbed for immunotherapeutics. We began with an *in vitro* experiment screening the immunomodulatory potential of ATRA and several other treatments including vitamin E, anti-CXCR2 antibodies, and anti-TSPAN33 antibodies, all of which have been shown to diminish MDSCs or alter their immunosuppressive function.[230–232] Peripheral blood immune cells derived from rats with bone and muscle polytrauma were cultured *in vitro* for 24 hours with each therapeutic and analyzed via flow cytometry. ATRA was the only therapeutic tested to successfully deplete MDSCs, and it was therefore investigated further in subsequent *in vivo* experiments. In these experiments, a group of rats with bone and muscle polytrauma received multiple intraperitoneal injections with ATRA in the early days after injury when MDSCs have been shown to be most elevated. The critically-sized bone defects (8mm) were also treated with bone morphogenetic protein-2 (BMP-2) at a low dose (2.5μg) which has previously been shown to result in a range of healing responses after 12 weeks of healing.[233] Circulating immune cells were quantified after ATRA

treatment via cell staining and flow cytometry, and bone healing was assessed via radiographs, micro-computed tomography (micro-CT) scans, and torsional testing of regenerated femurs. Finally, after observing only partial depletion of MDSCs following ATRA treatment, an additional *in vivo* experiment was performed to examine the effects of ATRA delivered at higher doses. Overall, the resulting data demonstrate a novel strategy for modulating the immune response to musculoskeletal trauma and highlight a need for deeper characterization of the post-trauma immune response and its relationship with healing.

4.2 Materials and Methods

4.2.1 Nanofiber Mesh Preparation

Poly(ε-caprolactone) (PC-12 PURASORB, Corbion, USA) was dissolved at 15% (w/v) in dichloromethane (DCM) and dimethylformamide (both Sigma-Aldrich, USA) at a 3:2 ratio for approximately 16 hours under gentle agitation. The polymer solution was loaded into a 3 mL syringe (FORTUNA, USA) with a 32-gauge metal nozzle (McMaster-Carr, USA). A custom-based solution electrospinning system was used to create a polymer coating onto a 5 mm diameter cylindrical collector (McMaster-Carr). The collector was placed at a distance of 13 cm from the printer head, and a voltage of 20 kV and a pressure of 1 bar were applied. The mandrel movement was programmed with Mach3 software (Artsoft, USA). Then, a solder was used to melt through the scaffold by setting the solder tip to 400°C, and programming solder movements with AeroBasic software (Aerotech, USA). Scaffolds were removed from the mandrel by spraying them with 70% ethanol to detach the polymer from the aluminum mandrel. Scaffolds were then gently perforated to yield 1-mm diameter perforations, which have been shown to

benefit vascular invasion during the repair process.[202] The resulting scaffolds were 13 mm in length, with alternating 3 and 2 openings of 1 mm on each row.

4.2.2 Alginate BMP-2 Preparation

Alginate hydrogel constructs were prepared under sterile conditions inside a laminar flow hood. The constructs were made by first creating a 3% w/v alginate solution by dissolving arginine-glycine-aspartic acid-functionalized alginate (FMC BioPolymer) in sterile alpha-MEM (Corning). Recombinant BMP-2 (Pfizer) was reconstituted in a solution containing 0.1% rat serum albumin (Sigma-Aldrich) in sterile 4 mM hydrochloric acid, which was then mixed with the alginate solution to yield 2.5 μg BMP-2 per 150 μL of final solution. The solution was cross-linked in an excess of calcium sulfate by thorough mixing, allowed to gel at room temperature for 30 minutes, and then stored in sterile syringes at 4°C before use in surgeries the next day.

4.2.3 Animal Model

For all experiments, female Sprague Dawley rats (Charles River Laboratories) were used, which were 14 weeks old at the time of surgeries. Rats were housed in pairs in individually ventilated caging with a PVC tunnel and nylon bones for enrichment. Food and filtered water were provided *ad libitum*. All animals were allowed to acclimate in cages for at least one week after arrival before any procedures were performed. Following surgeries, animals were individually housed for two weeks for better monitoring of postoperative recovery. Animals were randomly allocated to treatment and control groups. At experimental endpoints, euthanasia was performed with either CO_2 asphyxiation or exsanguination via cardiac puncture and was confirmed by secondary cervical dislocation.

4.2.4 Surgical Procedures

All animal care and experimental procedures were approved by the University of Oregon IACUC (Protocol 20-10). Surgical procedures have been previously described.[229] Anesthesia was induced and maintained via isoflurane (VetOne) inhalation for all animal procedures. For analgesia, all animals received a single subcutaneous injection of buprenorphine HCL (0.03 mg/kg, Hikma Pharmaceuticals) and a subcutaneous injection of sustained-release buprenorphine (0.65 mg/kg, Ethiqa) prior to surgical procedures. Animals also received 3 mL of Lactated Ringer's solution (VetOne) subcutaneously prior to surgical procedures. Briefly, an anterolateral skin incision was made in the thigh, followed by separation of the overlaying muscles to reach the femur. A radiolucent polysulfone internal fixation plate was placed to stabilize the femur, and a critically sized 8 mm defect was created in the mid-diaphysis of the femur using an oscillating saw. Additionally, an 8 mm diameter full-thickness muscle defect was created in the adjacent quadricep using a biopsy punch. For all rats used in the *in vivo* MDSC depletion experiments, a 6-mm diameter poly-caprolactone nanofiber mesh was then placed within the femur defect around the bone ends, and alginate containing BMP-2 was injected via syringe into the perforations in the mesh. For rats used in the *in vitro* MDSC depletion experiments, the bone defects were left untreated. In both groups, the muscle defects were also left untreated. The muscle was then closed with Vicryl suture, and the skin was closed with a subcutaneous Monocryl suture layer and wound clips. Following surgery, the incision sites were cleaned with sterile saline, and a mixture of Metronidazole (Viona Pharmaceuticals) and New-Skin Liquid Bandage was applied. Animals were provided DietGel® Boost (Clear H_2O) and Nutri-Cal gel (Vetoquinol) for post-operative nutritional support. All animals were monitored for post-operative complications for at least three days following surgeries.

4.2.5 In Vitro MDSC Depletion Experiments

For our *in vitro* experiments, blood was collected from rats with polytrauma at day 7 post-injury via cardiac puncture. Approximately 3-5 mL of blood was collected from each rat into Vacutainer lithium-heparin-coated tubes (BD Biosciences). Red blood cells were lysed using RBC Lysis Buffer (eBioscience) according to the manufacturer's instructions. Cells were then washed, counted, pooled, and resuspended in RPMI media supplemented with 10% FBS (Gibco) and 1% penicillin-streptomycin (Gibco) before being plated in a 96-well plate with 1,000,000 cells per well. For the treatment groups, ATRA, vitamin E, anti-CXCR2 antibodies, or anti-TSPAN33 antibodies were added to the respective wells. Another group was left untreated. Cells were incubated at 37°C for 24 hours before being collected for subsequent cell characterization via fluorescent antibody staining and flow cytometry. This experiment was also repeated with ATRA using blood from uninjured, naive animals.

4.2.6 In Vivo MDSC Depletion Experiments

For *in vivo* experiments, ATRA was delivered to rats at a dose of 1 mg/kg body weight, 5 mg/kg body weight, or 10 mg/kg body weight. For delivery of ATRA at the 1 mg/kg dose, ATRA was dissolved using olive oil (US Pharmacopeia) as the solvent. For delivery of ATRA at the higher doses, ATRA was dissolved in dimethyl sulfoxide (DMSO, US Pharmacopeia). ATRA solutions were administered via intraperitoneal injection. Blood was collected from rats after ATRA treatment via the ventral tail artery using Vacuette blood collection needles (Greiner Bio-One). Approximately 400 µL of blood was collected into Microtainer lithium-heparin coated tubes (BD Biosciences) for analysis via flow cytometry. Red blood cells were lysed using RBC Lysis Buffer (eBioscience) according to the manufacturer's instructions. Cells were subsequently fixed using Flow Cytometry Fixation Buffer (R&D Systems) and resuspended in FACS buffer

containing 2% fetal bovine serum in phosphate-buffered saline for subsequent cell characterization via fluorescent antibody staining and flow cytometry.

4.2.7 Immune Characterization

For immune cell characterization in both the *in vitro* and *in vivo* experiments, cells were first incubated for 10 minutes at 4°C with anti-rat CD32 to prevent non-specific antibody binding with Fc receptors. Next, cells were stained by incubation for 25 minutes at 4°C with various surface antibodies before being washed with FACS buffer. Cells were then permeabilized using Cytofix/Cytoperm buffer (BD Biosciences) and washed with Perm/Wash buffer (BD Biosciences) according to the manufacturer's instructions before staining with intracellular antibodies. Flow cytometry was then performed using a BD Accuri C6 flow cytometer. Data were analyzed using FlowJo software with gates positioned based on fluorescence minus one controls allowing <1% noise. The immune cell populations analyzed included myeloid-derived suppressor cells (HIS48+CD11b/c+), monocytes (CD68+CD11b/c+), B cells (B220+), T cells (CD3+), cytotoxic T cells (CD3+CD8+), helper T cells (CD3+CD4+), and regulatory T cells (CD3+CD4+FOXP3+).

4.2.8 Radiography and Micro-Computed Tomography

In our first *in vivo* experiment, longitudinal bone regeneration at weeks 4, 8, and 12 post-trauma was assessed both qualitatively and quantitatively via radiographic imaging and micro-computed tomography (micro-CT), respectively. Digital radiographs were captured at 40 kV with an exposure time of 7 seconds using an UltraFocus digital radiography system (Faxitron). Micro-CT was performed on femurs using a vivaCT80 (Scanco Medical) both *in vivo* at weeks 4 and 8 and *ex vivo* at week 12 post-injury. The volume of interest consisted of the central 6.5 mm of the 8 mm femoral defect. Scans were performed with a 55-kVp voltage and a 145-μA current,

and the voxel size was set to 48μm for *in vivo* scans and 24μm for *ex vivo* scans. To identify newly regenerated bone, a threshold corresponding to 50% of native cortical bone density was applied.

4.2.9 Biomechanical Testing

Torsional testing to failure was also performed to quantitatively assess functional bone regeneration. Rats were sacrificed at week 12 post-injury and femurs were excised, wrapped in PBS-soaked gauze, and frozen for three days at -20°C after micro-CT imaging. On the day of testing, femurs were thawed, their fixation plates were removed, and their native bone ends were potted in Wood's metal (Alfa Aesar). The femurs were then tested to failure in torsion at a rotation rate of 3 degrees per second using the ElectroForce 3220 testing system (TA Instruments). Failure strength was identified as the peak torque within the first 90 degrees of rotation, and torsional stiffness was calculated as the slope of the linear region before failure in the torque-rotation plot.

4.2.10 Statistical Analysis

All data are reported as mean ± SEM. Statistical significance was determined using either the t test or ANOVA with appropriate post hoc tests. Significance was determined by p values less than 0.05. All statistical calculations were performed using GraphPad Prism software (Version 10.4.2, La Jolla, CA, USA).

4.3 Results

4.3.1 Rat Polytrauma-Derived MDSCs are Depleted In Vitro Following ATRA Treatment

In vitro experiments were conducted using peripheral blood mononuclear cells derived from either our rat polytrauma model or naive, uninjured rats. Blood samples were cultured for

24 hours either untreated or treated with all-trans retinoic acid (ATRA) at one of three concentrations, vitamin E, anti-CXCR2 antibodies, or anti-TSPAN33 antibodies.

Treatment with ATRA at all three tested concentrations diminished myeloid-derived suppressor cells (MDSCs) in the polytrauma-derived blood as compared to untreated controls (Figure 10A). Monocytes were also significantly depleted in two of the three ATRA-treated groups (Figure 1B). Meanwhile, B cells, total T cells, helper T cells, cytotoxic T cells, and regulatory T cells (Tregs) were unaffected by ATRA treatment (Figure 10C–G).

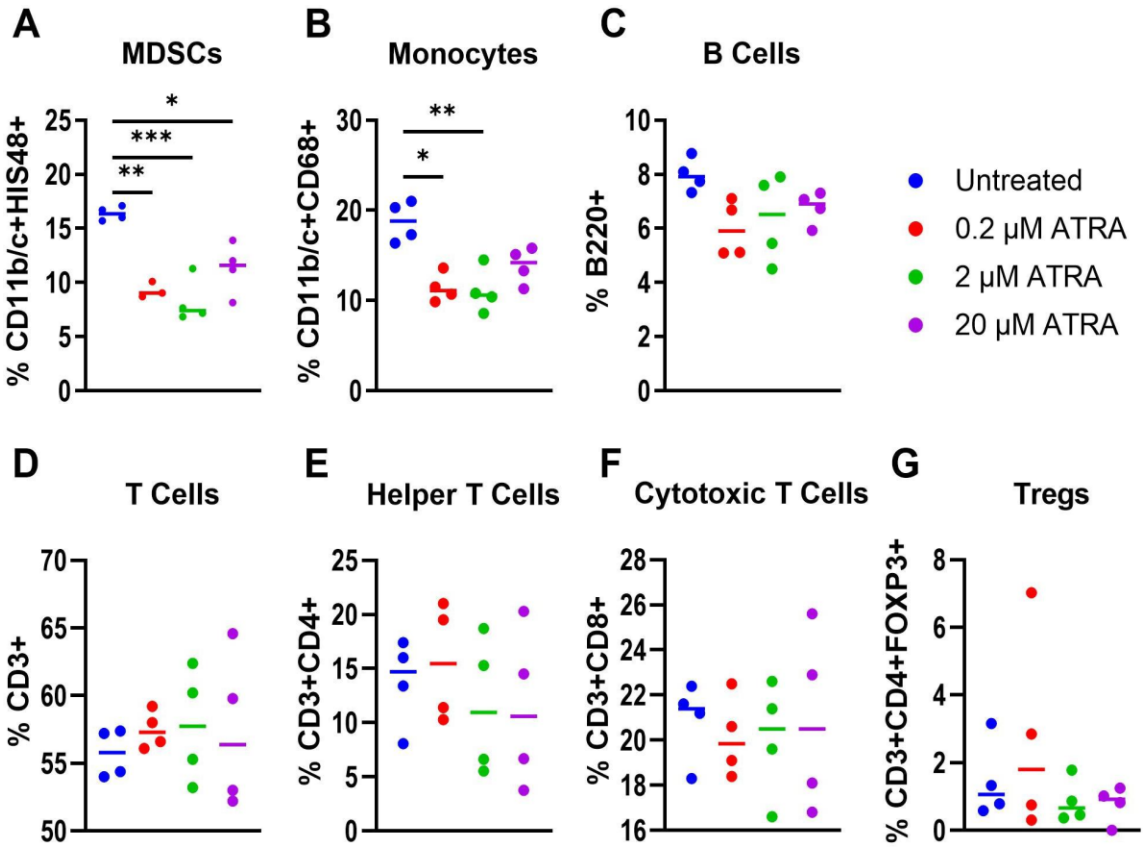


Figure 10. ATRA altered relative immune cell frequencies *in vitro* in blood derived from rats with polytrauma. (A) MDSCs were significantly depleted by treatment with ATRA at three different concentrations as compared to untreated controls. (B) Monocytes were significantly depleted by ATRA treatment at two of the concentrations tested. (C–G) Other immune cell populations were unaltered. Significance was determined using one-way ANOVA with Dunnett's post-hoc test. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 as indicated.

Besides ATRA, none of the other therapeutics tested *in vitro* resulted in altered blood MDSC levels (Figure 11A). However, anti-TSPAN33 antibodies interestingly decreased the relative frequencies of monocytes and T cells while increasing the relative frequency of B cells, and anti-CXCR2 antibodies also increased the B cell frequency (Figure 11B–D).

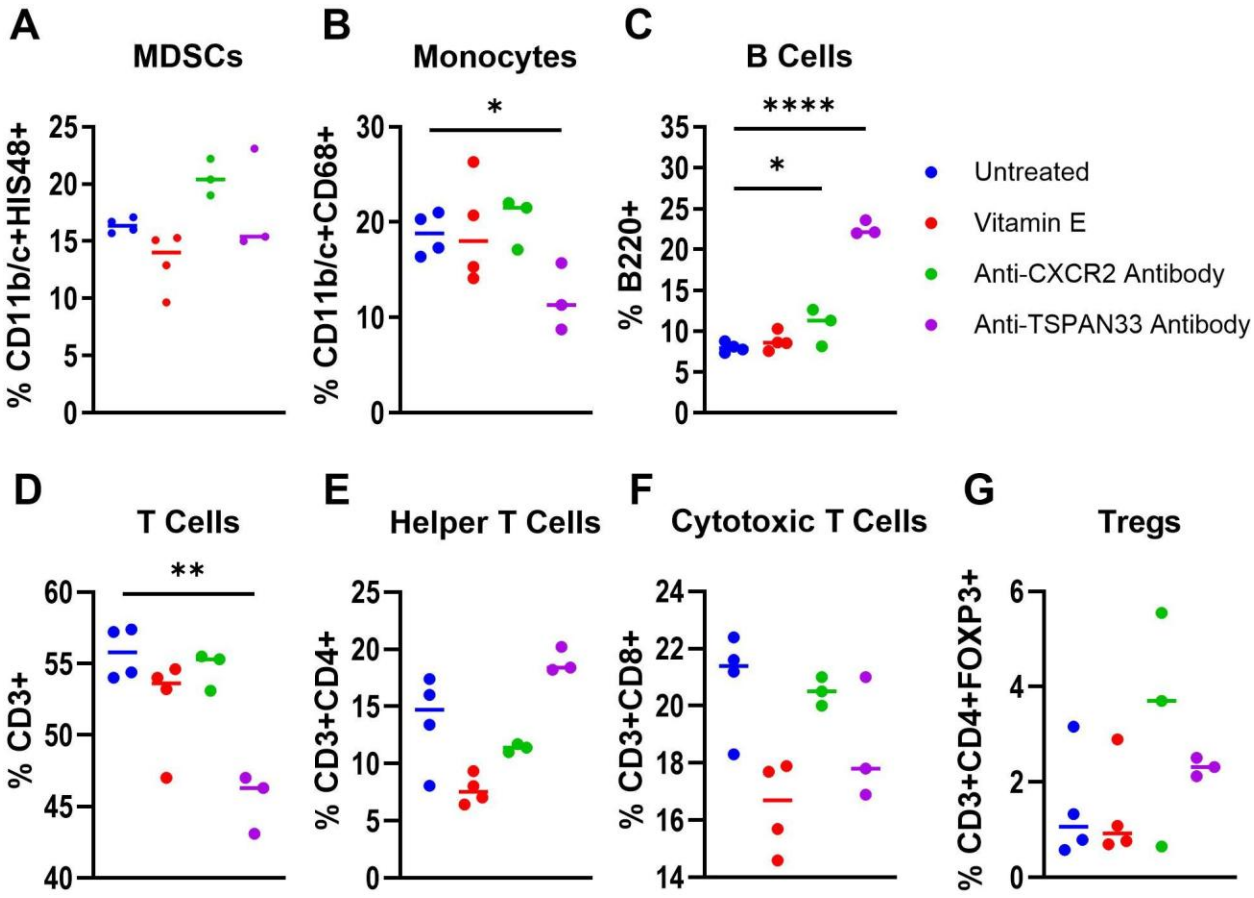


Figure 11. Immunomodulatory therapeutics did not alter MDSCs *in vitro* in blood derived from rats with polytrauma. (A) MDSCs were not affected by *in vitro* treatment with vitamin E, anti-CXCR2 antibodies, or anti-TSPAN33 antibodies. (B) Monocytes were depleted following treatment with anti-TSPAN33 antibodies. (C) B cells were elevated following treatment with both anti-CXCR2 antibodies and anti-TSPAN33 antibodies. (D) T cells were depleted following treatment with anti-TSPAN33 antibodies. (E–G) Other immune cell populations were not significantly altered by any of the treatments. Significance was determined using one-way ANOVA with Dunnett's post-hoc test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ as indicated.

Lastly, blood from uninjured animals was also treated with ATRA at the middle concentration (2 µM) tested in the trauma blood. Here, ATRA treatment was again shown to result in diminished MDSCs, although no other immune cell populations were significantly altered (Figure 12).

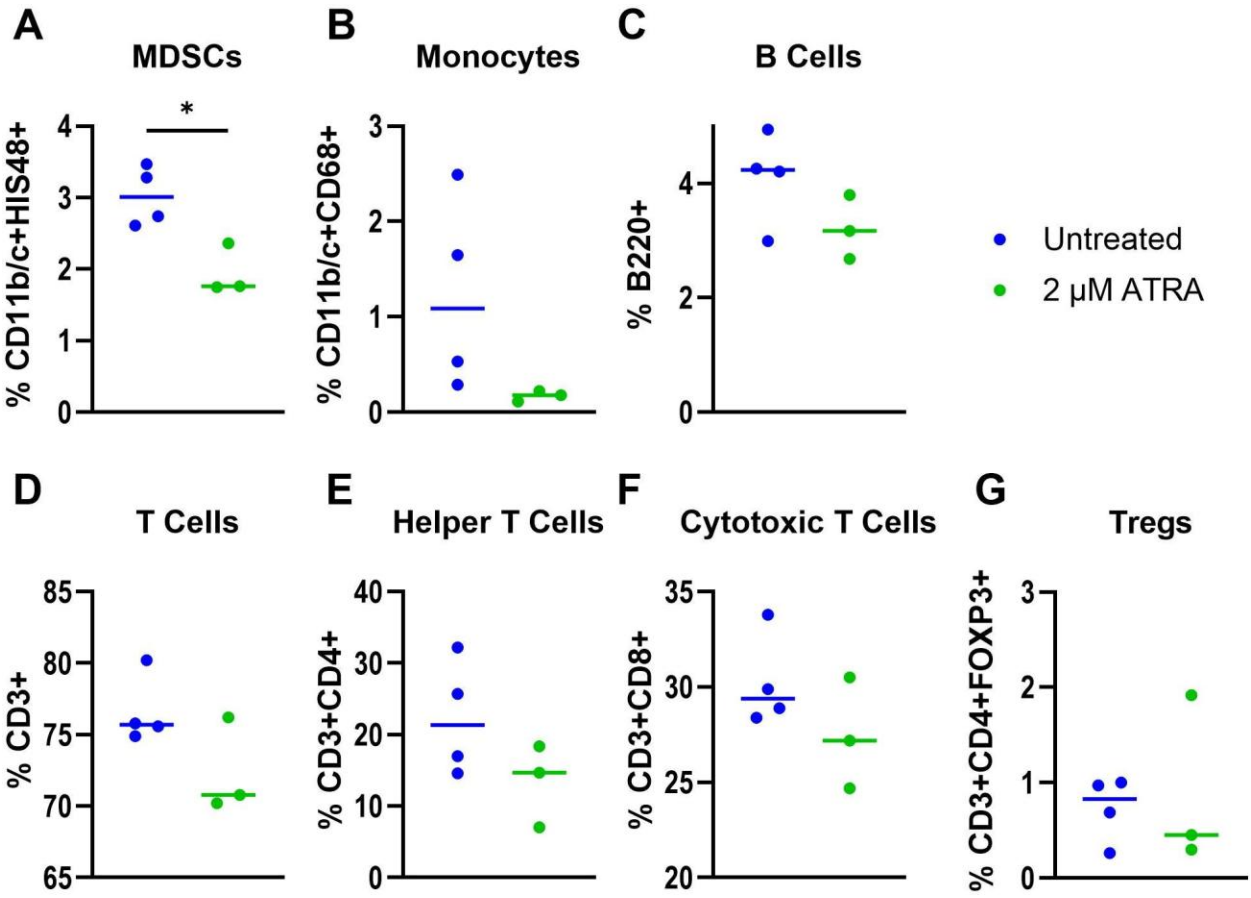


Figure 12. ATRA depleted MDSCs *in vitro* in blood derived from naive, uninjured rats. (A) MDSCs were significantly depleted following *in vitro* treatment with ATRA at a 2 µM concentration. (B–G) Other immune cell populations were not significantly altered. Significance was determined using a two-tailed t test for each comparison. *P < 0.05 as indicated.

