

INTEGRATING REHABILITATION MECHANICS AND IMMUNE
PROFILING TO INFORM OUTCOMES IN
ORTHOPAEDIC TRAUMA

by

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A THESIS

Presented to the Department of Human Physiology
and the Robert D. Clark Honors College
in partial fulfillment of the requirements for the degree of
Bachelor of Science

March 2026

An Abstract of the Thesis of

Auveen Hajarizadeh for the degree of Bachelor of Science
in the Department of Human Physiology to be taken March 2026

Title: Integrating Rehabilitation Mechanics and Immune Profiling to Inform Outcomes in
Orthopaedic Trauma

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Fracture healing is a complex, multiscale process governed by tightly coordinated interactions between mechanical stimuli and biological responses. Despite bone's intrinsic regenerative capacity, approximately 5-10% of fractures progress to nonunion, often resulting in prolonged disability, repeated surgical intervention, and substantial healthcare costs. Current clinical approaches rely primarily on radiographic assessment and nonspecific inflammatory markers, which lack sensitivity for early detection of impaired healing or infection. This diagnostic delay limits opportunities for timely intervention and highlights the need for prognostic tools capable of identifying healing trajectories at earlier stages. The objective of this thesis was to leverage predictive modeling and systemic immune profiling to identify mechanistic determinants of healing outcomes and develop translational frameworks for prognostic assessment in orthopaedic trauma.

This work integrates preclinical and clinical investigations across three interconnected aims. First, rehabilitation parameters were systematically evaluated in a rat segmental bone defect model to determine how mechanical loading profiles influence regeneration. Continuous activity monitoring combined with nonlinear predictive modeling revealed that healing outcomes depend not solely on cumulative mechanical load, but on the temporal structure of loading, with

rest emerging as a critical determinant. Genetic programming identified nonlinear relationships between activity parameters and bone formation, demonstrating the existence of an optimal rehabilitation zone characterized by moderate loading and sufficient recovery.

Second, longitudinal immune profiling was performed using flow cytometry to characterize systemic immune dynamics following injury and rehabilitation. Distinct temporal patterns in circulating immune cell populations—including monocytes, myeloid-derived suppressor cells, and T cell subsets—were observed across healing trajectories. Rehabilitation protocols incorporating structured rest were associated with immune profiles indicative of more favorable regenerative outcomes, suggesting that mechanical loading modulates systemic immune responses that may influence healing success.

Finally, these mechanistic insights were translated to a clinical population of orthopaedic trauma patients. Peripheral blood samples were analyzed using high-dimensional spectral flow cytometry to quantify immune cell populations longitudinally. Multivariate modeling using sparse partial least squares discriminant analysis identified coordinated immune signatures capable of distinguishing patients who developed fracture-related infection from those who healed successfully. These findings demonstrate the feasibility of using systemic immune profiling and predictive modeling to identify prognostic biomarkers of healing outcomes.

Collectively, this thesis establishes a translational framework integrating rehabilitation mechanics, immune biology, and multivariate modeling to improve prognostic assessment in orthopaedic trauma. By identifying immune signatures and mechanical parameters associated with healing outcomes, this work provides foundational evidence supporting the development of personalized rehabilitation strategies and early diagnostic tools aimed at improving patient recovery and reducing complications.

ACKNOWLEDGEMENTS

I would first like to express my deep gratitude to the Guldberg Lab for providing not only an incredible research environment, but also a community where I have made lasting friendships. I am especially thankful to Dr. Kylie Williams and Kelly Leguineche, whose mentorship has been invaluable. They have taught me so much, constantly pushed me to grow, and generously offered their time and support at every step. This experience and culminating thesis project would not have been possible without them.

I am also deeply appreciative of Dr. Robert Guldberg for his unwavering support of my development as a researcher, from thoughtful feedback on my work to opportunities to attend conferences and engage with the broader scientific community. My experience in the Guldberg Lab has unequivocally been the most cathartic and consequential experience I have had in college, and I feel forever indebted to Dr. Guldberg for allowing me to learn and grow so much.

I would also like to thank my Honors College thesis advisor, Dr. Tobin Hansen, for his guidance and support throughout this process, and for being an exceptional professor and mentor during my time at the Honors College. Working with him both in class and on projects beyond the classroom has had a lasting impact on my growth as a student and thinker.

I would also like to thank Karl Reasoner and the Knight Campus Undergraduate Scholar (KCUS) and Hui Undergraduate Research Scholar (HURS) communities. These programs provided unique spaces to grow professionally, gain insightful feedback, and connect with peers who have become close friends and collaborators.

I am also immensely grateful to the Accelerated General Biology teaching staff, especially Dr. Laurel Pfeifer-Meister, whose support, mentorship, and inspiring teaching made

her the best professor I've had in the sciences here at the UO. I've learned so much from being part of her teaching team.