4.3.2 Circulating Immune Cells are Altered In Vivo Following ATRA Treatment

Given the results of the *in vitro* immunomodulatory experiments, ATRA was selected from the therapeutics tested for further study *in vivo* using our rat model of bone and muscle

polytrauma. Here, a subset of injured rats received daily intraperitoneal injections with ATRA dissolved in olive oil at a dose of 1 mg/kg on days 3 through 7 post-injury. Blood was collected from rats in both the ATRA-treated and untreated groups at day 4, week 1, week 2, week 4, week 9, and week 12 after injury, and immune cells were characterized via flow cytometry. A naive control group consisting of age-matched uninjured rats was also included. The relative frequency of MDSCs in the blood was not significantly altered by ATRA treatment at any time point (Figure 13A). Tregs were significantly depleted in the ATRA-treated group at both day 4 and week 1 post-injury (Figure 13G). Compared to naive controls, both the ATRA-treated and untreated groups of injured rats had elevated MDSCs and decreased T cells, helper T cells, and cytotoxic T cells at day 4 and week 1 post-injury (Figure 13A, B, D–F).

Additionally, a more extensive analysis of the MDSCs revealed that while total MDSCs were not depleted by ATRA treatment, a sub-population of the cells decreased. MDSCs possess two subsets in rats, which can be classified as CD11b/c^{+}HIS48^{hi} and CD11b/c^{+}HIS48^{lo}. Here, the CD11b/c^{+}HIS48^{hi} cells were depleted at day 4 post-injury (Figure 3A), while the CD11b/c^{+}HIS48^{lo} cells were not altered (Figure 14B).

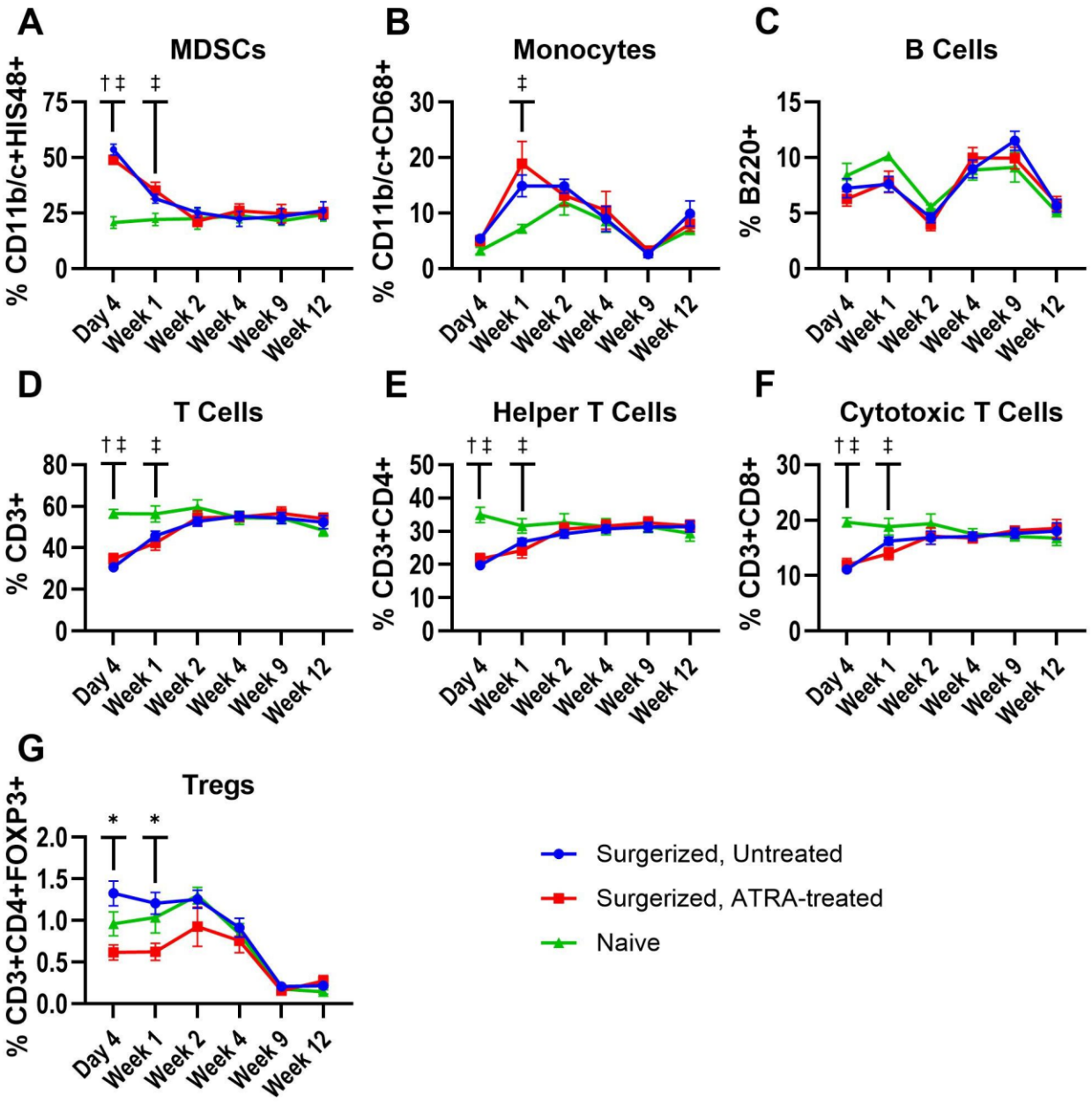


Figure 13. Peripheral blood immune cells were differentially regulated by polytrauma and by ATRA treatment *in vivo*. (A) MDSCs were elevated in rats with polytrauma as compared to uninjured rats at day 4 and week 1 post-injury. (B) Monocytes were also significantly elevated in one of the polytrauma groups at week 1 post-injury. (D–F) Total T cells, helper T cells, and cytotoxic T cells were diminished at day 4 and week 1 post-injury as compared to uninjured rats. (G) Tregs were significantly diminished following ATRA treatment in rats with polytrauma as compared to the untreated polytrauma group. Asterisks (*) indicate a significant difference between the ATRA-treated and untreated groups. Daggers (†) indicate a significant difference between the untreated and naive groups. Double daggers (‡) indicate a significant difference between the ATRA-treated and naive groups. n = 4 to 7 per group. Significance was determined using one-way ANOVA with Tukey's post-hoc test. $P<0.05$ for all significant differences shown.

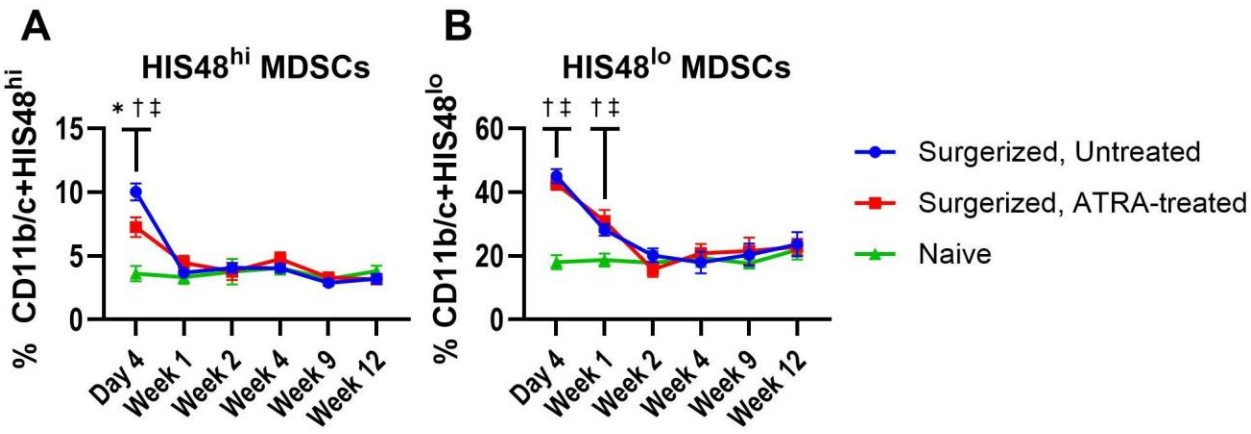


Figure 14. MDSC sub-populations were elevated by polytrauma and differentially regulated by ATRA treatment. (A) HIS48^{hi} MDSCs were elevated in both polytrauma groups as compared to the naive group and were diminished by ATRA treatment. (B) HIS48^{lo} MDSCs were also elevated in both polytrauma groups but were not diminished by ATRA treatment. Asterisks (*) indicate a significant difference between the ATRA-treated and untreated groups. Daggers (†) indicate a significant difference between the untreated and naive groups. Double daggers (‡) indicate a significant difference between the ATRA-treated and naive groups. n = 4 to 7 per group. Significance was determined using one-way ANOVA with Tukey's post-hoc test. $P<0.05$ for all significant differences shown.

4.3.3 Treatment with ATRA did not Alter Functional Bone Regeneration

Bone healing was assessed by *in vivo* radiographic imaging and micro-computed tomography (micro-CT) at weeks 4 and 8 post-injury as well as *ex vivo* micro-CT and biomechanical testing at week 12 post-injury. Bone volume and bone mineral density were not significantly altered by ATRA treatment at any time point (Figure 15A, B). Similarly, endpoint torsional strength and torsional stiffness were not significantly altered (Figure 15C, D).

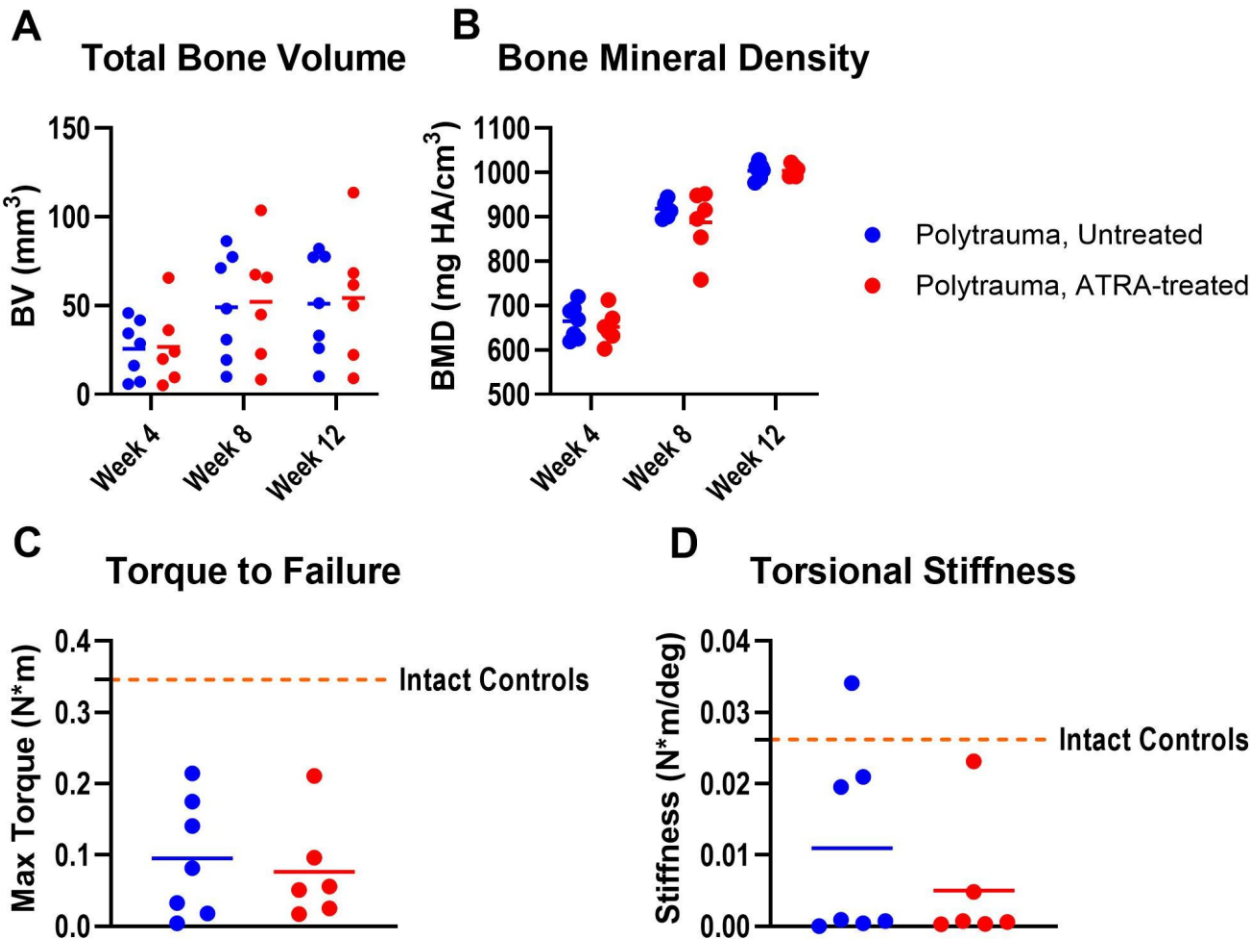


Figure 15. Functional bone regeneration was not significantly altered by treatment with ATRA. (A,B) Total bone volume (A) and bone mineral density (B) of regenerated bone as quantified by micro-CT. (C,D) Mechanical strength (C) and stiffness (D) of regenerated femurs as determined by *ex vivo* torsional testing to failure at week 12. Dotted lines indicate mechanical properties of intact femurs from the contralateral limbs of the polytrauma rats. Significance was assessed using a two-tailed *t* test for each comparison.

4.3.4 Circulating Tregs Correlate with Bone Regeneration

Linear regression analyses were performed using data from all rats in the polytrauma groups to identify systemic immune cell populations that correlated with bone regeneration. The relative frequency of circulating Tregs at week 2 post-injury correlated negatively with endpoint bone volume as measured by *ex vivo* micro-CT scans (Figure 16).

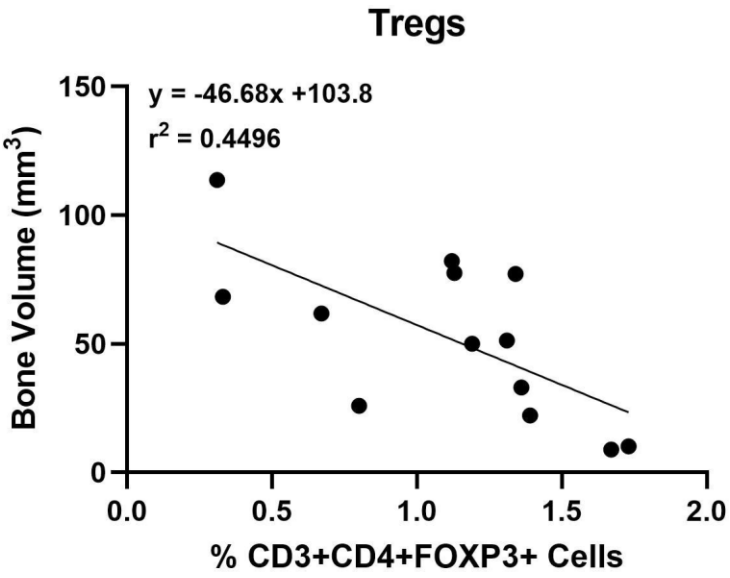


Figure 16. Tregs measured at two weeks post-injury correlate with bone regeneration. Week 2 Tregs correlated negatively with endpoint bone volume. Slope of linear regression significantly nonzero (P = 0.0121).

4.3.5 High Dose ATRA Increased Circulating MDSCs in a Rat Model of Trauma

Given that ATRA diminished a subset of MDSCs and Tregs in the previous *in vivo* study, a subsequent study was performed to determine whether ATRA at a higher dose could yield greater depletion of total MDSCs and modulate the healing response. Here, rats with the same bone and muscle injuries as in the previous study received intraperitoneal injections with ATRA at a dose of 5 mg/kg on days 3 and 7 post-injury. Notably, in this study, the higher ATRA dose necessitated the solvent DMSO rather than olive oil. Blood draws were performed 12 hours after each injection and immune cells were analyzed via flow cytometry. Total MDSCs were once again not altered following the first day of ATRA injections, while Tregs were again depleted (Figure 17A, G). Interestingly, however, MDSCs were increased on day 7 in the ATRA-treated group as compared to the untreated group following the second set of injections. Monocytes also increased significantly in the ATRA-treated group at this time point, while the relative

frequencies of T cells, helper T cells, and cytotoxic T cells were diminished as compared to the untreated controls (Figure 17B, D –F).

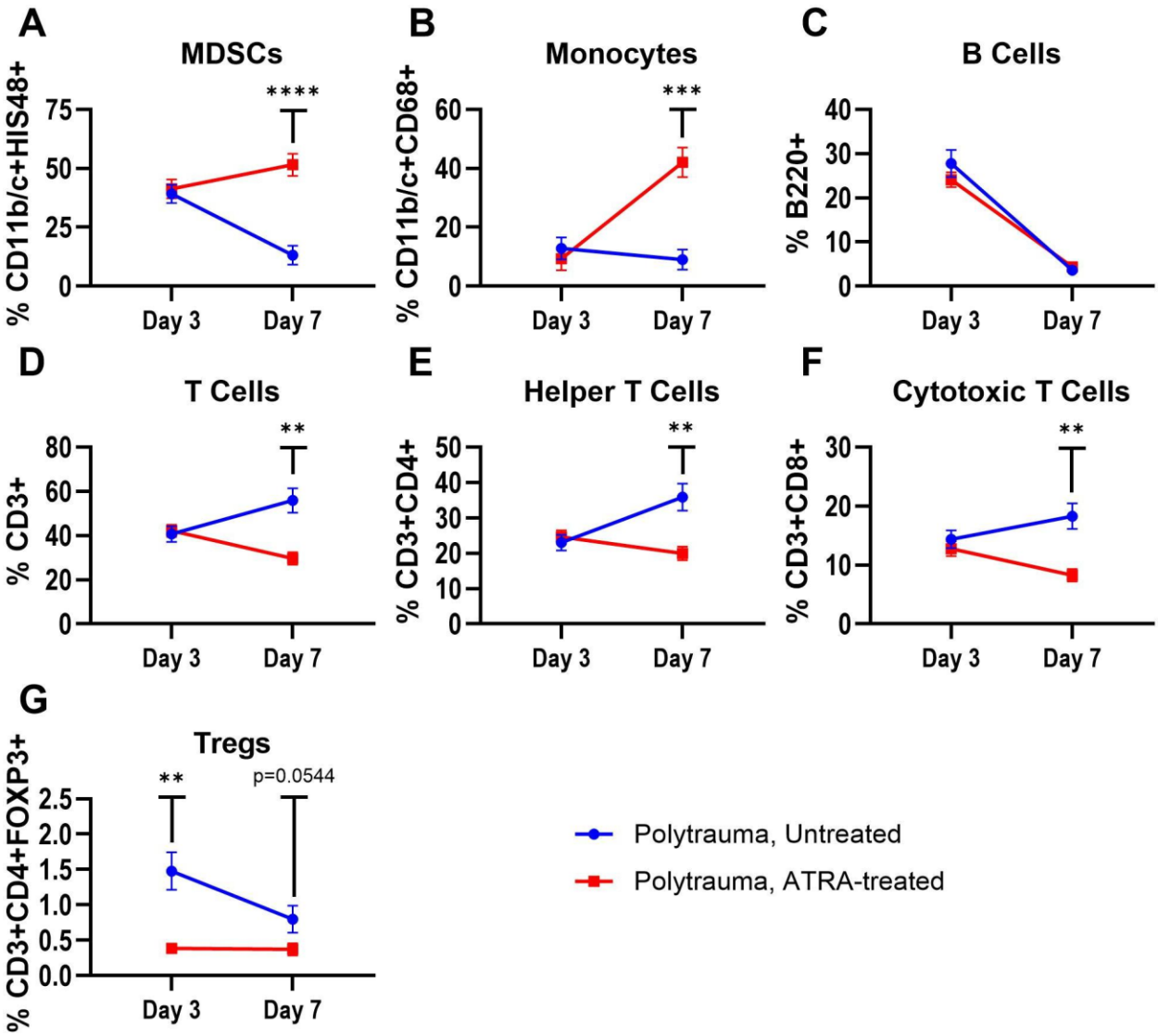


Figure 17. Treatment with ATRA at a higher dose resulted in elevated MDSCs. (A) MDSCs were not altered at day 3 following a single injection with ATRA at a dose of 5 mg/kg, but they unexpectedly increased following a second ATRA injection at day 7, as compared to untreated controls. (B) Monocytes were also elevated at day 7. (D–F) Relative frequencies of total T cells, helper T cells, and cytotoxic T cells decreased at day 7 in the ATRA-treated group as compared to the untreated control group. (G) Tregs were significantly diminished by ATRA treatment at day 3. n = 6 to 7 per group. Significance was determined using a two-tailed *t* test for each comparison. **P < 0.01; ***P < 0.001; ****P < 0.0001 as indicated.

Analysis of MDSC sub-populations demonstrated that both HIS48^{hi} and HIS48^{lo} MDSCs were significantly elevated on day 7, although the total increase in MDSCs appeared to be driven predominantly by the elevation of the HIS48^{lo} sub-population (Figure 18).