Finally, I want to extend my warmest thanks to my family and close friends. To my parents, Farin and Saied, thank you for teaching me the importance of science at an early age, remaining so dedicated to my success throughout my entire life, and being my biggest cheerleaders. To my brother, Armaan, thank you for being my closest ally and for standing beside me through every step of this journey. To my late Maman Fakhry, thank you for being a rock in all of our lives and for instilling in our family a deep and unwavering commitment to education. And to my closest friends, thank you for being there through my countless late nights. I have so much to be grateful for and feel incredibly lucky to have you all in my life!

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CHAPTER 1: INTRODUCTION

Despite bone's endogenous regenerative capacity, severe extremity trauma frequently results in high complication rates and limited functional recovery. In the United States, around 6 million extremity fractures occur annually, with 5-10% progressing to nonunions [1]. While substantial progress has been made using preclinical models to understand the cellular and molecular responses to bone injury, there remains a pressing need to translate these findings into tools that can improve patient outcomes. Motivated by this gap, we designed a two-pronged research strategy that first leverages animal models to investigate how rehabilitation strategies modulate bone repair and systemic immunity, and then applies these mechanistic insights to analyze human clinical samples. This integrative approach allowed us to identify immune signatures relevant to healing outcomes and to explore their diagnostic potential in patients at risk for nonunion. In this work, we explored this through three specific aims.

1.1 Specific Aim 1: Determine the role of different rehabilitation regimens on enhancing bone regeneration in an animal model.

Despite decades of research demonstrating that bone is a mechanoresponsive tissue, clinical rehabilitation protocols following fracture remain largely subjective, often relying on patient-reported pain, static imaging, and provider intuition. This subjectivity leads to conservative weight-bearing recommendations that are not tailored to individual healing responses and may limit optimal recovery. To address this, we employed a segmental bone defect model in rodents, using internally fixed bilateral femur injuries to investigate the multifactorial effects of different rehabilitation profiles on bone regeneration. We tracked six distinct rehabilitation parameters—running distance, duration, velocity, bout frequency, bout

duration, and daily rest—to generate subject-specific activity profiles. Initial nonlinear modeling revealed rest as a critical determinant of healing, which prompted additional studies designed to isolate and evaluate the effects of structured rest on bone regeneration. Our analyses revealed that bone formation follows a “Goldilocks” phenomenon: moderate levels of activity paired with sufficient rest promoted bone bridging, while both excessive and insufficient loading impaired healing. These findings underscore the inadequacy of one-size-fits-all protocols and support a shift toward individualized rehabilitation strategies that balance mechanical loading with adequate recovery time.

1.2 Specific Aim 2: Characterize major cell populations in an animal model of traumatic injury to define the cellular landscape underlying the injury response.

In the clinical setting, nonunion is often diagnosed only after 6 to 9 months of failed healing, leaving patients without early intervention strategies [2]. This delay stems from a lack of early-stage biomarkers that can distinguish normal healing from trajectories likely to result in nonunion. To begin addressing this challenge, we used an animal model to explore the systemic immune response to traumatic bone injury under varying rehabilitation conditions. Using flow cytometry, we profiled key immune cell populations—T cells, helper T cells, cytotoxic T cells, monocytes, and MDSCs—longitudinally after injury and rehabilitation. We found that certain rehabilitation regimens, particularly those incorporating intermittent rest, not only improved bone formation but also accelerated the normalization of immune signatures associated with healthy healing. These immune shifts—such as early reductions in circulating monocytes and MDSCs—preceded radiographic signs of union, suggesting that the systemic immune landscape may contain early indicators of healing success or failure. This foundational work provides

critical insight for developing future diagnostic tools aimed at predicting nonunion risk much earlier in the clinical timeline.

1.3 Specific Aim 3: Perform comprehensive cellular profiling to identify immune signatures associated with fracture-related infection.

Given the prevalence of infection as a major driver of delayed healing and nonunion, we extended our work to clinical samples to investigate whether systemic immune signatures could distinguish infection outcomes in orthopaedic trauma patients. Building on preclinical findings demonstrating systemic immune dysregulation in the presence of local infection, peripheral blood was collected longitudinally from patients with open tibia fractures. Spectral flow cytometry was used to quantify over 16 circulating immune cell populations relevant to innate and adaptive immune function. Multivariate modeling using sparse partial least squares discriminant analysis (sPLS-DA) was then applied to identify coordinated immune signatures that distinguish patients who developed infection from those who healed successfully. This approach aims to overcome the limitations of current diagnostics (e.g., CRP, WBC count) by providing early, noninvasive infection detection tools, which could support timely clinical intervention and improve patient outcomes.

CHAPTER 2: BACKGROUND

2.1 Clinical Burden of Bone Injury and Nonunion

Nonunion represents a significant clinical failure in which a fracture fails to heal within the expected timeframe, often requiring secondary interventions to restore structural integrity and limb function [2]. Long bone fractures are particularly prone to this complication due to size, load-bearing role, and frequent involvement in high-energy trauma. Although surgical fixation is often used to stabilize complex injuries, such as those involving comminution or segmental bone loss, mechanical stabilization alone does not guarantee successful regeneration [3]. Biological processes such as revascularization, progenitor cell recruitment, and inflammation must occur in a coordinated sequence, and disruptions at any stage—whether due to mechanical instability, systemic inflammation, or infection—can derail the regenerative process (Figure 1) [4].