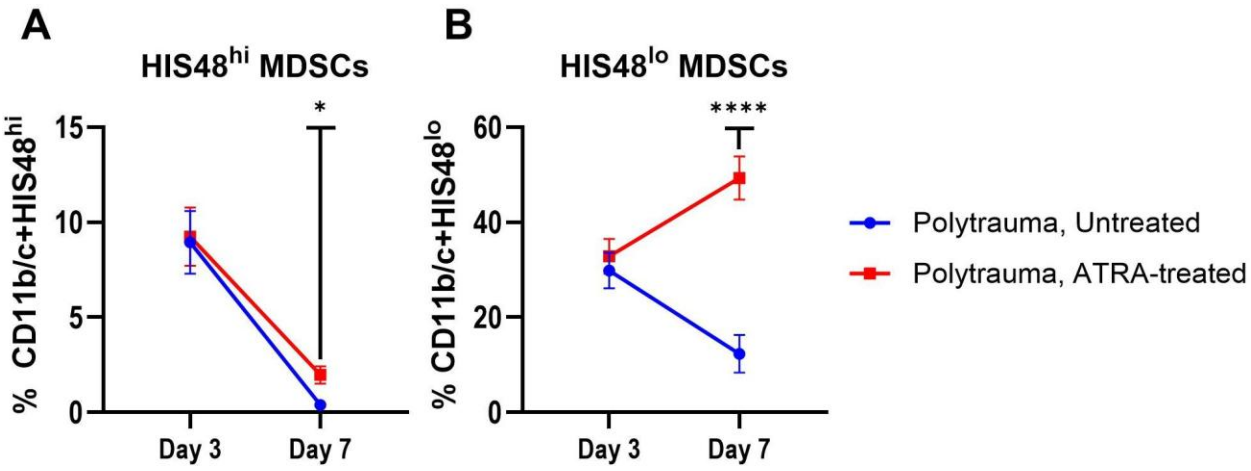


Figure 18. MDSC sub-populations were elevated following treatment with ATRA at a higher dose. (A–B) HIS48^{hi} MDSCs and HIS48^{lo} MDSCs were not altered at day 3 following a single injection with ATRA at a dose of 5 mg/kg, but they increased following a second ATRA injection at day 7. n = 6 to 7 per group. Significance was determined using a two-tailed t test for each comparison. *P < 0.05; ****P < 0.0001 as indicated.

Lastly, to determine whether these effects were caused by the higher dose of ATRA or by the DMSO in which it was dissolved, an additional experiment was performed wherein a group of naive, uninjured rats received an intraperitoneal injection with either ATRA at a high dose of 10 mg/kg dissolved in DMSO or DMSO alone containing no ATRA. Blood was drawn from both groups 12 hours after injections and immune cells were characterized by flow cytometry. The group that received ATRA in DMSO had significantly higher MDSCs than the group that received DMSO only (Figure 19A). Other immune cell populations were not significantly affected (Figure 19B–F).

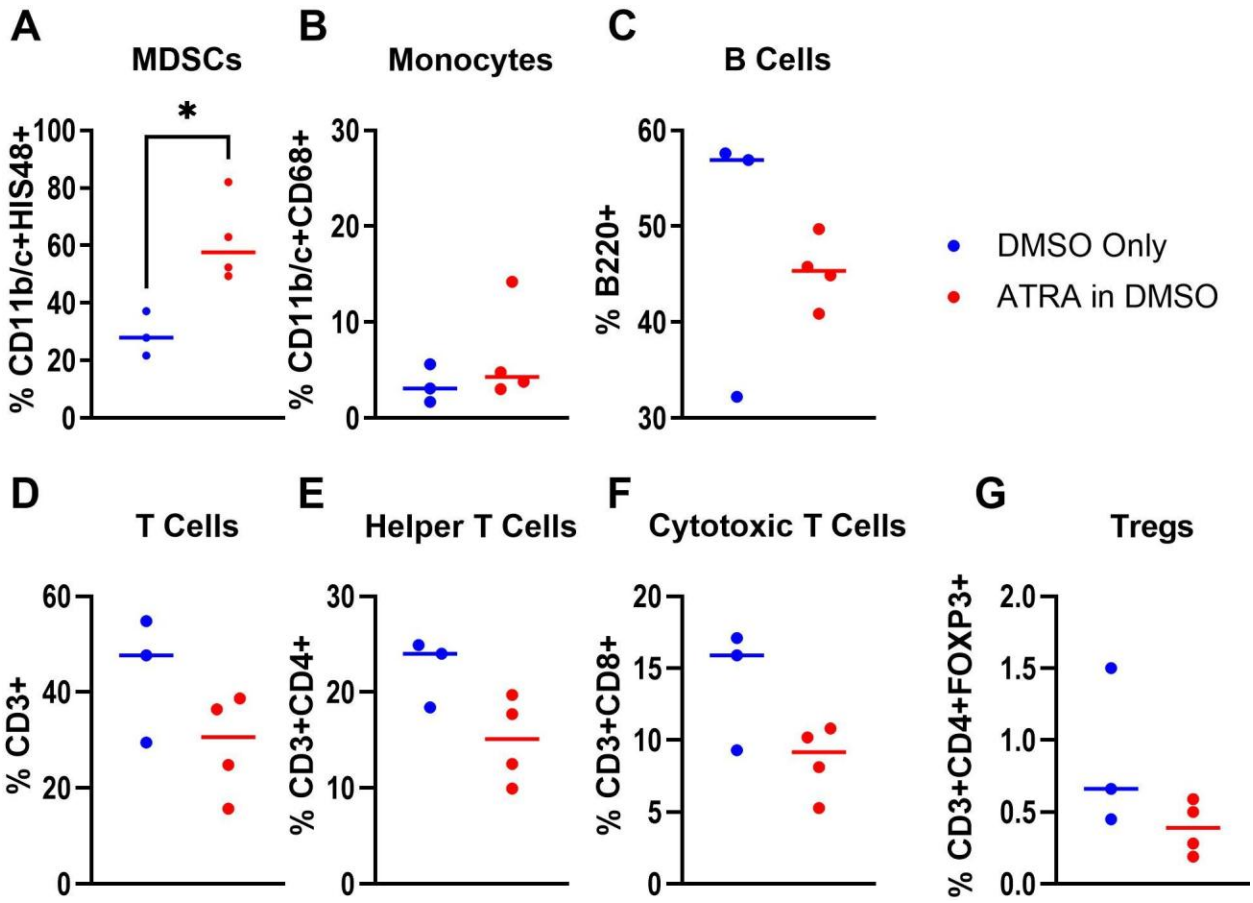


Figure 19. Intraperitoneal delivery of high-dose ATRA in DMSO alters MDSCs as compared to delivery of DMSO alone. (A) MDSCS were significantly elevated in uninjured rats injected with ATRA at a dose of 10mg/kg dissolved in DMSO as compared to rats injected with DMSO alone. (B–G) Other immune cell populations were not significantly altered. Significance was determined using a two-tailed *t* test for each comparison. *$P < 0.05$ as indicated.

Analysis of MDSC sub-populations demonstrated that the increase in total MDSCs observed here was driven by an increase specifically in HIS48^{lo} MDSCs, while HIS48^{hi} MDSCs were unaltered (Figure 20).

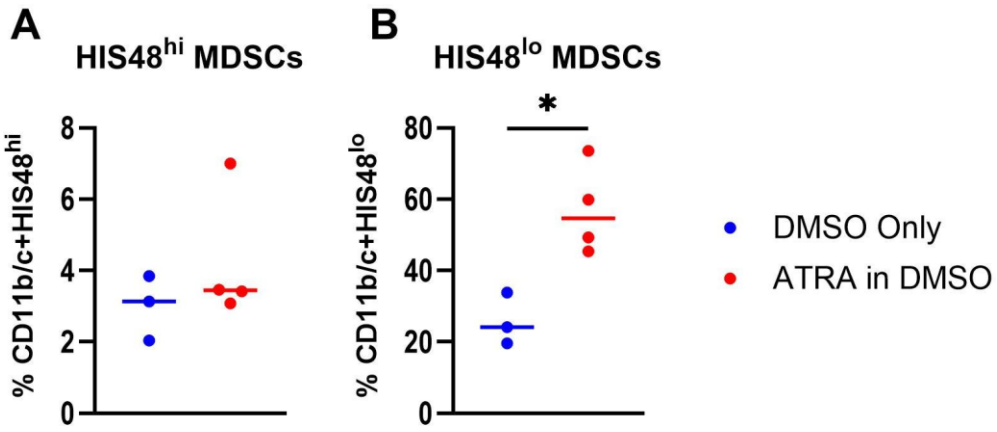


Figure 20. Intraperitoneal delivery of high-dose ATRA specifically alters HIS48lo MDSCs in uninjured rats. (A) Circulating HIS48^{hi} MDSCs were not altered by treatment with ATRA in DMSO as compared to DMSO alone. (B) Circulating HIS48lo MDSCs were elevated following treatment with ATRA in DMSO. Significance was determined using a two-tailed t test for each comparison. *$P < 0.05$ as indicated.

4.4 Discussion

Considerable advances have been made in acute trauma care in recent decades, which have significantly increased the rates of survival and limb salvage. However, this progress has sadly coincided with a resultant increase in late-emerging complications,[3] highlighting a need for new and improved treatment strategies. Previous work by our lab has consistently demonstrated a correlation between the systemic immune response to trauma and bone regenerative outcomes. In particular, circulating levels of myeloid-derived suppressor cells (MDSCs) at multiple time points post-injury have been found to correlate negatively with endpoint bone volume.[112] We also found that a nanoparticle-based immunomodulatory treatment in a rat trauma model led to both an expansion in the total number of circulating MDSCs and poorer bone healing, with levels of MDSCs in the treatment group again correlating negatively to endpoint regenerated bone volume.[179] Multiple cytokines known to be expressed by MDSCs such as IL-10 and IP-10 have also been negatively correlated to healing in our previous studies.[112,194] Meanwhile, T cells correlate positively to healing in rat models of trauma,[112,179] and specific T cell subsets are

known to promote bone formation and facilitate early fracture healing.[74] As such, their suppression by MDSCs may be detrimental to successful bone repair. Altogether, these results demonstrate a myriad of correlations between the systemic immune response to trauma and bone regeneration and highlight MDSCs as a promising potential therapeutic target. Based on our findings, we propose that strategies aimed at combating systemic immunosuppression and restoring homeostasis to the systemic immune environment may yield therapeutic benefits after musculoskeletal trauma and therefore warrant additional study.

To begin to explore such strategies, we performed an initial *in vitro* investigation of several therapeutics that have been previously used to target MDSCs for cancer treatment. Blood immune cells derived from our rat trauma model were treated *in vitro* with all-trans retinoic acid (ATRA) at three different concentrations. ATRA at all three concentrations resulted in a decrease in total MDSCs as compared to untreated controls. The other therapeutics tested *in vitro* did not diminish the relative frequency of MDSCs in this study. However, these were not exhaustively tested at a variety of concentrations or at later time points than 24 hours in culture, so future work would be necessary to determine whether altering these experimental conditions might result in successful MDSC depletion. Furthermore, because our analyses were limited to flow cytometric characterization with a small group of antibodies, it is possible that these drugs modulated the immune cells in ways that were not detectable here. Another interesting finding in this study was that ATRA diminished the relative frequency of MDSCs *in vitro* in blood derived from naive, uninjured rats as well. Normally, MDSCs in healthy humans and in healthy mice are rarely detected and only in very small numbers among peripheral blood mononuclear cells.[234,235] In uninjured rats, however, cells expressing the recognized rat markers for MDSCs comprise a substantial portion of the immune cells in the blood. Markers for MDSCs notably differ among

species, with the granulocytic marker HIS48 being used to identify MDSCs in rats while Gr-1 is used in mice and various other markers are used in humans.[131] Therefore, whether there are inherent differences in the relative frequencies of MDSCs between species or whether any observed differences are instead a result of the different markers used for characterizing the cells remains unclear. Nonetheless, the observation that ATRA diminished MDSCs derived from the naive rats as well as those derived from the trauma rats showed initial promise for its therapeutic potential in our model.

Interestingly, a significant decrease in monocytes was also observed for two of the ATRA concentrations tested *in vitro* in blood derived from the trauma model. This effect was unexpected, as ATRA has been shown to increase differentiation of MDSCs into mature myeloid cell types including monocytes.[151] ATRA has been shown in some models to exert anti-inflammatory effects on monocytes and macrophages, decreasing toll-like receptor 2 expression, downregulating the production of proinflammatory cytokines by monocytes, and shifting macrophages from the M1 pro-inflammatory phenotype to the M2 anti-inflammatory or pro-regenerative phenotype.[236] A decrease in the relative frequency of monocytes was unexpected however. It is also worth noting that circulating monocytes in our rat model of musculoskeletal trauma have also been previously correlated to poor bone regeneration, so a decrease in both populations could still yield therapeutic benefits.[112]

Given these positive *in vitro* results, we next aimed to investigate the effects of ATRA on immune cells in our rat polytrauma model *in vivo*. Following injury, both the ATRA-treated and untreated groups exhibited altered levels of circulating immune cells compared to naive controls that were indicative of trauma-induced immune dysregulation and immunosuppression. Higher levels of MDSCs and diminished levels of T cells and T cell subsets were observed in both

groups at day 4 and week 1 post-injury before returning to baseline values by week 2, similar to previous results using a rat trauma model.[179] Monocytes were also elevated in one of the trauma groups only at week 1 and not at day 4, suggesting a delayed influence of injury on this immune cell population.

Comparing the two polytrauma groups, daily intraperitoneal injections with ATRA between days 3 and 7 post-injury did not result in a decrease in total blood MDSCs, contrary to the results observed *in vitro*. Notably, however, a significant decrease in one sub-population of MDSCs was observed in the blood at day 4 post-injury, 24 hours after the first ATRA injections . MDSCs are most commonly identified in rats as expressing both granulocyte (HIS48) and monocyte (CD11b/c) markers, but two distinct subsets of the cells can also be distinguished based on their varying degrees of HIS48 expression. These two subsets, which we refer to here as $HIS48^{hi}$ MDSCs and $HIS48^{lo}$ MDSCs, may parallel the two subsets which have previously been distinguished in mice and humans, known as granulocytic and monocytic MDSCs.[237] Here, only $HIS48^{hi}$ MDSCs were significantly depleted after ATRA treatment, and only at the first time point after injections. Interestingly, a similar result was observed in a previous study in which we attempted to deplete MDSCs in a trauma model using another therapeutic platform called Synthetic Nanoparticle Antibodies.[179] These nanoparticles were used to target the calcium binding protein S100A8/A9 which is involved in MDSC signaling and accumulation.[150] Synthetic Nanoparticle Antibodies selectively depleted the $HIS48^{hi}$ MDSCs, possibly owing to higher levels of S100A8/A9 expression by this subset. Besides MDSCs, regulatory T cells (Tregs) were also diminished in the ATRA-treated group at both day 4 and day 7 post-injury as compared to the untreated polytrauma group. Given that Tregs are expanded by MDSCs, this

result could have been a secondary effect of the ATRA modulating the function of MDSCs, although additional assays would be necessary to confirm this. Despite a subset of MDSCs and Tregs being diminished, no changes were observed in the relative frequencies of the T cell populations measured after ATRA treatment.

Additionally, ATRA treatment did not improve functional bone regeneration, as no differences were detected in the average bone volume, bone mineral density, torsional strength, or torsional stiffness between the treatment and untreated control groups. Given that total MDSCs were not significantly depleted and the HIS48^{hi} MDSC subset was only depleted at one time point, it remains unclear whether systemically targeting and successfully depleting MDSCs may yield a therapeutic benefit for bone regeneration. Notably, linear regression analysis revealed a negative correlation between Tregs and endpoint bone volume across all injured rats. Although this could be interpreted as evidence that Tregs might have potential as a therapeutic target, the fact that Tregs were diminished in the ATRA-treated group in this study while the group's bone healing remained unchanged somewhat contradicts this interpretation. Still, it is also possible that Tregs were not diminished significantly enough or for a long enough duration to yield a therapeutic benefit. It is also worth noting that some recent studies have indicated pro-regenerative roles of local Tregs in the healing of bone and other tissues,[164,238] but the relationship between systemic Tregs and healing has not been studied as extensively. Moreover, depletion of immunosuppressive cells such as Tregs and MDSCs could yield other benefits besides improving healing, such as combating infections.

Finally, given the diminished subset of MDSCs and Tregs following ATRA treatment in the previous *in vivo* study, a follow-up study was conducted to assess the effects of a higher dose of ATRA on circulating immune cells. To facilitate administration of a higher ATRA dose, the

ATRA was dissolved in DMSO rather than in olive oil. Rats with polytrauma were injected with ATRA twice, at days 3 and 7 post-injury, and blood was collected 12 hours after each injection. We chose to collect blood sooner after injections to investigate whether the effects of ATRA were more potent over shorter time horizons. Once again, Tregs were diminished in the ATRA-treated group as compared to the untreated group, while total MDSCs remained unchanged after the first injections. Unexpectedly, however, MDSCs were elevated in the ATRA-treated group at day 7, 12 hours after the second round of ATRA injections. It is worth noting that DMSO possesses immunomodulatory properties, with both inflammatory and anti-inflammatory effects of the compound reported in humans and in rodents.[239,240] Therefore, to further investigate whether the observed elevation of MDSCs was due to the higher dose of ATRA or the use of DMSO, a final experiment was conducted using naive rats injected with either ATRA in DMSO or DMSO alone. Here, the ATRA-treated group possessed higher MDSCs 12 hours after injections than the DMSO-only group, suggesting that the high dose of ATRA itself was the likely contributor to the unexpected MDSC elevation observed in the trauma rats. It is worth noting the doses of ATRA used in this study were similar to those used previously to successfully suppress MDSCs in mouse cancer models,[228,241] with considerations made for differences in size and metabolic rates between species. This apparent contradiction between our results and previous literature likely underscores the heterogeneity of MDSCs and potential differences in the cells across models and species.

One opportunity for further study is to assess the immunosuppressive capacity of MDSCs before and after treatment with ATRA. Previous studies of ATRA in cancer models have employed T cell proliferation assays and other methods for quantifying the suppressive effects of MDSCs on other immune cells, whereas this study was limited to examining only the relative

frequencies of circulating MDSCs and other immune cells by flow cytometry. As such, although total MDSCs were not reduced after *in vivo* ATRA treatment in this study, it is possible that their immunosuppressive effects on other cells were altered in ways that could not be detected using the methods here. Still, specific mechanisms of MDSC-mediated immunosuppression have not been previously correlated to bone healing outcomes, and therefore assays for such mechanisms were somewhat beyond the scope and purposes of this study. Furthermore, the ability of ATRA to diminish the immunosuppressive functions of MDSCs has already been reported extensively in cancer studies.

Another critical area of future work is to develop improved methods for characterizing the highly heterogeneous population of myeloid derived immune cells termed MDSCs, particularly in rats. While extensive research has been performed on MDSCs in mice and in humans, literature on MDSCs in rats is scarce, making broad comparisons between the species difficult. For example, while it has been speculated that the $HIS48^{hi}$ and $HIS48^{lo}$ MDSC populations observed in rats may mirror the granulocytic and monocytic MDSC subsets previously defined in mice and humans, no study to date has provided considerable evidence to link these subsets across the species. Finally, it should be acknowledged that bone morphogenetic protein-2 (BMP-2) has been shown to have immunomodulatory effects such as macrophage stimulation and upregulation of cytokines involved in angiogenesis.[209] Therefore, all groups of injured rats in our *in vivo* studies were treated with BMP-2 to eliminate the confounding factor of its immunomodulatory effects. The bone defects in this study were critically sized, and therefore a local treatment such as BMP-2 was necessary for any significant regeneration of bone volume to occur. It is unlikely that a systemic immunomodulatory therapeutic strategy alone would result in the healing of a critically-sized bone defect. However,

when used in conjunction with a local treatment, we posit that a systemic immunomodulatory treatment might augment the therapeutic benefit of a local treatment, resulting in greater healing. This might, for example, allow smaller effective doses of BMP-2 to be administered while still yielding successful bone regeneration, which could be beneficial given the adverse side effects that have been reported for use of BMP-2, especially at higher doses.[71]

In conclusion, this study examined the effects of ATRA on circulating MDSCs and other systemic immune cells in a polytrauma model. This strategy demonstrated depletion of MDSCs *in vitro* using cells derived from rats with polytrauma. Subsequent *in vivo* studies indicated that while ATRA treatment was unable to deplete total circulating MDSCs, a subset of MDSCs and Tregs were diminished. Functional bone regeneration was not altered by ATRA treatment, but a negative correlation was identified between endpoint bone volume and circulating Tregs measured in the blood 2 weeks after injury. In subsequent experiments, when rats received high doses of ATRA, Tregs were again depleted, but total MDSCs were unexpectedly elevated. This result was observed both in rats with polytrauma and in uninjured, naive rats. Altogether, these data demonstrate that ATRA is capable of systemic immune modulation, but a challenge remains in achieving successful *in vivo* depletion of total circulating MDSCs, as has been observed previously in mouse cancer models. Further research would be necessary to determine whether this challenge is a result of phenotypic differences between rat MDSCs and MDSCs in other species, or whether this result is unique to MDSCs in the context of trauma. Moreover, it remains unclear whether successful depletion of both sub-populations of MDSCs in the peripheral blood of rats could improve trauma healing outcomes. Ultimately, further studies are needed to more adequately characterize the role of systemic immune dysregulation in trauma outcomes and to develop immunomodulatory interventions that enhance bone regeneration.

CHAPTER 5: SPECTRAL FLOW CYTOMETRY REVEALS DYSREGULATED IMMUNE CELL POPULATIONS IN LOCAL TISSUES FOLLOWING SEVERE POLYTRAUMA

Contents of this chapter were adapted from Guyer T., Gill D., Link K., Strait L.K., Leguineche K.E., O'Neill K.L., and Guldberg R.E. (manuscript in preparation).[270]

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Conceptualization: TG, KEL, REG

Data collection: TG, DG, KL, LKS, KEL, KLO, REG

Data analysis: TG, KEL

Interpretation: TG, REG

Writing and editing: TG, REG

5.1 Introduction

Treatment of large bone defects often resulting from severe trauma, tumor resection, or congenital deformity continues to present challenges for orthopedic surgeons. With over a century of successful clinical use, and despite intensive research of potential alternatives, autologous bone grafts remain the gold standard for treatment of large bone defects.[64] Nevertheless, bone graft surgeries face drawbacks including limited supply, increased time and complexity of surgeries, increased pain, and donor site morbidity.[242–244] As such, there remains a need to investigate novel strategies to replace or complement current bone grafting methods. Notably, bone morphogenetic proteins (BMPs) are a group of signaling molecules that play critical roles in bone repair, and BMP-2 has previously been approved by the U.S. Food and Drug Administration for use as a bone graft substitute.[245] This approach also faces drawbacks however, such as a high risk for complications including postoperative inflammation, ectopic bone formation, and inappropriate adipogenesis.[71,246–249] Novel therapeutic approaches to

supplement the use of BMPs could allow for successful bone regeneration using smaller BMP doses, thereby mitigating risks. Osteoimmunology represents one ongoing area of research which has potential for the development of such strategies.

The onset of bone healing is highly dependent on the initial inflammatory response to injury and its timely resolution.[250] Numerous cellular mediators play roles in this response and subsequent bone repair. Macrophages derived from circulating monocytes are critical to the pro-inflammatory phase in the immediate aftermath of injury before later transitioning to an anti-inflammatory, pro-regenerative phenotype that helps limit excessive, chronic inflammation.[251,252] Neutrophils and other granulocytic cells help recruit monocytes to the injury site via the release of mediators such as monocyte chemoattractant protein 1.[94] Neutrophils also contribute more directly to fracture healing by rapidly synthesizing fibronectin positive extracellular matrix early after injury before stromal cells infiltrate the fracture hematoma.[55] Lymphocytes play roles throughout multiple stages of bone repair as well. T cells express the pro-osteoclastogenic cytokine Receptor Activator of Nuclear Factor-κB Ligand (RANKL), and they support osteoblast maturation via inteleukin-17F.[74,105,253] B cells express osteoprotegerin (OPG), which blocks RANKL activity, and they also assist in osteoblast maturation and retention.[104,254] Altogether, these immune cells and others are instrumental in establishing a permissive, pro-regenerative environment within the injury site. However, dysregulation of these cells for sustained periods of time may also compromise healing. For example, prolonged neutrophil activation within the fracture site has been linked to diminished osteogenic activity, reduced mineralization, and impaired bone regeneration.[76]

Recently, we have longitudinally quantified the relative frequencies of several immune cell populations in the peripheral blood following injury in a rat model of severe musculoskeletal

trauma. Our previous findings have indicated that multiple immune cell populations become systemically elevated or depressed after trauma and furthermore correlate with bone regenerative outcomes.[112] For example, circulating T cells and T cell subpopulations at various time points after injury correlate positively with endpoint bone volume, while circulating monocytes correlate negatively with healing. Notably, we have also identified another population of circulating myeloid cells that are elevated after injury and correlate negatively with healing in rats. These cells are referred to as myeloid-derived suppressor cells (MDSCs), and they are characterized in rats as expressing both CD11b/c and HIS48 markers. Overall, these observations of systemically dysregulated immune cell frequencies post-trauma that correlate to bone healing outcomes highlight the potential for immune cells as targets to modulate healing. However, these studies were also limited to characterizing immune cell populations within the peripheral blood. Given that immune cells local to the site of injury are already known to be crucial to repair, a better understanding of the changes to these cells in the trauma microenvironment could provide essential insight for the development of immunomodulatory therapeutic strategies at the local level.

To address this need, here, we employed spectral flow cytometry to characterize immune cells in local tissues using a rat model of bone and muscle polytrauma. Rats received composite injuries consisting of both a critically sized segmental femur defect and a full-thickness muscle defect in the adjacent quadricep. Rats were then sacrificed at day 3, week 1, or week 2 post-injury for immune cell analysis. Tissues harvested included soft tissue derived directly from the bone defect space, bone marrow from the femur ends adjacent to the bone defect, muscle dissected from the muscle defect in the adjacent quadricep, and peripheral blood. The solid tissues were then dissociated both chemically and mechanically to yield single cell suspensions,

and all samples were stained using antibodies to identify various immune cell populations, including MDSCs, monocytes and macrophages, B cells, T cells, helper T cells, cytotoxic T cells, and T regulatory cells (Tregs). Spectral flow cytometry was then performed to quantify the cells. Relative immune cell frequencies from each tissue were compared across each of the time points to assess the time-dependent relationship between trauma and the immune response. Blood and bone marrow were also collected from a group of naive rats to establish baseline values for the relative frequencies of each immune cell population. Finally, a subset of rats also received treatment for their bone injuries with BMP-2 delivered in an alginate hydrogel, and their immune cells across all of the tissue compartments were compared to those of untreated rats to assess whether the BMP-2 treatment altered the immunological response to trauma. Broadly characterizing the immune response to polytrauma over time across multiple tissue compartments is a crucial step toward developing a deeper understanding of its roles in tissue repair. Further, this may help provide insight into novel immunomodulatory strategies at the local level to optimize trauma patient outcomes.

5.2 Materials and Methods

5.2.1 Nanofiber Mesh Preparation

Poly(ε-caprolactone) (PCL) (PC-12 PURASORB, Corbion, USA) was dissolved at 15% (w/v) in a solution comprised of a 3:2 ratio of dichloromethane (DCM) and dimethylformamide (DMF) (both Sigma-Aldrich, USA) for 16 hours under gentle agitation. The polymer solution was loaded into a 3 mL syringe (FORTUNA, USA) with a 32-gauge metal nozzle (McMaster-Carr, USA). A custom solution electrospinning (SES) system was used to create a polymer coating on a 5 mm diameter cylindrical collector (McMaster-Carr). The collector was placed 13

centimeters from the printer head, and a voltage of 20 kV and a pressure of 1 bar were applied. The mandrel movement was programmed with Mach3 software (Artsoft, USA). Next, the scaffold was melted through using a solder with the solder tip set to 400°C. The solder movement was programmed with AeroBasic software (Aerotech, USA). Scaffolds were removed from the mandrel by spraying them with 70% ethanol to detach the polymer from the aluminum mandrel. Scaffolds were then given 1-mm diameter perforations to promote vascular invasion during the repair process. The resulting scaffolds were 13 mm in length, with alternating 3 and 2 openings of 1 mm diameter on each row.

5.2.2 Alginate BMP-2 Preparation

Alginate hydrogel constructs containing bone morphogenetic protein 2 (BMP-2) were produced as described previously.[202] All methods were performed under sterile conditions within a laminar flow hood. Arginine-glycine-aspartic acid (RGD)-functionalized alginate (FMC BioPolymer) was dissolved in sterile alpha-MEM (Corning) to yield a 3% w/v alginate solution. A solution containing 0.1% rat serum albumin (Sigma-Aldrich) in sterile 4 mM hydrochloric acid was produced, and recombinant BMP-2 (Pfizer) was added to yield 2.5 μg BMP-2 per 150 μL of final solution. The solution was cross-linked via thorough mixing with an excess of calcium sulfate using sterile syringes. The solution was allowed to gel at room temperature for 30 minutes, and it was then stored at 4°C until use in surgeries the next day.

5.2.3 Animal Model

All animal care and experimental procedures were approved by the University of Oregon IACUC (Protocol 20-10). Female Sprague Dawley rats (Charles River Laboratories) were used for all experiments. The rats were aged 14-15 weeks old at the time of surgical procedures. Prior to surgeries, rats were housed in pairs in individually ventilated caging. After surgeries, rats were

housed individually for better monitoring of their post-operative recovery. Food and filtered water were made available *ad libitum*, and PVC tunnels and nylon bones were provided for enrichment. Rats were randomly assigned to treatment and control groups. Following their arrival, all animals were allowed to acclimate for at least one week in their cages prior to being subjected to any experimental procedures.

5.2.4 Surgical Procedures

Surgical methods have been described previously.[229] Isoflurane (VetOne) inhalation was used to induce and maintain anesthesia during all animal procedures. Rats received a subcutaneous injection of buprenorphine HCL (0.03 mg/kg, Hikma Pharmaceuticals) and a subcutaneous injection of sustained-release buprenorphine (0.65 mg/kg, Ethiqa) prior to surgical procedures for pain management. Rats also received 3 mL of Lactated Ringer's solution (VetOne) subcutaneously before surgical procedures. An anterolateral skin incision was then made in the thigh, and the overlaying muscles were separated to expose the femur. The femur was stabilized by placement of a radiolucent polysulfone internal fixation plate, and then an oscillating saw was used to create an 8-mm segmental defect in the mid-diaphysis of the femur. An 8-mm full-thickness muscle defect was then created in the adjacent quadricep using a biopsy punch. A 6-mm diameter PCL mesh was placed within the femur defect space around the exposed bone ends. In one group of rats, alginate containing BMP-2 was then injected into the perforations in the mesh using a syringe. In another group of rats, the mesh was left empty. The muscle defects were also left untreated in both experimental groups. Vicryl suture was then used to close the muscle, and the skin was closed with both a subcutaneous Monocryl suture layer and wound clips. After surgery, sterile saline was applied to clean the incision site. A mixture of Metronidazole (Viona Pharmaceuticals) and New-Skin Liquid Bandage was also applied. For

post-operative nutritional support, rats were provided DietGel® Boost (Clear H_2O) and Nutri-Cal gel (Vetoquinol). Rats were closely monitored for at least three days after surgery to observe for post-operative complications.

5.2.5 Experimental Endpoints and Tissue Collection

Blood, muscle tissue, bone defect tissue, and bone marrow were harvested from rats in the polytrauma group at day 3, week 1, or week 2 post-injury. Approximately 400 μL of blood was collected via the ventral tail artery from each rat into Microtainer lithium-heparin coated tubes (BD) using Vacuette blood collection needles (Greiner Bio-One). Euthanasia was then performed with exsanguination via cardiac puncture and was confirmed by secondary cervical dislocation. The skin was incised on the operated limb to expose the quadricep adjacent to the femur, and approximately 300 mg of muscle was collected from the quadricep using an 8-mm biopsy punch. Femurs were then excised, PCL meshes containing tissue from the bone defects were extracted, and bone marrow was collected by removing the bone ends and flushing the marrow cavities on either side of the defect with approximately 5 mL of PBS into a 15 mL conical using a syringe with a 16-gauge needle. Blood and bone marrow were similarly harvested from a group of age-matched naive rats.

5.2.6 Tissue Processing and Immune Cell Characterization

Muscle samples and bone defect tissue samples were dissociated using a Skeletal Muscle Dissociation Kit (Miltenyi Biotec) according to the manufacturer's instructions. Briefly, the tissues were first thoroughly minced with scissors and were then transferred to gentleMACS C tubes (Miltenyi Biotec) containing the kit's standard enzymes in solution with DMEM. Samples were then digested for 30 minutes at 37°C, and the tubes were then attached to a gentleMACS dissociator (Miltenyi Biotec) for mechanical dissociation. The incubation and mechanical

dissociation steps were then repeated once, and then each sample was strained twice using first a 200 μm cell strainer and then a 70 μm cell strainer. Bone marrow samples were also strained once using a 70 μm cell strainer.

Blood, muscle, bone marrow, and bone defect tissue samples were then all resuspended in RBC Lysis Buffer (eBioscience) to remove red blood cells according to the manufacturer's instructions. Afterward, samples were centrifuged at 500 × *g* for 5 min and washed twice using approximately 5 mL of PBS. Samples were then incubated for 10 minutes at 4°C with 50 μL of anti-rat CD32 to block Fc receptors. Following this incubation, samples were stained using the Zombie UV™ Fixable Viability Kit (Cytek) according to the manufacturer's instructions. Next, the samples were resuspended in Flow Cytometry Fixation Buffer (R&D Systems) for 10 minutes at room temperature to fix the cells. The samples were then resuspended in 50 μL of Brilliant Stain Buffer (BD Biosciences) and stained for 25 minutes at 4°C with the following antibody panel: B220 RY775, CD3 BV605, CD4 BUV661, CD8 RB780, CD11b/c RB613, CD45 BV786, and HIS48 FITC. Afterward, samples were permeabilized with Cytofix/Cytoperm buffer (BD Biosciences) and washed twice with Perm/Wash buffer (BD Biosciences) before staining for 25 minutes at 4°C with 50 μL of Brilliant Stain Buffer and the following antibody panel: CD68 PE, and FOXP3 BV421. Finally, samples were washed and resuspended in 250 μL of FACS buffer containing 2% fetal bovine serum in phosphate-buffered saline without calcium and magnesium.

Samples were collected using a 5 Laser Cytek® Aurora Spectral Flow Cytometer. Spectral unmixing was performed using the Cytek® SpectroFlo® Software with single-color reference bead controls prepared according to the manufacturer's instructions. Subsequent analysis was performed using FlowJo (BD, Version 10.10.0) software. Gates were positioned

based on fluorescence minus one (FMO) controls allowing <1% noise. The immune cell populations analyzed included myeloid-derived suppressor cells ($HIS48^+CD11b/c^+$), monocytes and macrophages ($CD68^+CD11b/c^+$), B cells ($B220^+$), T cells ($CD3^+$), helper T cells ($CD3^+CD4^+$), cytotoxic T cells ($CD3^+CD8^+$), and regulatory T cells ($CD3^+CD4^+FOXP3^+$).

5.2.7 Statistical Analysis

Data were analyzed and graphed using GraphPad Prism software (Version 10.4.2, La Jolla, CA, USA). All data are displayed as mean ± SEM. The relevant statistical tests performed are listed in the captions below each figure. Significance was determined by p values less than 0.05.

5.3 Results

5.3.1 Circulating MDSCs are Elevated and Effector Cells are Depressed Following Trauma

To assess the systemic immune response to trauma, blood was collected from rats with polytrauma at terminal endpoints on day 3, week 1, or week 2 post-injury. Blood was also collected from a control group of age-matched naive rats. Multiple significant differences were observed in the relative frequencies of blood immune cell populations across the post-injury time points. MDSCs and monocytes were elevated at day 3 post-injury as compared to week 1 and week 2 post-injury (Figure 21A, B). T cells, including helper T cell and cytotoxic T cell sub-populations, were depressed in the blood at day 3 post-injury compared to week 1 and week 2 post-injury (Figure 21D–F). Tregs were elevated at all three time points post-injury as compared to the naive control group (Figure 21G). The relative frequency of B cells was not altered in the blood at any time point they were measured after injury (Figure 21C).

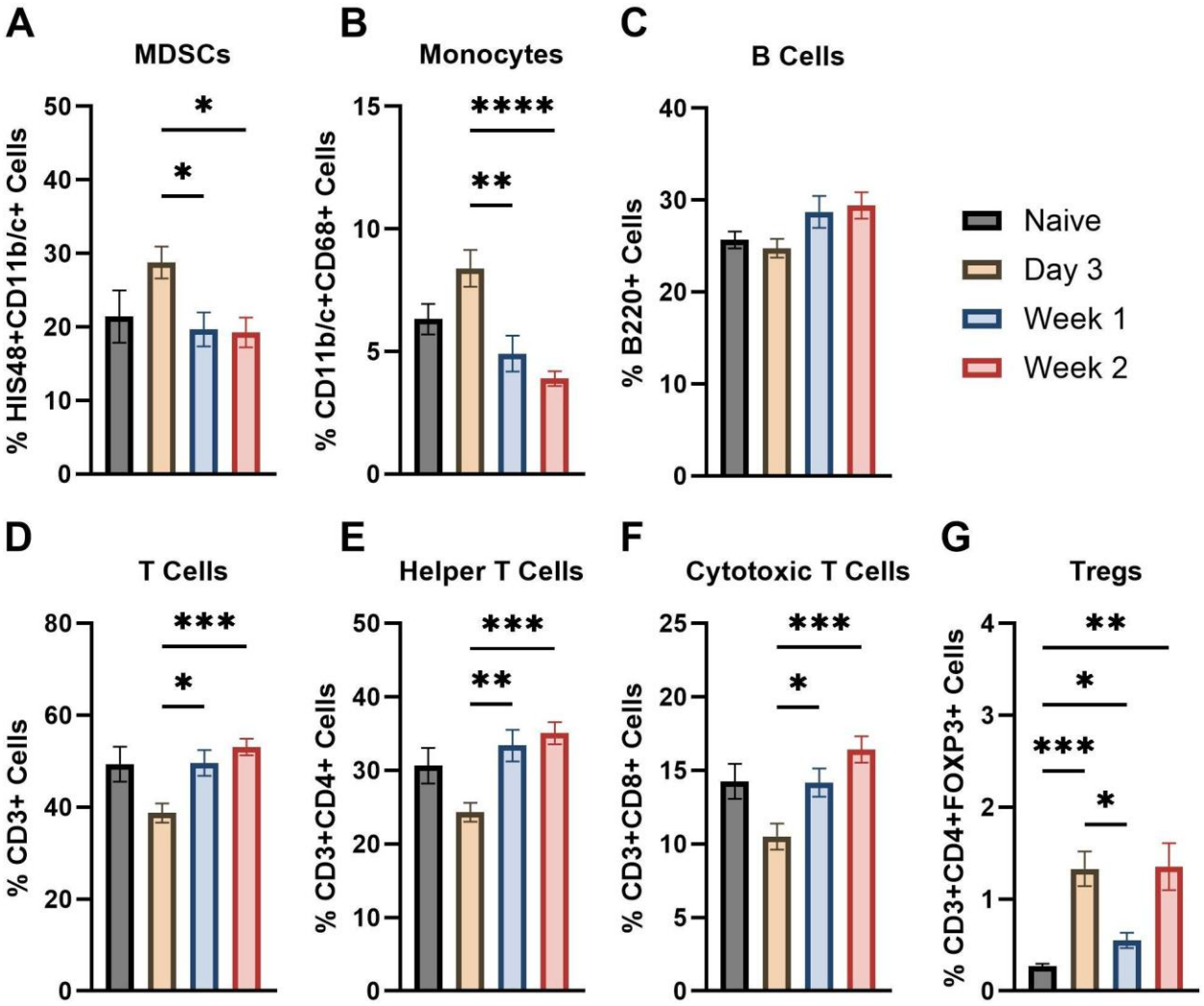


Figure 21. Peripheral blood immune cell populations were differentially regulated after severe polytrauma. MDSCs and monocytes were elevated at day 3 post-injury compared to later time points (A, B), while T cells and their subsets were depressed at day 3 post-injury compared to later time points (D–F). Tregs were elevated at all time points post-injury as compared to naive controls (G). Significance was determined using a one-way ANOVA with Tukey's multiple comparisons test or a Welch ANOVA with Dunnett's T3 multiple comparisons test when variances were significantly different between groups. n=6 for the naive group, n=12 for all other groups. *P<0.05 **P<0.01; ***P<0.001; ****P<0.0001 as indicated.

5.2.2 MDSCs Expand Early After Injury in the Bone Defect Tissue and the Muscle Defect Tissue

Immune cells in the tissue harvested from the bone defect site at day 3, week 1, or week 2 post-trauma were also characterized via spectral flow cytometry. MDSCs notably comprised

approximately 80% of all immune cells on average at day 3 before declining at week 1 and again at week 2 (Figure 22A). Macrophages and B cells increased between day 3 and week 1 (Figure 22B, C), and T cells, helper T cells, and cytotoxic T cells increased across each subsequent time point (Figure 22D–F). Tregs also increased between week 1 and week 2 (Figure 22G). Notably, Tregs were not detectable in the bone defect tissue samples at day 3 post-injury, likely due to their low relative frequency coupled with the small amount of tissue present in the bone defect space at this early time point.

Immune cells in the muscle defect samples followed a similar pattern to those in the bone defect. MDSCs in the muscle were highest at day 3 before decreasing at week 1 and then remaining approximately stable through week 2 (Figure 23A). Macrophages and B cells both increased between day 3 and week 1, although B cells notably decreased again between week 1 and week 2 (Figure 23B, C). T cells, helper T cells, and cytotoxic T cells increased between day 3 and week 1 in the muscle (Figure 23D–F). Tregs were significantly higher at week 2 than at day 3 (Figure 23G).

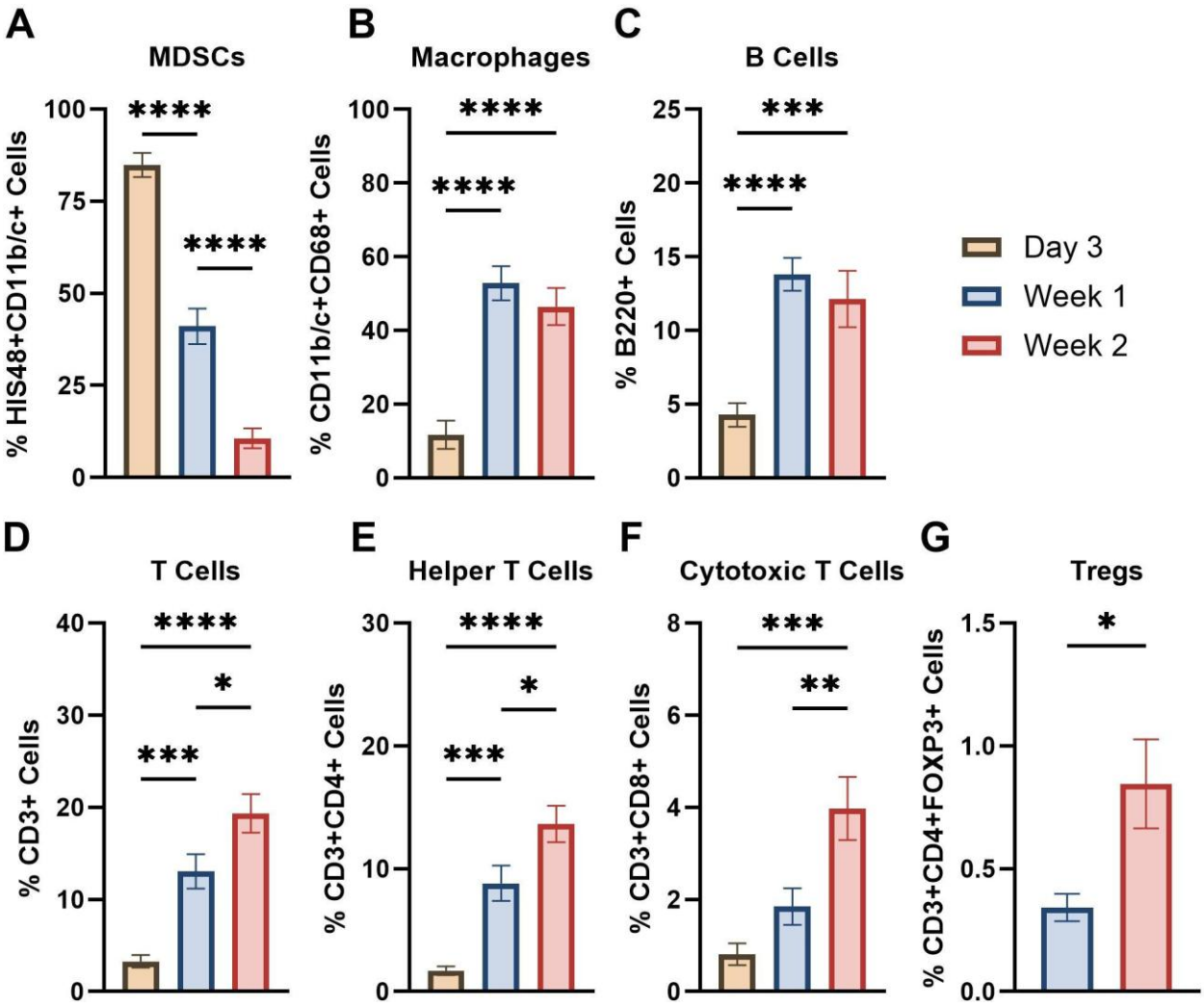


Figure 22. Immune cell frequencies in soft tissue local to the bone defect site underwent changes over time following severe polytrauma. MDSCs comprised the majority of immune cells in the defect tissue at day 3 post-injury and decreased at week 1 and week 2 post-injury (A), while macrophages and B cells increased between day 3 and week 1 (B, C), and T cells, helper T cells, and cytotoxic T cells increased across each subsequent time point (D–F). Tregs were not detected at day 3 but increased between week 1 and week 2 (G). Significance was determined using a one-way ANOVA with Tukey's multiple comparisons test or a Welch ANOVA with Dunnett's T3 multiple comparisons test when variances were significantly different between groups. n=10 to 12 per group. *P<0.05 **P<0.01; ***P<0.001; ****P<0.0001 as indicated.

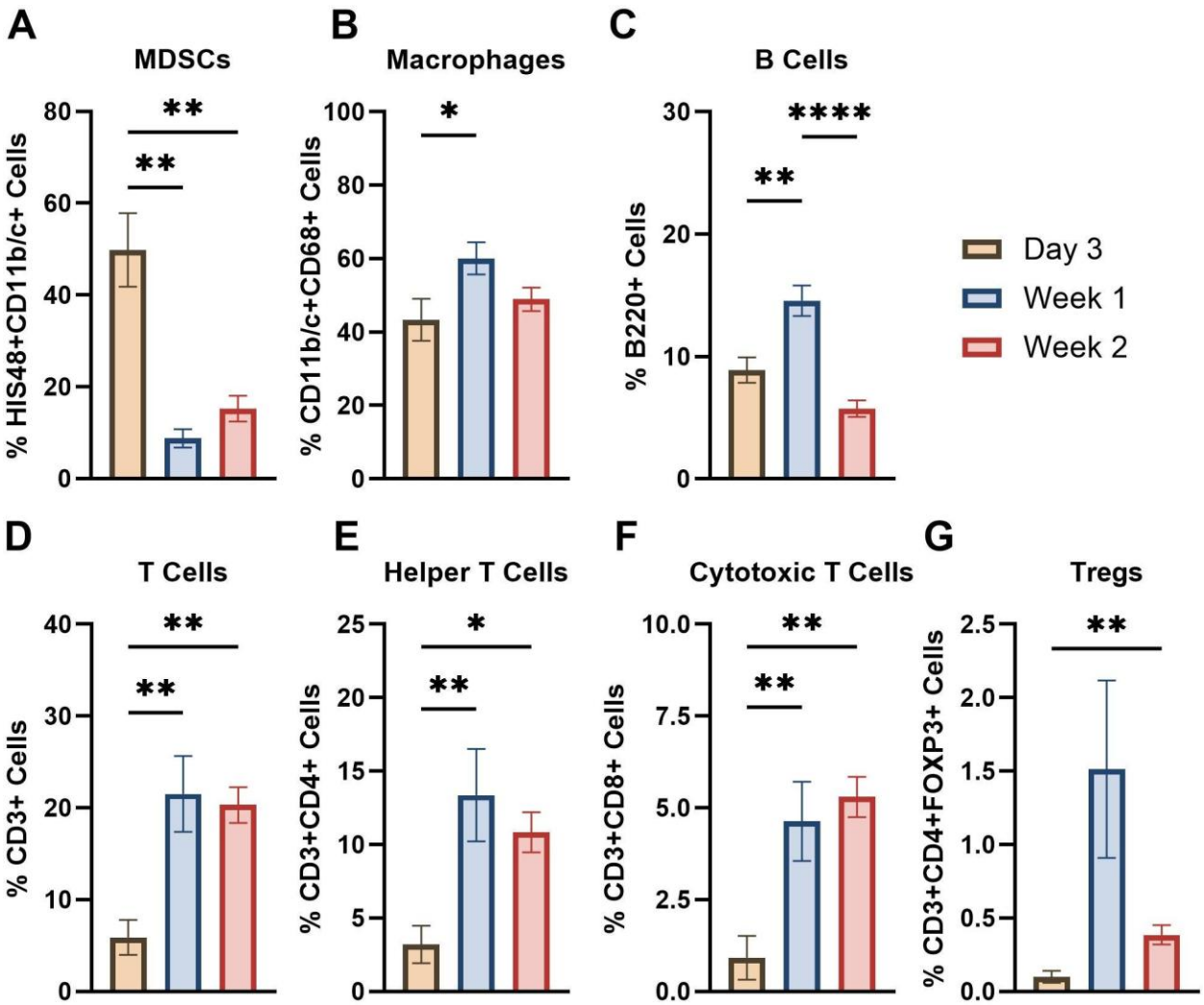


Figure 23. Immune cell frequencies within the muscle defect space underwent changes over time following severe polytrauma. MDSCs were the largest immune cell population at day 3 post-injury but decreased by week 1 (A). Macrophages and B cells conversely increased between day 3 and week 1, but B cells decreased again by week 2 (B, C). T cells, helper T cells, and cytotoxic T cells increased between day 3 and week 1 (D–F). Tregs were significantly higher at week 2 than at day 3 (G). Significance was determined using a one-way ANOVA with Tukey's multiple comparisons test or a Welch ANOVA with Dunnett's T3 multiple comparisons test when variances were significantly different between groups. n=10 to 12 per group. *P<0.05 **P<0.01; ***P<0.001; ****P<0.0001 as indicated.

5.3.3 Monocytes, B Cells, and T Cells Were Dysregulated in Local Bone Marrow After Injury

Bone marrow was collected from the femur ends adjacent to the bone defect space for immune cell characterization via spectral flow cytometry. Bone marrow was similarly collected from the femurs of naive rats for a control comparison. The relative frequency of MDSCs in the

bone marrow was not altered at any time point post-injury as compared to the naive controls (Figure 24A). Monocytes were depressed at day 3 post-injury compared to the naive group before increasing between day 3 and week 1 post-injury and then decreasing again between week 1 and week 2 post-injury (Figure 24B). B cells were depressed from naive levels at day 3 post-injury before recovering partially by week 1 and further increasing even above naive levels by week 2 (Figure 24C). The frequency of T cells in the bone marrow was elevated compared to naive controls at week 1 post-injury before returning to naive levels by week 2 (Figure 24D). Helper T cells increased between day 3 and week 1 before decreasing again by week 2, although they were not significantly elevated or depressed compared to the naive controls at any time point (Figure 24E). Cytotoxic T cell frequencies were not significantly altered in the bone marrow at any time point (Figure 24F). Tregs in the bone marrow were elevated at all three time points post-injury as compared to the naive controls, although they peaked at week 1 post-injury (Figure 24G).

5.3.4 Treatment With BMP-2 Altered Immune Cell Populations in the Bone Defect Tissue

Finally, we collected blood, bone defect tissue, muscle defect tissue, and bone marrow from rats treated with BMP-2 in an alginate hydrogel at the time of their injuries and from rats that were left untreated to assess the effects of BMP-2 on immune cells across the tissue compartments. Several significant differences were observed in the frequencies of the immune cell populations within the bone defect space one week post-injury. Macrophages were found to be elevated in defect tissue from the BMP-2 treated group at this time point (Figure 25B), while T cells, including both helper T cells and cytotoxic T cells, were depressed (Figure 25D–F).

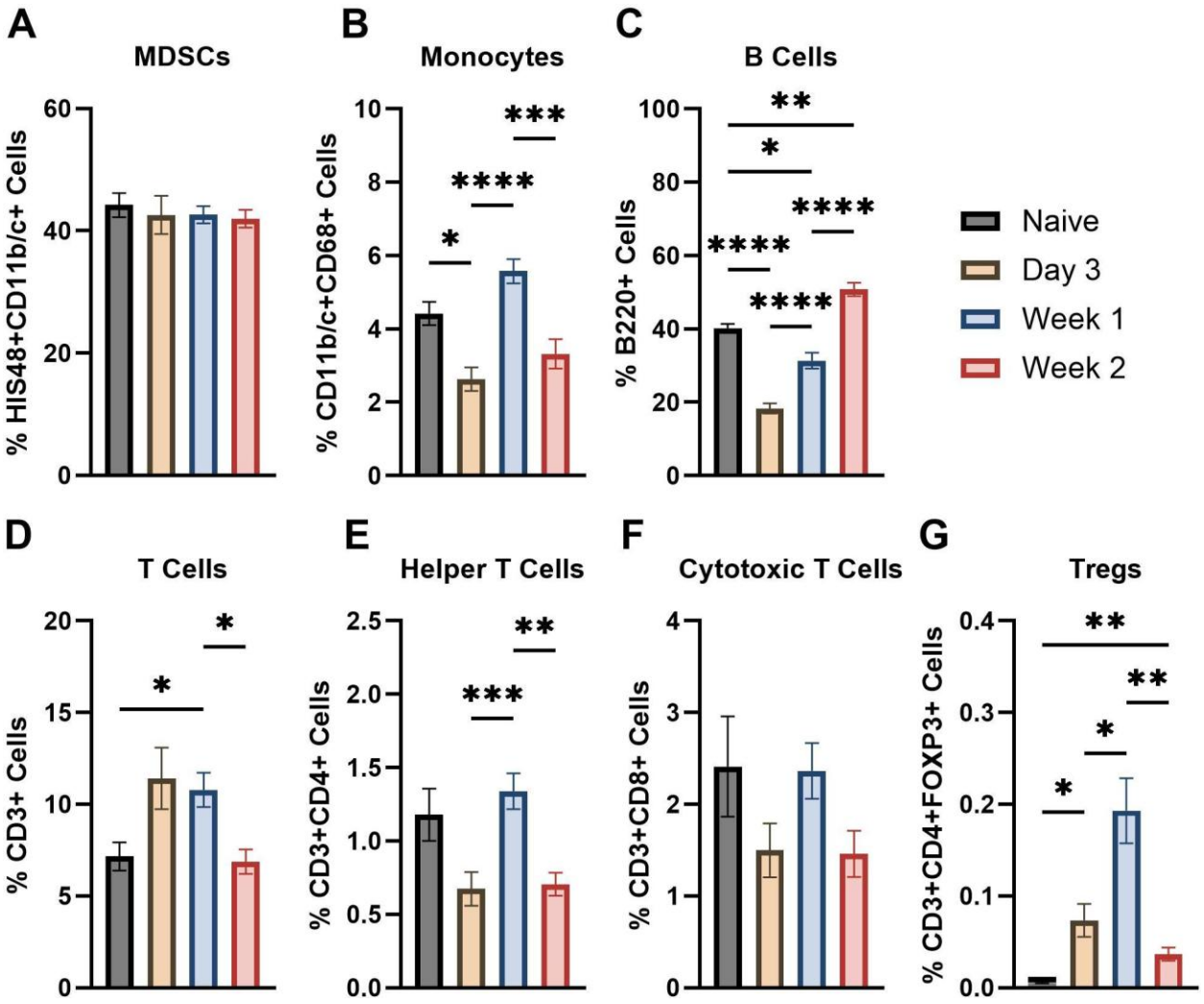


Figure 24. Immune cell populations in bone marrow from bone ends adjacent to the injury site were dysregulated after severe polytrauma. MDSC frequencies were not significantly different from naive levels at any time point post-injury (A). Monocytes decreased from naive levels by day 3 post-injury before increasing by week 1 and decreasing again by week 2 (B). B cells were depressed from naive levels by day 3 post-injury increased by week 1 and again by week 2 (C). Total T cells in the bone marrow were elevated from naive levels at week 1 post-injury, and post-injury helper T cells peaked at week 1 (D, E). Cytotoxic T cells were not significantly altered at any time point post-injury (F). Tregs were significantly elevated from naive levels at all time points post-injury, although they peaked at week 1. Significance was determined using a one-way ANOVA with Tukey's multiple comparisons test or a Welch ANOVA with Dunnett's T3 multiple comparisons test when variances were significantly different between groups. n=10 to 12 per group. *P<0.05 **P<0.01; ***P<0.001; ****P<0.0001 as indicated.

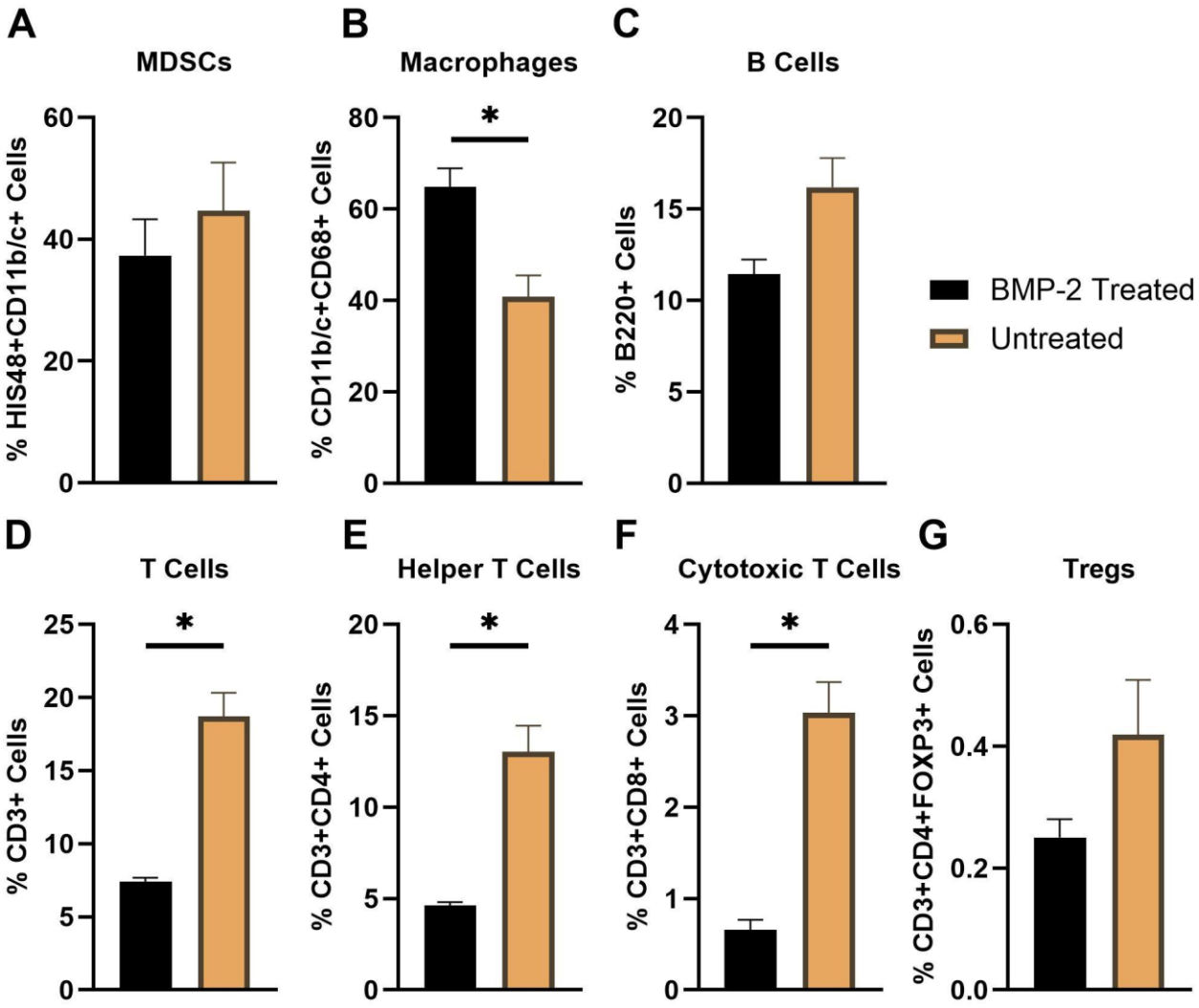


Figure 25. Immune cell profiles within the bone defect site at week 1 post-injury were altered by BMP-2 treatment. (A, C, G) Relative frequencies of MDSCs, B cells, and Tregs in the defect site were not significantly affected by BMP-2 treatment. (B, D-F) Macrophages were elevated while T cells and T cell subsets were diminished in the bone defect site at week 1 post-injury in BMP-2 treated rats. Significance was determined using t tests corrected for multiple comparisons using the Holm-Šídák method with $\alpha = 0.05$. n = 4 to 6 per group. *P<0.05 as indicated.

5.4 Discussion

Although acute trauma care has seen considerable advances in recent decades, late-emerging complication rates remain high,[3] warranting the need for additional investigation of novel therapeutic strategies. The recent advent of osteoimmunology has highlighted the relationship between bone healing and the immune response to trauma at both the local and

systemic levels.[43,112] This study aimed to characterize the local cellular immune response in a rat model of bone and muscle polytrauma by quantifying relative frequencies of various immune cell populations in multiple tissue compartments using spectral flow cytometry.

Immune cell analysis in the peripheral blood revealed that MDSCs and monocytes were elevated and T cells were depressed at day 3 post-injury as compared to later time points. These findings agree with our previous results in a similar model of rat polytrauma.[179]

In both the bone defect and muscle defect tissues, MDSCs were found to be the most prevalent cell type at day 3 post-injury, notably making up approximately 80% of all immune cells on average in the bone defect region. This population of immune cells remains understudied, especially in the context of rat trauma. MDSCs, which are characterized in rats as $HIS48^{+}CD11b/c^{+}$ cells, have been described as a heterogeneous population of immature, immunosuppressive myeloid cells.[132,255] MDSCs are known to expand in many injury and disease states including cancer, sepsis, and acute trauma.[216,256,257] Importantly, however, HIS48 is only used as a marker for these cells in rats. In mice, MDSCs are instead characterized as expressing CD11b and Gr-1 markers, and in humans, markers for MDSCs include CD11b, CD14, CD15, CD33, CD66b, and HLA-DR.[131] In 2010, *Jia et al.* identified $HIS48^{+}CD11b/c^{+}$ cells in a rat tumor model as MDSCs based on their phenotype, origin, and ability to suppress T cell effector functions *in vitro*, which mimicked observations seen previously in mouse and human MDSCs.[132] More recently however, *Maniar et al.* performed single cell RNA sequencing on $HIS48^{+}CD11b/c^{+}$ cells isolated from blood in a rat trauma model and identified both immunosuppressive cells expressing gene markers characteristic of MDSCs as well as neutrophils and monocytes lacking such markers.[258] This suggests that while the

HIS48^{+}CD11b/c^{+} cell population seen in rats with bone trauma likely includes MDSCs, it also appears to contain other mature myeloid cell populations. Additionally, previous studies in mice have demonstrated that neutrophils and other granulocytes comprise the predominant immune cell populations present in the bone defect site in the early days after injury,[55,259] further evidencing that HIS48^{+}CD11b/c^{+} cells include more than MDSCs. Still, for simplicity, these cells will continue to be referred to as MDSCs throughout this document.

Immune cell frequencies in the bone defect and muscle defect tissues largely followed similar patterns of progression over time. The relative frequency of MDSCs decreased between day 3 and week 1 post-injury in both tissues while the frequencies of macrophages, B cells, and T cells increased in both tissues across the same time span. Notably, MDSCs continued to decrease between week 1 and week 2 in the bone defect tissue, while these populations appeared to have already stabilized by this time in the muscle defect tissue, possibly indicating that acute inflammation resolved more quickly in the muscle than in the bone. This result generally agrees with previous studies which have demonstrated that local inflammation tends to resolve around one week post-injury in muscle, while inflammation in the bone defect site can be prolonged until as late as two weeks post-trauma particularly in the context of a severe bone injury.[260] Further, the pattern of elevated MDSCs and depressed T cells in the muscle and bone defect sites at day 3 as compared to later time points follows a similar trend to the observations made in the blood in this study and in previous studies.[179] Immune cell frequencies in the bone marrow adjacent to the injury site interestingly did not follow these same trends, with no changes in MDSCs observed at any time point and T cells being elevated earlier on and then decreasing between week 1 and week 2 for example.

Treatment with BMP-2 in this study resulted in an increase in the frequency of macrophages and a decrease in the frequency of T cells in the bone defect tissue at week 1 post-injury. The elevation of macrophages in this tissue agrees with previous research that has demonstrated the capacity for BMP-2 to induce recruitment of macrophages to the bone injury site.[209] It is worth noting that BMP-2 did not significantly alter any immune cell frequencies in the bone defect tissue at either of the other time points they were measured, nor did it alter immune cell frequencies in any of the other tissues assessed at any time point. This may have been due in part to the low dose of 2.5 micrograms of BMP-2 used for treatment in this study.

In conclusion, here we sought to characterize the immune response to bone and muscle polytrauma by quantifying relative immune cell frequencies systemically and in tissues local to the injury sites via spectral flow cytometry. Our findings revealed that MDSCs are elevated in multiple tissue compartments early after injury and are the predominant immune cell population present at both the bone and muscle injury sites. Polytrauma also resulted in temporal changes to levels of macrophages, B cells, T cells, and T regulatory cells in multiple tissues. Treatment with BMP-2 further altered relative immune cell frequencies in the bone defect space one week after injury, notably increasing recruitment of macrophages to the injury site. Altogether, these results help to establish the time course of the local immune response in a rat model of polytrauma. Still, this study was limited to only three post-injury time points and the characterization of only seven immune cell populations. There therefore remains an opportunity for future studies to expand on this work by quantifying other relevant immune cell populations in the local trauma environment, such as dendritic cells and natural killer cells, and by characterizing the immune response at additional time points, especially within the first two weeks post-injury. Ultimately, a deeper and more comprehensive understanding of the immune system's state throughout the

crucial early stages of bone repair is likely to generate novel methods for targeting and utilizing the immune system to improve patient outcomes.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Primary Conclusions & Contributions to the Field

The earliest medical description of a bone grafting procedure can be traced back to 1668, when Job van Meekeren transplanted a heterologous graft of a dog skull fragment into the skull of a wounded soldier. Despite more than 350 years of medical advancements since then, bone grafts remain the standard of care for the treatment of severe bone injuries today. Modern advances in acute trauma care have increased survival rates following severe trauma, but complications including bone nonunion continue to prove challenging for clinicians to address. This work aimed to ameliorate this problem by investigating the immune response to severe trauma and subsequent bone healing outcomes. Our ultimate objectives were to identify biomarkers associated with trauma-induced immune dysregulation and poor outcomes, and to investigate the efficacy of immunomodulatory strategies to supplement existing treatment modalities for bone fractures.

6.1.1 Immunomodulation Using Nanoparticles Targeting Myeloid-Derived Suppressor Cells

To begin, we investigated the effects of a previously-established therapeutic platform, Synthetic Nanoparticle Antibodies (SNAbs), on immune dysregulation and healing outcomes in a rat model of bone and muscle polytrauma.[179] SNAbs were used to target S100A8/A9, a protein complex produced by myeloid-derived suppressor cells (MDSCs). This strategy had previously been shown to deplete MDSCs from a rat trauma model *ex vivo* as well as MDSCs from a mouse cancer model *in vivo*.[196]

Interestingly, we found that when SNAbs were systemically injected *in vivo* in our rat polytrauma model, they did not result in depletion of total MDSCs, but instead caused their

expansion. While the reason for this expansion is unknown, a rapid increase in myeloid cells could be attributed to a form of emergency myelopoiesis which ordinarily results from interactions between pathogen-associated or damage-associated molecular patterns and pattern recognition receptors such as toll-like receptors. Notably, certain S100 proteins including S100A8/A9 have been identified as damage-associated molecular patterns.[261] It is possible, then, that by targeting and killing cells expressing S100A8/A9, the SNAbs in this study caused the release of S100 proteins into the extracellular environment, which interacted with pattern recognition receptors triggering emergency myelopoiesis. If the resulting expansion of myeloid cells outpaced the depletion of myeloid cells by the SNAbs, this could have had a net positive effect on circulating myeloid cell frequencies, thereby explaining the observed increase in MDSCs. If this was the case, then a key takeaway from this study is that immunomodulatory strategies should take into consideration the rapid and dynamic response of the immune system to sudden changes in large cell populations, which could hinder attempted immune therapies.

A closer look at specific MDSC subpopulations in this study revealed that SNAbs did result in a decrease in the relative frequency of HIS48^{hi} MDSCs, while HIS48^{lo} MDSCs increased considerably and drove the expansion of total MDSCs. While the exact reason for these distinct effects remains unknown, single cell RNA sequencing revealed differences in the expression of S100A8/A9 among MDSC subpopulations, which could be at least partially responsible for differences in the impacts of SNAbs on the two subpopulations. This result exemplifies how the heterogeneity of a cell population can hinder the efficacy of immunomodulatory therapies targeting those cells.

A third important finding in this study was that the expanded MDSCs in the SNAb-treated groups correlated negatively with endpoint bone volume. Although this effect was

unintentional, this result provides further evidence of a link between aberrant levels of these myeloid cells and impaired bone regeneration. As such, additional studies are warranted to explore this cell population as a potential therapeutic target.

Finally, changes to several cytokine populations were observed after SNAb treatment, and these changes were slightly delayed compared to the observed changes in immune cell frequencies, which could indicate that the alteration of the cytokine populations was a byproduct of immune cell modulation. This result demonstrates how cell-based immunomodulatory strategies can have far wider-reaching effects than strategies targeting individual cytokine or chemokine populations. This could give strategies targeting cells rather than proteins the potential for greater efficacy, but it also warrants additional consideration of unexpected or unwanted adverse effects that could be accompanied by cell-based immunomodulation.

6.1.2 Immunomodulation Using All-Trans Retinoic Acid in a Rat Model of Trauma

In our next study, we employed all-trans retinoic acid (ATRA) as an immunomodulatory therapeutic in our rat model of composite bone and muscle trauma. ATRA has been shown previously to modulate MDSCs by promoting their differentiation into mature myeloid cell types such as monocytes and dendritic cells, thereby reducing their immunosuppressive functions.

One key finding of this study was that ATRA successfully depleted MDSCs derived from both naive and injured rats *in vitro*, even when used at three different concentrations across three orders of magnitude. However, *in vivo* systemic delivery of ATRA to rats with trauma did not deplete total circulating MDSCs, and at higher doses, ATRA delivery even resulted in an increase in total MDSCs. This result, alongside our earlier finding that SNAbs increased total MDSCs when delivered systemically, exemplifies the potential difficulty of translating *in vitro*

immunomodulatory strategies into successful strategies *in vivo*, where dynamic changes in the immune system such as emergency myelopoiesis can alter outcomes.

Also similar to a previous finding related to SNAbs, systemic ATRA treatment resulted in a decrease in the relative frequency of one subpopulation of MDSCs, although the other, larger subpopulation remained unchanged by ATRA treatment. This result further highlights the heterogeneity of these myeloid cells and the need for additional characterization of their subpopulations to identify methods for effectively targeting and depleting all of them.

Interestingly, ATRA treatment also resulted in a decrease in circulating regulatory T cells (Tregs). Tregs are induced by MDSCs,[123] and therefore this effect could have been a secondary result of MDSC modulation. Given that total MDSCs were not significantly depleted however, this could indicate that ATRA modulated MDSCs in ways that could not be detected by flow cytometry analysis alone. One possibility is that ATRA successfully caused MDSCs to differentiate and consequently lose their immunosuppressive capacity, but the resultant mature myeloid cells still largely expressed the surface markers used in the identification of rat MDSCs, HIS48 and CD11b/c. One recent study that performed single cell RNA sequencing on circulating $HIS48^{+}CD11b/c^{+}$ rat cells found that these cells were highly heterogeneous, containing subpopulations identified as both neutrophils and monocytes, and only a small subset of these cells significantly expressed genes characteristic of immunosuppression.[258] This appears to support the notion that the cell population identified by flow cytometry as MDSCs in rats actually contains mature myeloid cells as well as immature, immunosuppressive cells, and as a result, deeper characterization methods are likely needed to distinguish these subpopulations and properly assess the effects of immunomodulatory treatments targeting them.

Finally, the observed changes to circulating Tregs and one MDSC subpopulation following systemic delivery of ATRA did not improve healing. This could suggest that depletion of only one subpopulation of MDSCs was insufficient to improve healing while the other, larger subpopulation remained not significantly diminished. Another possibility is that while circulating MDSCs have previously been found to correlate with impaired bone regeneration, they may only be predictive markers of poor healing rather than its cause. Further studies would be necessary to confirm this however, ideally by depleting total MDSCs more effectively, rather than just individual subpopulations of the cells.

6.1.3 Characterization of the Local Immune Response to Trauma via Spectral Flow Cytometry

In our final study, we characterized the cellular immune response to trauma in tissues local to the bone and muscle injury sites in our rat model using spectral flow cytometry. Tissues harvested from the bone defect site, the muscle defect site, and the bone marrow adjacent to the bone defect were stained for various immune cell populations and analyzed by spectral flow cytometry along with peripheral blood. We harvested these tissues from rats at either day 3, week 1, or week 2 post-injury, and blood and bone marrow were also collected from a group of naive rats for comparison. We also treated a subset of rats locally with bone morphogenetic protein 2 (BMP-2) and alginate at the time of their injuries while leaving another subset of rats untreated, thereby allowing us to investigate the effects of BMP-2 treatment on the local immune response.

Certain trends were observed in local tissues that were consistent with observations made in the peripheral blood. For example, MDSCs were most elevated at day 3 in the blood, muscle, and bone defect tissue before decreasing at later time points. Bone marrow was an exception to this trend, however, as MDSCs remained steady across all time points and compared to the naive

controls. T cells were similarly most depressed at day 3 post-injury in the blood, bone defect tissue, and bone marrow. Other trends occurred in local tissues that were not observed in peripheral blood, such as an increase of B cells in the muscle, bone defect tissue, and bone marrow between day 3 and week 1 post-injury. Altogether, these results provided new knowledge of the cellular immune response over time in tissues local to the injury site following severe musculoskeletal trauma.

Lastly, BMP-2 was found to alter immune cell populations, including macrophages and T cell populations, in the bone defect tissue week 1 post-injury. Interestingly, this effect was not observed at day 3 or at week 2 post-injury, nor was it observed in other tissues, possibly due in part to the low dose of BMP-2 used in this study. Still, this finding contributes to our understanding of the immunomodulatory effects of BMP-2 on the local osteoimmune environment during the early stages of bone repair.

6.2 Future Directions

Much work remains to be done to improve our understanding of trauma-induced immune dysregulation and the roles of immune mediators in bone regeneration, and to develop and translate immunomodulatory strategies to target trauma-induced immune dysregulation and improve healing outcomes.

6.2.1 Characterization of the Immune Response to Trauma

While this work characterized the immune response in multiple tissues in a rat model of composite bone and muscle injury, our results still possess multiple limitations. First, our characterization of immune cells was limited to only a select few immune cell populations, including MDSCs, monocytes, B cells, T cells, and T cell subtypes. Neutrophils, dendritic cells,

natural killer cells, and numerous other immune cell populations and subpopulations remain entirely unexplored in our trauma model, although these cells likely play roles in healing and could correlate to bone regenerative outcomes. The advent of recent technologies such as spectral flow cytometry and the ever-growing market of immunofluorescent markers for phenotyping cell populations present a novel opportunity to characterize the cellular immune response to trauma to a greater degree than has been previously accomplished. In rats alone, one study recently developed and validated a 24-marker spectral flow cytometry panel for the immunologic analysis of tissue,[262] which would considerably expand upon the cell populations examined in our studies.

Beyond flow cytometry, other methods of immune characterization would be beneficial to more deeply characterize cell phenotypes and stratify heterogeneous cell populations after injury. In particular, the heterogeneity of MDSCs throughout this work was a seemingly important factor that may have affected outcomes of immunomodulatory strategies on more than one occasion. Limited antibody markers exist for these cells in rats, however, so further characterization of MDSCs will likely require other methods. One recent study employed both fluorescence-activated cell sorting and single cell RNA sequencing to isolate MDSCs derived from a rat trauma model and examine their gene expression profiles.[258] Other methods have been used previously in the study of MDSCs in cancer models to assess their immunosuppressive capacity. For example, co-culturing of MDSCs with T cells and then measuring subsequent T cell proliferation is a commonly used method for assessing MDSC immunosuppressive function.[263] This could help us determine whether immunomodulatory therapeutic strategies may alter MDSCs in ways that cannot be detected by cell staining and flow cytometry.

One other limitation to current immune characterization methods is that analysis of immune cells in local tissues typically requires sacrificing the animal, which makes it impossible to directly correlate early immune cell infiltration in the local injury environment with later healing. The development of non-invasive methods for tracking immune cells in the bone injury niche *in vivo* would overcome this limitation and allow stronger connections between local immune cell populations and bone healing to be drawn. Multiple molecular imaging techniques have been developed in recent years that enable non-invasive tracking of immune cells in soft tissues,[264] but the highly mineralized nature of bone creates additional challenges for these methods.

6.2.2 Development and Translation of Immunomodulatory Strategies for Treatment of Trauma

While a great variety of immunomodulatory strategies have been explored for the purpose of improving bone regenerative outcomes, such strategies have not seen much successful translation yet into clinical settings. This is likely in part due to numerous challenges that must be overcome and considerations that must be made for immunomodulatory interventions to be successful.

One such challenge arises from the considerable variation among the immune responses of patients with musculoskeletal trauma. Patients with trauma may follow one of several inflammatory trajectories, including normal progression and uncomplicated resolution of inflammation, insufficient local inflammation to initiate repair, excessive acute inflammation either locally or systemically, or chronic inflammation that does not resolve in a timely manner. Optimal immunomodulatory strategies may therefore not follow a one-size-fits-all approach, but rather they may need to be tailored to each individual patient's needs based on their immune status. Importantly, a major goal in the use of immunomodulatory strategies for treatment of

bone fractures is to help prevent or treat bone nonunions. However, only a relatively small percentage of bone fractures actually proceed to nonunion,[6] and in patients who are already on track to heal properly, improper immunomodulation may actually jeopardize successful bone regeneration or cause other adverse effects. Indeed, while timely resolution of inflammation is considered crucial to successful bone repair, certain anti-inflammatory strategies investigated in past studies resulted in negative, and sometimes severe outcomes. The corticosteroid randomization after significant head injury (CRASH) trial, a clinical trial with over 10,000 head trauma patients enrolled, had to be stopped early due to significantly higher mortality among patients in the corticosteroid treatment group compared to the placebo control group.[265] Some other immunosuppressive strategies, such as the use of TNF-α blockers to limit inflammation, have been shown to potentially increase the risk of patients developing opportunistic infections.[175] Considering these findings, and given the small percentage of fractures that actually progress to nonunion, many patients stand to gain limited to no benefit from the administration of immunomodulatory treatments, while the risks of such treatments may be significant. As such, a crucial first step in the translation of immunomodulatory therapeutics to the clinic will be the development of diagnostic tools to properly characterize the status of each patient's immune system after injury, identify patients in need of immunomodulatory interventions to help prevent nonunion or other poor outcomes, and create patient-specific treatment plans that are optimized based on the patient's immune status to ameliorate immune dysregulation without inducing adverse side effects. Adding to the challenge of this endeavor are the myriad factors that can alter a patient's immune profile even prior to injury, such as age, sex, and health status, all of which may require additional consideration in developing novel treatments.[266–268]

Injury type, injury severity, and complications such as infections are also important considerations for the development of immunomodulatory therapeutics. The immune response to trauma can vary considerably depending on the extent of the injury or injuries, and this can also affect healing outcomes. For example, one previous study demonstrated that femoral bone regeneration in a rat model of trauma was impaired when the rats also had blunt chest trauma, which induced considerable systemic inflammation.[110] Comorbidities such as infections can also alter a patient's immune status and increase their risk for developing a bone nonunion.[20]

Timing of treatment represents another crucial consideration for the development of immunomodulatory interventions in trauma. During the normal course of successful bone healing, the inflammatory phase lasts for several days to one week post-injury.[30] Around this time, pro-inflammatory mediators such as M1 macrophages should begin to subside while anti-inflammatory and pro-regenerative mediators such as M2 macrophages and mesenchymal stem cells (MSCs) should expand within the fracture site. Prospective immunomodulatory strategies should take this normal time course of local inflammation into account, helping to facilitate the timely transition from a pro-inflammatory to an anti-inflammatory state without overly inhibiting early inflammation that is still crucial for laying the foundations for repair. Understanding the time course over which potential immunomodulatory targets are altered both in the normal course of healing and in complicated healing can help guide the development of treatment strategies.

Despite all of the challenges that remain to be overcome, the prospect of employing immunomodulation to improve bone healing remains an exciting possibility. The development and optimization of patient-specific strategies that leverage pro-regenerative immune mediators or treat trauma-induced immune dysregulation to improve healing will provide powerful new

tools to supplement and help overcome the limitations of current bone trauma treatment modalities.

REFERENCES CITED

1. DiMaggio, C. *et al.* Traumatic injury in the United States: In-patient epidemiology 2000-2011. *Injury* **47**, 1393–1403 (2016).

2. Abe, T. *et al.* Trauma complications and in-hospital mortality: failure-to-rescue. *Crit. Care Lond. Engl.* **24**, 223 (2020).

3. Harris, A. M. *et al.* Complications following limb-threatening lower extremity trauma. *J. Orthop. Trauma* **23**, 1–6 (2009).

4. Brinker, M. R. & O'Connor, D. P. Nonunions: Evaluation and Treatment. in *Skeletal Trauma: Basic Science, Management, and Reconstruction* 637–718 (WB Saunders, 2009).

5. Tzioupis, C. & Giannoudis, P. V. Prevalence of long-bone non-unions. *Injury* **38 Suppl 2**, S3-9 (2007).

6. Mills, L. A., Aitken, S. A. & Simpson, A. H. R. W. The risk of non-union per fracture: current myths and revised figures from a population of over 4 million adults. *Acta Orthop.* **88**, 434–439 (2017).

7. Miranda, M. A. & Moon, M. Treatment Strategy for Nonunions and Malunions. in *Surgical Treatment of Orthopaedic Trauma* 77–100 (Thieme, 2007).

8. Antonova, E., Le, T. K., Burge, R. & Mershon, J. Tibia shaft fractures: costly burden of nonunions. *BMC Musculoskelet. Disord.* **14**, 42 (2013).

9. MacKenzie, E. J. *et al.* Health-care costs associated with amputation or reconstruction of a limb-threatening injury. *J. Bone Joint Surg. Am.* **89**, 1685–1692 (2007).

10. Melcer, T. *et al.* A Comparison of Four-Year Health Outcomes following Combat Amputation and Limb Salvage. *PloS One* **12**, e0170569 (2017).

11. Doukas, W. C. *et al.* The Military Extremity Trauma Amputation/Limb Salvage (METALS) study: outcomes of amputation versus limb salvage following major lower-extremity trauma. *J. Bone Joint Surg. Am.* **95**, 138–145 (2013).

12. Chandra, A. & Rajawat, J. Skeletal Aging and Osteoporosis: Mechanisms and Therapeutics. *Int. J. Mol. Sci.* **22**, 3553 (2021).

13. O'Halloran, K. *et al.* Will My Tibial Fracture Heal? Predicting Nonunion at the Time of Definitive Fixation Based on Commonly Available Variables. *Clin. Orthop.* **474**, 1385–1395 (2016).

14. Zura, R. *et al.* Bone fracture nonunion rate decreases with increasing age: A prospective inception cohort study. *Bone* **95**, 26–32 (2017).

15. Szczęsny, G. *et al.* Genetic factors responsible for long bone fractures non-union. *Arch. Orthop. Trauma Surg.* **131**, 275–281 (2011).

16. Brinker, M. R., O'Connor, D. P., Monla, Y. T. & Earthman, T. P. Metabolic and endocrine abnormalities in patients with nonunions. *J. Orthop. Trauma* **21**, 557–570 (2007).

17. Gorter, E. A., Krijnen, P. & Schipper, I. B. Vitamin D status and adult fracture healing. *J. Clin. Orthop. Trauma* **8**, 34–37 (2017).

18. Al Farii, H., Farahdel, L., Frazer, A., Salimi, A. & Bernstein, M. The effect of NSAIDs on postfracture bone healing: a meta-analysis of randomized controlled trials. *OTA Int. Open Access J. Orthop. Trauma* **4**, e092 (2021).

19. Bishop, J. A., Palanca, A. A., Bellino, M. J. & Lowenberg, D. W. Assessment of compromised fracture healing. *J. Am. Acad. Orthop. Surg.* **20**, 273–282 (2012).

20. Hackl, S. *et al.* The role of low-grade infection in the pathogenesis of apparently aseptic tibial shaft nonunion. *Injury* **52**, 3498–3504 (2021).

21. Thomas, J. D. & Kehoe, J. L. Bone Nonunion. in *StatPearls* (StatPearls Publishing, Treasure Island (FL), 2025).

22. Kolar, P. *et al.* The early fracture hematoma and its potential role in fracture healing. *Tissue Eng. Part B Rev.* **16**, 427–434 (2010).

23. Wise, J. K. *et al.* Temporal gene expression profiling during rat femoral marrow ablation-induced intramembranous bone regeneration. *PloS One* **5**, e12987 (2010).

24. Hadjiargyrou, M. *et al.* Transcriptional profiling of bone regeneration. Insight into the molecular complexity of wound repair. *J. Biol. Chem.* **277**, 30177–30182 (2002).

25. Zhong, N., Gersch, R. P. & Hadjiargyrou, M. Wnt signaling activation during bone regeneration and the role of Dishevelled in chondrocyte proliferation and differentiation. *Bone* **39**, 5–16 (2006).

26. Marsell, R. & Einhorn, T. A. The biology of fracture healing. *Injury* **42**, 551–555 (2011).

27. Sm, P. Physical and biological aspects of fracture healing with special reference to internal fixation. *Clin. Orthop.* (1979).

28. Cs, B. *et al.* Cellular biology of fracture healing. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* **37**, (2019).

29. Kaderly, R. E. Primary bone healing. *Semin. Vet. Med. Surg. (Small Anim.)* **6**, 21–25 (1991).

30. F, L. *et al.* Inflammation, fracture and bone repair. *Bone* **86**, (2016).

31. Shiu, H. T., Leung, P. C. & Ko, C. H. The roles of cellular and molecular components of a hematoma at early stage of bone healing. *J. Tissue Eng. Regen. Med.* **12**, e1911–e1925 (2018).

32. Schell, H. *et al.* The haematoma and its role in bone healing. *J. Exp. Orthop.* **4**, 5 (2017).

33. Ito, H. Chemokines in mesenchymal stem cell therapy for bone repair: a novel concept of recruiting mesenchymal stem cells and the possible cell sources. *Mod. Rheumatol.* **21**, 113–121 (2011).

34. X, W. *et al.* Role of mesenchymal stem cells in bone regeneration and fracture repair: a review. *Int. Orthop.* **37**, (2013).

35. Z, M. *et al.* Differentiation of adipocytes and osteocytes from human adipose and placental mesenchymal stem cells. *Iran. J. Basic Med. Sci.* **18**, (2015).

36. Sheen, J. R., Mabrouk, A. & Garla, V. V. Fracture Healing Overview. in *StatPearls* (StatPearls Publishing, Treasure Island (FL), 2025).

37. Somoza, R. A., Welter, J. F., Correa, D. & Caplan, A. I. Chondrogenic differentiation of mesenchymal stem cells: challenges and unfulfilled expectations. *Tissue Eng. Part B Rev.* **20**, 596–608 (2014).

38. Wang, H. *et al.* Advances in the role and mechanism of fibroblasts in fracture healing. *Front. Endocrinol.* **15**, 1350958 (2024).

39. ElHawary, H. *et al.* Bone Healing and Inflammation: Principles of Fracture and Repair. *Semin. Plast. Surg.* **35**, 198–203 (2021).

40. Beederman, M. *et al.* BMP signaling in mesenchymal stem cell differentiation and bone formation. *J. Biomed. Sci. Eng.* **6**, 32–52 (2013).

41. Bolamperti, S., Villa, I. & Rubinacci, A. Bone remodeling: an operational process ensuring survival and bone mechanical competence. *Bone Res.* **10**, 48 (2022).

42. Giannoudis, P. V., Einhorn, T. A. & Marsh, D. Fracture healing: the diamond concept. *Injury* **38 Suppl 4**, S3-6 (2007).

43. Molitoris, K. H., Huang, M. & Baht, G. S. Osteoimmunology of Fracture Healing. *Curr. Osteoporos. Rep.* **22**, 330–339 (2024).

44. Yao, Q., He, L., Bao, C., Yan, X. & Ao, J. The role of TNF-α in osteoporosis, bone repair and inflammatory bone diseases: A review. *Tissue Cell* **89**, 102422 (2024).

45. Sarahrudi, K. *et al.* Elevated levels of macrophage colony-stimulating factor in human fracture healing. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* **28**, 671–676 (2010).

46. Boyce, B. F. & Xing, L. Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Arch. Biochem. Biophys.* **473**, 139–146 (2008).

47. Charoenlarp, P., Rajendran, A. K. & Iseki, S. Role of fibroblast growth factors in bone regeneration. *Inflamm. Regen.* **37**, 10 (2017).

48. Bonewald, L. F. & Mundy, G. R. Role of transforming growth factor-beta in bone remodeling. *Clin. Orthop.* 261–276 (1990).

49. Caplan, A. I. & Correa, D. PDGF in bone formation and regeneration: new insights into a novel mechanism involving MSCs. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* **29**, 1795–1803 (2011).

50. Garrison, K. R. *et al.* Bone morphogenetic protein (BMP) for fracture healing in adults. *Cochrane Database Syst. Rev.* **2010**, CD006950 (2010).

51. Hu, K. & Olsen, B. R. The roles of vascular endothelial growth factor in bone repair and regeneration. *Bone* **91**, 30–38 (2016).

52. Liu, Y., Kuang, B., Rothrauff, B. B., Tuan, R. S. & Lin, H. Robust bone regeneration through endochondral ossification of human mesenchymal stem cells within their own extracellular matrix. *Biomaterials* **218**, 119336 (2019).

53. Tao, H., Han, Z., Han, Z. C. & Li, Z. Proangiogenic Features of Mesenchymal Stem Cells and Their Therapeutic Applications. *Stem Cells Int.* **2016**, 1314709 (2016).

54. Planat-Benard, V., Varin, A. & Casteilla, L. MSCs and Inflammatory Cells Crosstalk in Regenerative Medicine: Concerted Actions for Optimized Resolution Driven by Energy Metabolism. *Front. Immunol.* **12**, 626755 (2021).

55. Bastian, O. W., Koenderman, L., Alblas, J., Leenen, L. P. H. & Blokhuis, T. J. Neutrophils contribute to fracture healing by synthesizing fibronectin+ extracellular matrix rapidly after injury. *Clin. Immunol. Orlando Fla* **164**, 78–84 (2016).

56. Lin, X., Patil, S., Gao, Y.-G. & Qian, A. The Bone Extracellular Matrix in Bone Formation and Regeneration. *Front. Pharmacol.* **11**, 757 (2020).

57. Rubin, J., Rubin, C. & Jacobs, C. R. Molecular pathways mediating mechanical signaling in bone. *Gene* **367**, 1–16 (2006).

58. Jagodzinski, M. & Krettek, C. Effect of mechanical stability on fracture healing--an update. *Injury* **38 Suppl 1**, S3-10 (2007).

59. Wang, L., You, X., Zhang, L., Zhang, C. & Zou, W. Mechanical regulation of bone remodeling. *Bone Res.* **10**, 16 (2022).

60. Kutys, M. L. & Chen, C. S. Forces and mechanotransduction in 3D vascular biology. *Curr. Opin. Cell Biol.* **42**, 73–79 (2016).

61. Mammoto, A. *et al.* A mechanosensitive transcriptional mechanism that controls angiogenesis. *Nature* **457**, 1103–1108 (2009).

62. Hak, D. J., Toker, S., Yi, C. & Toreson, J. The influence of fracture fixation biomechanics on fracture healing. *Orthopedics* **33**, 752–755 (2010).

63. Taljanovic, M. S. *et al.* Fracture fixation. *Radiogr. Rev. Publ. Radiol. Soc. N. Am. Inc* **23**, 1569–1590 (2003).

64. Schmidt, A. H. Autologous bone graft: Is it still the gold standard? *Injury* **52 Suppl 2**, S18–S22 (2021).

65. Robinson, P. G., Abrams, G. D., Sherman, S. L., Safran, M. R. & Murray, I. R. Autologous Bone Grafting. *Oper. Tech. Sports Med.* **28**, 150780 (2020).

66. Miron, R. J. *et al.* Osteogenic potential of autogenous bone grafts harvested with four different surgical techniques. *J. Dent. Res.* **90**, 1428–1433 (2011).

67. Roberts, T. T. & Rosenbaum, A. J. Bone grafts, bone substitutes and orthobiologics: the bridge between basic science and clinical advancements in fracture healing. *Organogenesis* **8**, 114–124 (2012).

68. Ho-Shui-Ling, A. *et al.* Bone regeneration strategies: Engineered scaffolds, bioactive molecules and stem cells current stage and future perspectives. *Biomaterials* **180**, 143–162 (2018).

69. Battafarano, G. *et al.* Strategies for Bone Regeneration: From Graft to Tissue Engineering. *Int. J. Mol. Sci.* **22**, 1128 (2021).

70. Gautschi, O. P., Frey, S. P. & Zellweger, R. Bone morphogenetic proteins in clinical applications. *ANZ J. Surg.* **77**, 626–631 (2007).

71. James, A. W. *et al.* A Review of the Clinical Side Effects of Bone Morphogenetic Protein-2. *Tissue Eng. Part B Rev.* **22**, 284–297 (2016).

72. Jeffcoach, D. R. *et al.* Nonsteroidal anti-inflammatory drugs' impact on nonunion and infection rates in long-bone fractures. *J. Trauma Acute Care Surg.* **76**, 779–783 (2014).

73. Rapp, A. E. *et al.* Fracture Healing Is Delayed in Immunodeficient NOD/scid-IL2Rγcnull Mice. *PloS One* **11**, e0147465 (2016).

74. Ono, T. *et al.* IL-17-producing γδ T cells enhance bone regeneration. *Nat. Commun.* **7**, 10928 (2016).

75. Ishikawa, M. *et al.* MCP/CCR2 signaling is essential for recruitment of mesenchymal progenitor cells during the early phase of fracture healing. *PloS One* **9**, e104954 (2014).

76. Bastian, O. W. *et al.* Neutrophils Inhibit Synthesis of Mineralized Extracellular Matrix by Human Bone Marrow-Derived Stromal Cells In Vitro. *Front. Immunol.* **9**, 945 (2018).

77. Zhang, D. *et al.* Research Progress of Macrophages in Bone Regeneration. *J. Tissue Eng. Regen. Med.* **2023**, 1512966 (2023).

78. Sauaia, A., Moore, F. A. & Moore, E. E. Postinjury Inflammation and Organ Dysfunction. *Crit. Care Clin.* **33**, 167–191 (2017).

79. Xiao, W. *et al.* A genomic storm in critically injured humans. *J. Exp. Med.* **208**, 2581–2590 (2011).

80. Roth, S. *et al.* Post-injury immunosuppression and secondary infections are caused by an AIM2 inflammasome-driven signaling cascade. *Immunity* **54**, 648-659.e8 (2021).

81. Mira, J. C., Brakenridge, S. C., Moldawer, L. L. & Moore, F. A. Persistent Inflammation, Immunosuppression and Catabolism Syndrome. *Crit. Care Clin.* **33**, 245–258 (2017).

82. Jones, M. A. *et al.* Plasma interleukin responses as predictors of outcome stratification in patients after major trauma: a prospective observational two centre study. *Front. Immunol.* **14**, 1276171 (2023).

83. Heffernan, D. S. *et al.* Failure to normalize lymphopenia following trauma is associated with increased mortality, independent of the leukocytosis pattern. *Crit. Care Lond. Engl.* **16**, R12 (2012).

84. Baht, G. S., Vi, L. & Alman, B. A. The Role of the Immune Cells in Fracture Healing. *Curr. Osteoporos. Rep.* **16**, 138–145 (2018).

85. Capobianco, C. A., Hankenson, K. D. & Knights, A. J. Temporal dynamics of immune-stromal cell interactions in fracture healing. *Front. Immunol.* **15**, 1352819 (2024).

86. Peiseler, M. & Kubes, P. More friend than foe: the emerging role of neutrophils in tissue repair. *J. Clin. Invest.* **129**, 2629–2639 (2019).

87. Wang, J. Neutrophils in tissue injury and repair. *Cell Tissue Res.* **371**, 531–539 (2018).

88. Soehnlein, O., Lindbom, L. & Weber, C. Mechanisms underlying neutrophil-mediated monocyte recruitment. *Blood* **114**, 4613–4623 (2009).

89. Bastian, O. W. *et al.* Serum from the Human Fracture Hematoma Contains a Potent Inducer of Neutrophil Chemotaxis. *Inflammation* **41**, 1084–1092 (2018).

90. Chung, R., Cool, J. C., Scherer, M. A., Foster, B. K. & Xian, C. J. Roles of neutrophil-mediated inflammatory response in the bony repair of injured growth plate cartilage in young rats. *J. Leukoc. Biol.* **80**, 1272–1280 (2006).

91. Grøgaard, B., Gerdin, B. & Reikerås, O. The polymorphonuclear leukocyte: has it a role in fracture healing? *Arch. Orthop. Trauma Surg.* **109**, 268–271 (1990).

92. Kovtun, A. *et al.* The crucial role of neutrophil granulocytes in bone fracture healing. *Eur. Cell. Mater.* **32**, 152–162 (2016).

93. Pillay, J., Hietbrink, F., Koenderman, L. & Leenen, L. P. H. The systemic inflammatory response induced by trauma is reflected by multiple phenotypes of blood neutrophils. *Injury* **38**, 1365–1372 (2007).

94. Kasama, T., Strieter, R. M., Standiford, T. J., Burdick, M. D. & Kunkel, S. L. Expression and regulation of human neutrophil-derived macrophage inflammatory protein 1 alpha. *J. Exp. Med.* **178**, 63–72 (1993).

95. Rider, P. *et al.* IL-1α and IL-1β recruit different myeloid cells and promote different stages of sterile inflammation. *J. Immunol. Baltim. Md 1950* **187**, 4835–4843 (2011).

96. Leibovich, S. J. & Ross, R. The role of the macrophage in wound repair. A study with hydrocortisone and antimacrophage serum. *Am. J. Pathol.* **78**, 71–100 (1975).

97. Niu, Y., Wang, Z., Shi, Y., Dong, L. & Wang, C. Modulating macrophage activities to promote endogenous bone regeneration: Biological mechanisms and engineering approaches. *Bioact. Mater.* **6**, 244–261 (2021).

98. Spiller, K. L. *et al.* The role of macrophage phenotype in vascularization of tissue engineering scaffolds. *Biomaterials* **35**, 4477–4488 (2014).

99. Gordon, S. Alternative activation of macrophages. *Nat. Rev. Immunol.* **3**, 23–35 (2003).

100. Gou, M., Wang, H., Xie, H. & Song, H. Macrophages in guided bone regeneration: potential roles and future directions. *Front. Immunol.* **15**, 1396759 (2024).

101. Brown, B. N. *et al.* Macrophage phenotype as a predictor of constructive remodeling following the implantation of biologically derived surgical mesh materials. *Acta Biomater.* **8**, 978–987 (2012).

102. Claes, L., Recknagel, S. & Ignatius, A. Fracture healing under healthy and inflammatory conditions. *Nat. Rev. Rheumatol.* **8**, 133–143 (2012).

103. Parihar, A., Eubank, T. D. & Doseff, A. I. Monocytes and macrophages regulate immunity through dynamic networks of survival and cell death. *J. Innate Immun.* **2**, 204–215 (2010).

104. Könnecke, I. *et al.* T and B cells participate in bone repair by infiltrating the fracture callus in a two-wave fashion. *Bone* **64**, 155–165 (2014).

105. Kawai, T. *et al.* B and T lymphocytes are the primary sources of RANKL in the bone resorptive lesion of periodontal disease. *Am. J. Pathol.* **169**, 987–998 (2006).

106. Kong, Y. Y. *et al.* Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* **402**, 304–309 (1999).

107. Titanji, K. Beyond Antibodies: B Cells and the OPG/RANK-RANKL Pathway in Health, Non-HIV Disease and HIV-Induced Bone Loss. *Front. Immunol.* **8**, 1851 (2017).

108. Sun, G. *et al.* Regulatory B cell is critical in bone union process through suppressing proinflammatory cytokines and stimulating Foxp3 in Treg cells. *Clin. Exp. Pharmacol. Physiol.* **44**, 455–462 (2017).

109. Zaiss, M. M. *et al.* Treg cells suppress osteoclast formation: a new link between the immune system and bone. *Arthritis Rheum.* **56**, 4104–4112 (2007).

110. Recknagel, S. *et al.* Experimental blunt chest trauma impairs fracture healing in rats. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* **29**, 734–739 (2011).

111. Recknagel, S. *et al.* C5aR-antagonist significantly reduces the deleterious effect of a blunt chest trauma on fracture healing. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* **30**, 581–586 (2012).

112. Cheng, A. *et al.* Early systemic immune biomarkers predict bone regeneration after trauma. *Proc. Natl. Acad. Sci. U. S. A.* **118**, e2017889118 (2021).

113. Makarenkova, V. P., Bansal, V., Matta, B. M., Perez, L. A. & Ochoa, J. B. CD11b+/Gr-1+ myeloid suppressor cells cause T cell dysfunction after traumatic stress. *J. Immunol. Baltim. Md 1950* **176**, 2085–2094 (2006).

114. Fultang, N., Li, X., Li, T. & Chen, Y. H. Myeloid-Derived Suppressor Cell Differentiation in Cancer: Transcriptional Regulators and Enhanceosome-Mediated Mechanisms. *Front. Immunol.* **11**, 619253 (2020).

115. Malengier-Devlies, B., Metzemaekers, M., Wouters, C., Proost, P. & Matthys, P. Neutrophil Homeostasis and Emergency Granulopoiesis: The Example of Systemic Juvenile Idiopathic Arthritis. *Front. Immunol.* **12**, 766620 (2021).

116. Bergenfelz, C. & Leandersson, K. The Generation and Identity of Human Myeloid-Derived Suppressor Cells. *Front. Oncol.* **10**, 109 (2020).

117. Rodriguez, P. C. *et al.* Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes. *Cancer Res.* **69**, 1553–1560 (2009).

118. Li, Y.-N. *et al.* Inhibition of myeloid-derived suppressor cell arginase-1 production enhances T-cell-based immunotherapy against Cryptococcus neoformans infection. *Nat. Commun.* **13**, 4074 (2022).

119. Srivastava, M. K., Sinha, P., Clements, V. K., Rodriguez, P. & Ostrand-Rosenberg, S. Myeloid-derived suppressor cells inhibit T-cell activation by depleting cystine and cysteine. *Cancer Res.* **70**, 68–77 (2010).

120. Huang, J., Zhao, Y., Zhao, K., Yin, K. & Wang, S. Function of reactive oxygen species in myeloid-derived suppressor cells. *Front. Immunol.* **14**, 1226443 (2023).

121. Zhong, J., Yau, A. C. Y. & Holmdahl, R. Regulation of T Cell Function by Reactive Nitrogen and Oxygen Species in Collagen-Induced Arthritis. *Antioxid. Redox Signal.* **32**, 161–172 (2020).

122. Feng, S. *et al.* Myeloid-derived suppressor cells inhibit T cell activation through nitrating LCK in mouse cancers. *Proc. Natl. Acad. Sci. U. S. A.* **115**, 10094–10099 (2018).

123. Huang, B. *et al.* Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. *Cancer Res.* **66**, 1123–1131 (2006).

124. Efron, P. A. *et al.* The persistent inflammation, immunosuppression, and catabolism syndrome 10 years later. *J. Trauma Acute Care Surg.* **95**, 790–799 (2023).

125. Mathias, B. *et al.* Human Myeloid-derived Suppressor Cells are Associated With Chronic Immune Suppression After Severe Sepsis/Septic Shock. *Ann. Surg.* **265**, 827–834 (2017).

126. Cheng, L., Xu, J., Chai, Y., Wang, C. & Han, P. Dynamic changes in trauma-induced myeloid-derived suppressor cells after polytrauma are associated with an increased susceptibility to infection. *Int. J. Clin. Exp. Pathol.* **10**, 11063–11068 (2017).

127. Kawai, H. *et al.* Potential role of myeloid-derived suppressor cells in transition from reaction to repair phase of bone healing process. *Int. J. Med. Sci.* **18**, 1824–1830 (2021).

128. Schlundt, C. *et al.* Individual Effector/Regulator T Cell Ratios Impact Bone Regeneration. *Front. Immunol.* **10**, 1954 (2019).

129. Yu, M. *et al.* Regulatory T cells are expanded by Teriparatide treatment in humans and mediate intermittent PTH-induced bone anabolism in mice. *EMBO Rep.* **19**, 156–171 (2018).

130. Sander, L. E. *et al.* Hepatic acute-phase proteins control innate immune responses during infection by promoting myeloid-derived suppressor cell function. *J. Exp. Med.* **207**, 1453–1464 (2010).

131. Bronte, V. *et al.* Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. *Nat. Commun.* **7**, 12150 (2016).

132. Jia, W., Jackson-Cook, C. & Graf, M. R. Tumor-infiltrating, myeloid-derived suppressor cells inhibit T cell activity by nitric oxide production in an intracranial rat glioma + vaccination model. *J. Neuroimmunol.* **223**, 20–30 (2010).

133. Cuenca, A. G. *et al.* A paradoxical role for myeloid-derived suppressor cells in sepsis and trauma. *Mol. Med. Camb. Mass* **17**, 281–292 (2011).

134. Vantucci, C. E. *et al.* Development of systemic immune dysregulation in a rat trauma model of biomaterial-associated infection. *Biomaterials* **264**, 120405 (2021).

135. Ostrand-Rosenberg, S. & Sinha, P. Myeloid-derived suppressor cells: linking inflammation and cancer. *J. Immunol. Baltim. Md 1950* **182**, 4499–4506 (2009).

136. Fleming, V. *et al.* Targeting Myeloid-Derived Suppressor Cells to Bypass Tumor-Induced Immunosuppression. *Front. Immunol.* **9**, 398 (2018).

137. Hong, D. *et al.* AZD9150, a next-generation antisense oligonucleotide inhibitor of STAT3 with early evidence of clinical activity in lymphoma and lung cancer. *Sci. Transl. Med.* **7**, 314ra185 (2015).

138. Bitsch, R. *et al.* STAT3 inhibitor Napabucasin abrogates MDSC immunosuppressive capacity and prolongs survival of melanoma-bearing mice. *J. Immunother. Cancer* **10**, e004384 (2022).

139. Kortylewski, M. & Moreira, D. Myeloid cells as a target for oligonucleotide therapeutics: turning obstacles into opportunities. *Cancer Immunol. Immunother. CII* **66**, 979–988 (2017).

140. Ratner, M. Setback for JAK2 inhibitors. *Nat. Biotechnol.* **32**, 119 (2014).

141. Weed, D. T. *et al.* Tadalafil reduces myeloid-derived suppressor cells and regulatory T cells and promotes tumor immunity in patients with head and neck squamous cell carcinoma. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **21**, 39–48 (2015).

142. Jiang, H. H. *et al.* Sildenafil might impair maternal-fetal immunotolerance by suppressing myeloid-derived suppressor cells in mice. *J. Reprod. Immunol.* **142**, 103175 (2020).

143. Orillion, A. *et al.* Entinostat Neutralizes Myeloid-Derived Suppressor Cells and Enhances the Antitumor Effect of PD-1 Inhibition in Murine Models of Lung and Renal Cell Carcinoma. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **23**, 5187–5201 (2017).

144. Hossain, F. *et al.* Inhibition of Fatty Acid Oxidation Modulates Immunosuppressive Functions of Myeloid-Derived Suppressor Cells and Enhances Cancer Therapies. *Cancer Immunol. Res.* **3**, 1236–1247 (2015).

145. Banuelos, A. *et al.* CXCR2 inhibition in G-MDSCs enhances CD47 blockade for melanoma tumor cell clearance. *Proc. Natl. Acad. Sci. U. S. A.* **121**, e2318534121 (2024).

146. Hawila, E. *et al.* CCR5 Directs the Mobilization of CD11b+Gr1+Ly6Clow Polymorphonuclear Myeloid Cells from the Bone Marrow to the Blood to Support Tumor Development. *Cell Rep.* **21**, 2212–2222 (2017).

147. Heine, A. *et al.* The induction of human myeloid derived suppressor cells through hepatic stellate cells is dose-dependently inhibited by the tyrosine kinase inhibitors nilotinib, dasatinib and sorafenib, but not sunitinib. *Cancer Immunol. Immunother. CII* **65**, 273–282 (2016).

148. Eriksson, E., Wenthe, J., Irenaeus, S., Loskog, A. & Ullenhag, G. Gemcitabine reduces MDSCs, tregs and TGFβ-1 while restoring the teff/treg ratio in patients with pancreatic cancer. *J. Transl. Med.* **14**, 282 (2016).

149. Vincent, J. *et al.* 5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity. *Cancer Res.* **70**, 3052–3061 (2010).

150. Sinha, P. *et al.* Proinflammatory S100 proteins regulate the accumulation of myeloid-derived suppressor cells. *J. Immunol. Baltim. Md 1950* **181**, 4666–4675 (2008).

151. Nefedova, Y. *et al.* Mechanism of all-trans retinoic acid effect on tumor-associated myeloid-derived suppressor cells. *Cancer Res.* **67**, 11021–11028 (2007).

152. Haddad, R. & Saldanha-Araujo, F. Mechanisms of T-cell immunosuppression by mesenchymal stromal cells: what do we know so far? *BioMed Res. Int.* **2014**, 216806 (2014).

153. Ren, G. *et al.* Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell* **2**, 141–150 (2008).

154. Laing, A. G. *et al.* Mesenchymal stem cells inhibit T-cell function through conserved induction of cellular stress. *PloS One* **14**, e0213170 (2019).

155. Carreras-Planella, L., Monguió-Tortajada, M., Borràs, F. E. & Franquesa, M. Immunomodulatory Effect of MSC on B Cells Is Independent of Secreted Extracellular Vesicles. *Front. Immunol.* **10**, 1288 (2019).

156. Jung, Y.-J. *et al.* MSC-DC interactions: MSC inhibit maturation and migration of BM-derived DC. *Cytotherapy* **9**, 451–458 (2007).

157. Cho, D.-I. *et al.* Mesenchymal stem cells reciprocally regulate the M1/M2 balance in mouse bone marrow-derived macrophages. *Exp. Mol. Med.* **46**, e70 (2014).

158. Huang, S., Xu, L., Zhang, Y., Sun, Y. & Li, G. Systemic and Local Administration of Allogeneic Bone Marrow-Derived Mesenchymal Stem Cells Promotes Fracture Healing in Rats. *Cell Transplant.* **24**, 2643–2655 (2015).

159. Granero-Moltó, F. *et al.* Regenerative effects of transplanted mesenchymal stem cells in fracture healing. *Stem Cells Dayt. Ohio* **27**, 1887–1898 (2009).

160. Lin, W. *et al.* Mesenchymal stem cells homing to improve bone healing. *J. Orthop. Transl.* **9**, 19–27 (2017).

161. Al-Sharabi, N. *et al.* Osteogenic human MSC-derived extracellular vesicles regulate MSC activity and osteogenic differentiation and promote bone regeneration in a rat calvarial defect model. *Stem Cell Res. Ther.* **15**, 33 (2024).

162. Budoni, M. *et al.* The immunosuppressive effect of mesenchymal stromal cells on B lymphocytes is mediated by membrane vesicles. *Cell Transplant.* **22**, 369–379 (2013).

163. Mokarizadeh, A. *et al.* Microvesicles derived from mesenchymal stem cells: potent organelles for induction of tolerogenic signaling. *Immunol. Lett.* **147**, 47–54 (2012).

164. Nayer, B. *et al.* Local administration of regulatory T cells promotes tissue healing. *Nat. Commun.* **15**, 7863 (2024).

165. Wu, T. *et al.* Regulatory T cell-derived exosome mediated macrophages polarization for osteogenic differentiation in fracture repair. *J. Control. Release Off. J. Control. Release Soc.* **369**, 266–282 (2024).

166. Wang, Y. *et al.* M2 macrophage-derived exosomes promote diabetic fracture healing by acting as an immunomodulator. *Bioact. Mater.* **28**, 273–283 (2023).

167. Hachemi, Y. *et al.* Multimodal analyses of immune cells during bone repair identify macrophages as a therapeutic target in musculoskeletal trauma. *BioRxiv Prepr. Serv. Biol.* 2024.04.29.591608 (2024) doi:10.1101/2024.04.29.591608.

168. Reinke, S. *et al.* Terminally differentiated $CD8^+$ T cells negatively affect bone regeneration in humans. *Sci. Transl. Med.* **5**, 177ra36 (2013).

169. Waters, R. V. *et al.* Systemic corticosteroids inhibit bone healing in a rabbit ulnar osteotomy model. *Acta Orthop. Scand.* **71**, 316–321 (2000).

170. Gilley, R. S., Wallace, L. J., Bourgeault, C. A., Kidder, L. S. & Bechtold, J. E. OP-1 augments glucocorticoid-inhibited fracture healing in a rat fracture model. *Clin. Orthop.* **467**, 3104–3112 (2009).

171. Ho, M. L., Chang, J. K. & Wang, G. J. Antiinflammatory drug effects on bone repair and remodeling in rabbits. *Clin. Orthop.* 270–278 (1995).

172. Bhattacharyya, T., Levin, R., Vrahas, M. S. & Solomon, D. H. Nonsteroidal antiinflammatory drugs and nonunion of humeral shaft fractures. *Arthritis Rheum.* **53**, 364–367 (2005).

173. Giannoudis, P. V. *et al.* Nonunion of the femoral diaphysis. The influence of reaming and non-steroidal anti-inflammatory drugs. *J. Bone Joint Surg. Br.* **82**, 655–658 (2000).

174. Timmen, M. *et al.* Influence of antiTNF-alpha antibody treatment on fracture healing under chronic inflammation. *BMC Musculoskelet. Disord.* **15**, 184 (2014).

175. Kawakami, K. *et al.* Complications and features after joint surgery in rheumatoid arthritis patients treated with tumour necrosis factor-alpha blockers: perioperative interruption of tumour necrosis factor-alpha blockers decreases complications? *Rheumatol. Oxf. Engl.* **49**, 341–347 (2010).

176. Li, D., Li, X., Zhang, J., Tang, Z. & Tian, A. The immunomodulatory effect of IL-4 accelerates bone substitute material-mediated osteogenesis in aged rats via NLRP3 inflammasome inhibition. *Front. Immunol.* **14**, 1121549 (2023).

177. Lin, T. *et al.* Preconditioning of murine mesenchymal stem cells synergistically enhanced immunomodulation and osteogenesis. *Stem Cell Res. Ther.* **8**, 277 (2017).

178. Spiller, K. L. *et al.* Sequential delivery of immunomodulatory cytokines to facilitate the M1-to-M2 transition of macrophages and enhance vascularization of bone scaffolds. *Biomaterials* **37**, 194–207 (2015).

179. Vantucci, C. E. and Guyer T. *et al.* Systemic Immune Modulation Alters Local Bone Regeneration in a Delayed Treatment Composite Model of Non-Union Extremity Trauma. *Front. Surg.* **9**, 934773 (2022).

180. Zura, R. *et al.* Epidemiology of Fracture Nonunion in 18 Human Bones. *JAMA Surg.* **151**, e162775 (2016).

181. Wildemann, B. *et al.* Non-union bone fractures. *Nat. Rev. Dis. Primer* **7**, 57 (2021).

182. Low, E. E., Inkellis, E. & Morshed, S. Complications and revision amputation following trauma-related lower limb loss. *Injury* **48**, 364–370 (2017).

183. MacKenzie, E. J. *et al.* Long-term persistence of disability following severe lower-limb trauma. Results of a seven-year follow-up. *J. Bone Joint Surg. Am.* **87**, 1801–1809 (2005).

184. Caetano-Lopes, J., Canhão, H. & Fonseca, J. E. Osteoimmunology--the hidden immune regulation of bone. *Autoimmun. Rev.* **8**, 250–255 (2009).

185. Gentile, L. F. *et al.* Persistent inflammation and immunosuppression: a common syndrome and new horizon for surgical intensive care. *J. Trauma Acute Care Surg.* **72**, 1491–1501 (2012).

186. Rosenthal, M. D. & Moore, F. A. Persistent Inflammation, Immunosuppression, and Catabolism: Evolution of Multiple Organ Dysfunction. *Surg. Infect.* **17**, 167–172 (2016).

187. Huber-Lang, M., Lambris, J. D. & Ward, P. A. Innate immune responses to trauma. *Nat. Immunol.* **19**, 327–341 (2018).

188. Binkowska, A. M., Michalak, G. & Słotwiński, R. Current views on the mechanisms of immune responses to trauma and infection. *Cent.-Eur. J. Immunol.* **40**, 206–216 (2015).

189. Kimura, F., Shimizu, H., Yoshidome, H., Ohtsuka, M. & Miyazaki, M. Immunosuppression following surgical and traumatic injury. *Surg. Today* **40**, 793–808 (2010).

190. Vantucci, C. E., Roy, K. & Guldberg, R. E. Immunomodulatory strategies for immune dysregulation following severe musculoskeletal trauma. *J. Immunol. Regen. Med.* **2**, 21–35 (2018).

191. Lai, D., Qin, C. & Shu, Q. Myeloid-derived suppressor cells in sepsis. *BioMed Res. Int.* **2014**, 598654 (2014).

192. Zhu, X. *et al.* The central role of arginine catabolism in T-cell dysfunction and increased susceptibility to infection after physical injury. *Ann. Surg.* **259**, 171–178 (2014).

193. Condamine, T. & Gabrilovich, D. I. Molecular mechanisms regulating myeloid-derived suppressor cell differentiation and function. *Trends Immunol.* **32**, 19–25 (2011).

194. Cheng, A. *et al.* Impaired bone healing following treatment of established nonunion correlates with serum cytokine expression. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* **37**, 299–307 (2019).

195. Vantucci, C. E. *et al.* BMP-2 delivery strategy modulates local bone regeneration and systemic immune responses to complex extremity trauma. *Biomater. Sci.* **9**, 1668–1682 (2021).

196. Liu, J. *et al.* Bifunctional Janus Particles as Multivalent Synthetic Nanoparticle Antibodies (SNAbs) for Selective Depletion of Target Cells. *Nano Lett.* **21**, 875–886 (2021).

197. Bah, I., Kumbhare, A., Nguyen, L., McCall, C. E. & El Gazzar, M. IL-10 induces an immune repressor pathway in sepsis by promoting S100A9 nuclear localization and MDSC development. *Cell. Immunol.* **332**, 32–38 (2018).

198. Zhao, F. *et al.* S100A9 a new marker for monocytic human myeloid-derived suppressor cells. *Immunology* **136**, 176–183 (2012).

199. Cheng, P. *et al.* S100A9-induced overexpression of PD-1/PD-L1 contributes to ineffective hematopoiesis in myelodysplastic syndromes. *Leukemia* **33**, 2034–2046 (2019).

200. Silvin, A. *et al.* Elevated Calprotectin and Abnormal Myeloid Cell Subsets Discriminate Severe from Mild COVID-19. *Cell* **182**, 1401-1418.e18 (2020).

201. Wang, S. *et al.* S100A8/A9 in Inflammation. *Front. Immunol.* **9**, 1298 (2018).

202. Kolambkar, Y. M. *et al.* An alginate-based hybrid system for growth factor delivery in the functional repair of large bone defects. *Biomaterials* **32**, 65–74 (2011).

203. Sadtler, K. *et al.* Developing a pro-regenerative biomaterial scaffold microenvironment requires T helper 2 cells. *Science* **352**, 366–370 (2016).

204. Spitzer, M. H. *et al.* Systemic Immunity Is Required for Effective Cancer Immunotherapy. *Cell* **168**, 487-502.e15 (2017).

205. Raber, P., Ochoa, A. C. & Rodríguez, P. C. Metabolism of L-arginine by myeloid-derived suppressor cells in cancer: mechanisms of T cell suppression and therapeutic perspectives. *Immunol. Invest.* **41**, 614–634 (2012).

206. Elkabets, M. *et al.* IL-1β regulates a novel myeloid-derived suppressor cell subset that impairs NK cell development and function. *Eur. J. Immunol.* **40**, 3347–3357 (2010).

207. Tu, S. *et al.* Overexpression of interleukin-1beta induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice. *Cancer Cell* **14**, 408–419 (2008).

208. Gazon, H., Barbeau, B., Mesnard, J.-M. & Peloponese, J.-M. Hijacking of the AP-1 Signaling Pathway during Development of ATL. *Front. Microbiol.* **8**, 2686 (2017).

209. Wei, F., Zhou, Y., Wang, J., Liu, C. & Xiao, Y. The Immunomodulatory Role of BMP-2 on Macrophages to Accelerate Osteogenesis. *Tissue Eng. Part A* **24**, 584–594 (2018).

210. Lykissas, M. & Gkiatas, I. Use of recombinant human bone morphogenetic protein-2 in spine surgery. *World J. Orthop.* **8**, 531–535 (2017).

211. Conway, J. D., Shabtai, L., Bauernschub, A. & Specht, S. C. BMP-7 versus BMP-2 for the treatment of long bone nonunion. *Orthopedics* **37**, e1049-1057 (2014).

212. Wagner, R. K. *et al.* Long-term patient reported outcomes after revision surgery for lower extremity nonunion: A retrospective cohort study. *Injury* **55**, 111779 (2024).

213. Schmidt-Bleek, K. *et al.* Inflammatory phase of bone healing initiates the regenerative healing cascade. *Cell Tissue Res.* **347**, 567–573 (2012).

214. Ward, N. S., Casserly, B. & Ayala, A. The compensatory anti-inflammatory response syndrome (CARS) in critically ill patients. *Clin. Chest Med.* **29**, 617–625, viii (2008).

215. Goris, R. J. MODS/SIRS: result of an overwhelming inflammatory response? *World J. Surg.* **20**, 418–421 (1996).

216. Hüsecken, Y. *et al.* MDSCs are induced after experimental blunt chest trauma and subsequently alter antigen-specific T cell responses. *Sci. Rep.* **7**, 12808 (2017).

217. Raber, P. L. *et al.* Subpopulations of myeloid-derived suppressor cells impair T cell responses through independent nitric oxide-related pathways. *Int. J. Cancer* **134**, 2853–2864 (2014).

218. Schlecker, E. *et al.* Tumor-infiltrating monocytic myeloid-derived suppressor cells mediate CCR5-dependent recruitment of regulatory T cells favoring tumor growth. *J. Immunol. Baltim. Md 1950* **189**, 5602–5611 (2012).

219. Corzo, C. A. *et al.* Mechanism regulating reactive oxygen species in tumor-induced myeloid-derived suppressor cells. *J. Immunol. Baltim. Md 1950* **182**, 5693–5701 (2009).

220. Park, M.-J. *et al.* Interleukin-10 produced by myeloid-derived suppressor cells is critical for the induction of Tregs and attenuation of rheumatoid inflammation in mice. *Sci. Rep.* **8**, 3753 (2018).

221. Zhang, H. *et al.* Critical Role of Myeloid-Derived Suppressor Cells in Tumor-Induced Liver Immune Suppression through Inhibition of NKT Cell Function. *Front. Immunol.* **8**, 129 (2017).

222. Rodriguez, P. C. *et al.* Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. *Cancer Res.* **64**, 5839–5849 (2004).

223. Uhel, F. *et al.* Early Expansion of Circulating Granulocytic Myeloid-derived Suppressor Cells Predicts Development of Nosocomial Infections in Patients with Sepsis. *Am. J. Respir. Crit. Care Med.* **196**, 315–327 (2017).

224. Wu, Y., Yi, M., Niu, M., Mei, Q. & Wu, K. Myeloid-derived suppressor cells: an emerging target for anticancer immunotherapy. *Mol. Cancer* **21**, 184 (2022).

225. Tobin, R. P. *et al.* Targeting MDSC Differentiation Using ATRA: A Phase I/II Clinical Trial Combining Pembrolizumab and All-Trans Retinoic Acid for Metastatic Melanoma. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **29**, 1209–1219 (2023).

226. Tobin, R. P. *et al.* Targeting myeloid-derived suppressor cells using all-trans retinoic acid in melanoma patients treated with Ipilimumab. *Int. Immunopharmacol.* **63**, 282–291 (2018).

227. Kusmartsev, S. & Gabrilovich, D. I. Inhibition of myeloid cell differentiation in cancer: the role of reactive oxygen species. *J. Leukoc. Biol.* **74**, 186–196 (2003).

228. Liang, Y., Wang, W., Zhu, X., Yu, M. & Zhou, C. Inhibition of myeloid-derived suppressive cell function with all-trans retinoic acid enhanced anti-PD-L1 efficacy in cervical cancer. *Sci. Rep.* **12**, 9619 (2022).

229. Willett, N. J. *et al.* Attenuated human bone morphogenetic protein-2-mediated bone regeneration in a rat model of composite bone and muscle injury. *Tissue Eng. Part C Methods* **19**, 316–325 (2013).

230. Kang, T. H. *et al.* Treatment of tumors with vitamin E suppresses myeloid derived suppressor cells and enhances CD8+ T cell-mediated antitumor effects. *PloS One* **9**, e103562 (2014).

231. Highfill, S. L. *et al.* Disruption of CXCR2-mediated MDSC tumor trafficking enhances anti-PD1 efficacy. *Sci. Transl. Med.* **6**, 237ra67 (2014).

232. Sukhatme, V. P. & Husain, Z. Cancer therapy targeting tetraspanin 33 (tspan33) in myeloid derived suppressor cells. (2016).

233. Ruehle, M. A. *et al.* Effects of BMP-2 dose and delivery of microvascular fragments on healing of bone defects with concomitant volumetric muscle loss. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* **37**, 553–561 (2019).

234. Raychaudhuri, B. *et al.* Myeloid-derived suppressor cell accumulation and function in patients with newly diagnosed glioblastoma. *Neuro-Oncol.* **13**, 591–599 (2011).

235. Zhao, F. *et al.* Increase in frequency of myeloid-derived suppressor cells in mice with spontaneous pancreatic carcinoma. *Immunology* **128**, 141–149 (2009).

236. Vellozo, N. S. *et al.* All-Trans Retinoic Acid Promotes an M1- to M2-Phenotype Shift and Inhibits Macrophage-Mediated Immunity to Leishmania major. *Front. Immunol.* **8**, 1560 (2017).

237. Youn, J.-I., Nagaraj, S., Collazo, M. & Gabrilovich, D. I. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. *J. Immunol. Baltim. Md 1950* **181**, 5791–5802 (2008).

238. Wu, T. *et al.* A distinct 'repair' role of regulatory T cells in fracture healing. *Front. Med.* **18**, 516–537 (2024).

239. Xing, L. & Remick, D. G. Mechanisms of dimethyl sulfoxide augmentation of IL-1 beta production. *J. Immunol. Baltim. Md 1950* **174**, 6195–6202 (2005).

240. Elisia, I. *et al.* DMSO Represses Inflammatory Cytokine Production from Human Blood Cells and Reduces Autoimmune Arthritis. *PloS One* **11**, e0152538 (2016).

241. Rao, A. *et al.* All-trans retinoic acid induces durable tumor immunity in IDH-mutant gliomas by rescuing transcriptional repression of the CRBP1-retinoic acid axis. *BioRxiv Prepr. Serv. Biol.* 2024.04.09.588752 (2024) doi:10.1101/2024.04.09.588752.

242. Sanz-Sánchez, I., Sanz-Martín, I., Ortiz-Vigón, A., Molina, A. & Sanz, M. Complications in bone-grafting procedures: Classification and management. *Periodontol. 2000* **88**, 86–102 (2022).

243. Summers, B. N. & Eisenstein, S. M. Donor site pain from the ilium. A complication of lumbar spine fusion. *J. Bone Joint Surg. Br.* **71**, 677–680 (1989).

244. Becker, S. T., Warnke, P. H., Behrens, E. & Wiltfang, J. Morbidity after iliac crest bone graft harvesting over an anterior versus posterior approach. *J. Oral Maxillofac. Surg. Off. J. Am. Assoc. Oral Maxillofac. Surg.* **69**, 48–53 (2011).

245. McKay, W. F., Peckham, S. M. & Badura, J. M. A comprehensive clinical review of recombinant human bone morphogenetic protein-2 (INFUSE Bone Graft). *Int. Orthop.* **31**, 729–734 (2007).

246. Shahlaie, K. & Kim, K. D. Occipitocervical fusion using recombinant human bone morphogenetic protein-2: adverse effects due to tissue swelling and seroma. *Spine* **33**, 2361–2366 (2008).

247. Wong, D. A., Kumar, A., Jatana, S., Ghiselli, G. & Wong, K. Neurologic impairment from ectopic bone in the lumbar canal: a potential complication of off-label PLIF/TLIF use of bone morphogenetic protein-2 (BMP-2). *Spine J. Off. J. North Am. Spine Soc.* **8**, 1011–1018 (2008).

248. Deutsch, H. High-dose bone morphogenetic protein-induced ectopic abdomen bone growth. *Spine J. Off. J. North Am. Spine Soc.* **10**, e1-4 (2010).

249. Sottile, V. & Seuwen, K. Bone morphogenetic protein-2 stimulates adipogenic differentiation of mesenchymal precursor cells in synergy with BRL 49653 (rosiglitazone). *FEBS Lett.* **475**, 201–204 (2000).

250. Newman, H., Shih, Y. V. & Varghese, S. Resolution of inflammation in bone regeneration: From understandings to therapeutic applications. *Biomaterials* **277**, 121114 (2021).

251. Lu, L. Y. *et al.* Pro-inflammatory M1 macrophages promote Osteogenesis by mesenchymal stem cells via the COX-2-prostaglandin E2 pathway. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* **35**, 2378–2385 (2017).

252. Horwood, N. J. Macrophage Polarization and Bone Formation: A review. *Clin. Rev. Allergy Immunol.* **51**, 79–86 (2016).

253. Nam, D. *et al.* T-lymphocytes enable osteoblast maturation via IL-17F during the early phase of fracture repair. *PloS One* **7**, e40044 (2012).

254. Li, Y. *et al.* B cells and T cells are critical for the preservation of bone homeostasis and attainment of peak bone mass in vivo. *Blood* **109**, 3839–3848 (2007).

255. Cheng, L., Xu, J., Chai, Y., Want, C. & Han, P. Glucocorticoid release is involved in myeloid-derived suppressor cell accumulation in the spleen following polytrauma. *Int. J. Clin. Exp. Med.* **9**, 17850–17858 (2016).

256. Qu, P., Wang, L.-Z. & Lin, P. C. Expansion and functions of myeloid-derived suppressor cells in the tumor microenvironment. *Cancer Lett.* **380**, 253–256 (2016).

257. De Zuani, M. *et al.* Human myeloid-derived suppressor cell expansion during sepsis is revealed by unsupervised clustering of flow cytometric data. *Eur. J. Immunol.* **51**, 1785–1791 (2021).

258. Maniar, D. *et al.* Single-cell transcriptomic analysis identifies systemic immunosuppressive myeloid cells and local monocytes/macrophages as key regulators in polytrauma-induced immune dysregulation. *Bone Res*. **13**, 69 (2025).

259. Hoff, P. *et al.* Immunological characterization of the early human fracture hematoma. *Immunol. Res.* **64**, 1195–1206 (2016).

260. Muire, P. J., Mangum, L. H. & Wenke, J. C. Time Course of Immune Response and Immunomodulation During Normal and Delayed Healing of Musculoskeletal Wounds. *Front. Immunol.* **11**, 1056 (2020).

261. Vogl, T. *et al.* Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nat. Med.* **13**, 1042–1049 (2007).

262. Adusei, K. M. *et al.* Development of a High-Color Flow Cytometry Panel for Immunologic Analysis of Tissue Injury and Reconstruction in a Rat Model. *Cells Tissues Organs* **212**, 84–95 (2023).

263. Ferrer, G. *et al.* Myeloid-derived suppressor cell subtypes differentially influence T-cell function, T-helper subset differentiation, and clinical course in CLL. *Leukemia* **35**, 3163–3175 (2021).

264. Lee, H. W., Gangadaran, P., Kalimuthu, S. & Ahn, B.-C. Advances in Molecular Imaging Strategies for In Vivo Tracking of Immune Cells. *BioMed Res. Int.* **2016**, 1946585 (2016).

265. Roberts, I. *et al.* Effect of intravenous corticosteroids on death within 14 days in 10008 adults with clinically significant head injury (MRC CRASH trial): randomised placebo-controlled trial. *Lancet Lond. Engl.* **364**, 1321–1328 (2004).

266. Franceschi, C., Garagnani, P., Parini, P., Giuliani, C. & Santoro, A. Inflammaging: a new immune-metabolic viewpoint for age-related diseases. *Nat. Rev. Endocrinol.* **14**, 576–590 (2018).

267. Klein, S. L. & Flanagan, K. L. Sex differences in immune responses. *Nat. Rev. Immunol.* **16**, 626–638 (2016).

268. Shao, T. *et al.* Physical Activity and Nutritional Influence on Immune Function: An Important Strategy to Improve Immunity and Health Status. *Front. Physiol.* **12**, 751374 (2021).

269. Guyer, T., Gill, D., Leguineche, K.E., Link, K., Jones, K.A., Williams, K.E., Strait, L.K., O'Neill, K.L., Hastings, M.A., Lin, A., Maniar, D., Roy, K., & Guldberg, R.E. All-trans Retinoic Acid Modulates Circulating Immune Cells in a Preclinical Model of Musculoskeletal Trauma. *Manuscript in Preparation.*

270. Guyer, T., Gill, D., Link, K., Strait, L.K., Leguineche, K.E., O'Neill, K.L., & Guldberg, R.E. Spectral Flow Cytometry Reveals Dysregulated Immune Cell Populations in Local Tissues Following Severe Polytrauma. *Manuscript in Preparation.*