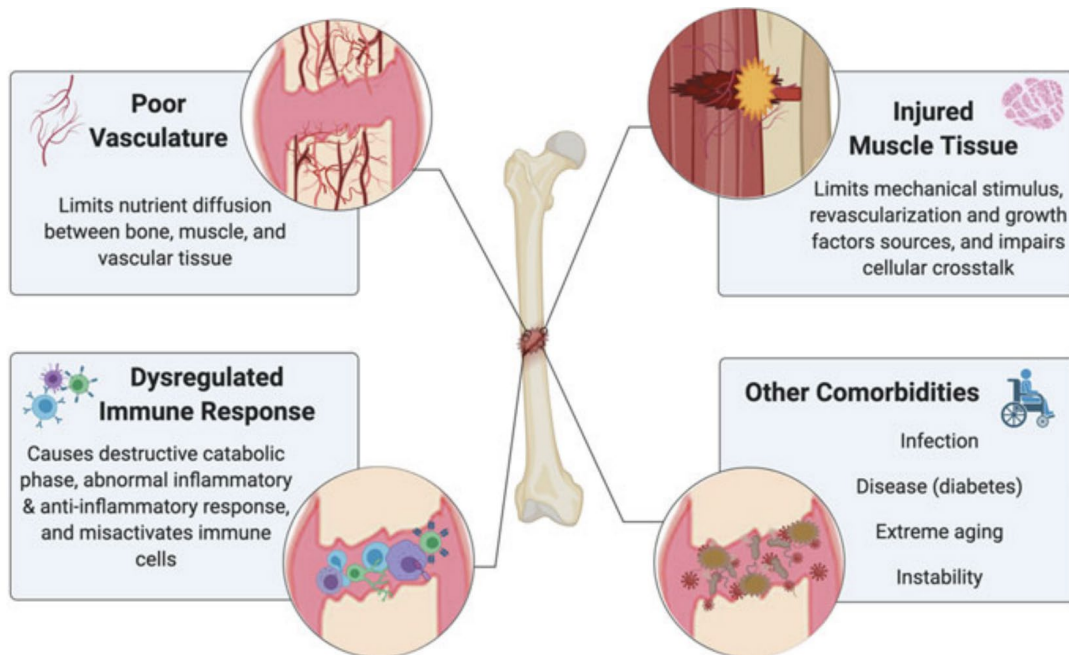


Figure 1. Factors that contribute to bone nonunion or healing complications following complex bone injuries. Figure reprinted from *Nash et al. (2022)* [4].

The consequences of delayed healing are profound. Patients experiencing nonunion often face months to years of pain, immobility, psychological stress, and loss of independence. Functionally, nonunion can lead to limb shortening, malalignment, or permanent disability. Financially, these cases contribute to substantial healthcare expenditures. Estimates suggest that fracture nonunion alone, without reoperation or infection, can cost a patient up to \$67,352, excluding lost wages, rehabilitation, and long-term disability [5].

Infection further increases both the likelihood and severity of nonunion. Fracture-related infection has been identified as a contributing factor in approximately 38% of cases progressing to nonunion [6]. These infections often present subclinically in early stages, evading detection until overt symptoms appear weeks or months postoperatively. Diagnosis is commonly delayed due to the low specificity of standard blood biomarkers such as white blood cell count (WBC), C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR), as well as the invasive nature of confirmatory tests like deep tissue sampling [7]. By the time clinical signs of infection manifest, significant tissue damage may already have occurred, often necessitating revision surgery, hardware removal, and prolonged antibiotic therapy.

Together, these challenges reflect a fundamental limitation in current fracture management: healing outcomes are governed not by mechanical or biological factors alone, but by their dynamic interaction. Mechanical loading shapes immune responses, and immune signaling influences tissue regeneration. Yet these domains remain largely studied and applied independently. Bridging this gap requires an integrated framework that simultaneously considers rehabilitation mechanics, systemic immune dynamics, and early predictive biomarkers.

2.2 Limitations of Current Rehabilitation Protocols

Although fracture stabilization is a cornerstone of surgical repair, postoperative rehabilitation also plays a critical role in functional recovery. Yet, despite the well-established mechanosensitivity of bone, clinical rehabilitation protocols remain largely conservative, non-standardized, and driven more by clinical intuition than by mechanobiological principles [8, 9]. Most commonly, patients are instructed to avoid weight-bearing or high-intensity activity for several weeks following injury, with the pace of reintroduction guided by subjective factors such as pain tolerance or visual inspection of radiographs. As a result, the rehabilitation process is often reactive rather than proactive, emphasizing complication avoidance rather than biological optimization.

This clinical practice stems in part from a lack of tools to objectively monitor and prescribe loading in real time. While clinicians acknowledge the importance of mechanical stimulation for healing, they are rarely able to quantify the dose, duration, or pattern of loading that a patient actually experiences. Consequently, loading prescriptions remain general rather than personalized, and they fail to account for the variability introduced by different injury types, fixation strategies, or patient activity levels.

Preclinical studies have consistently shown that bone regeneration is enhanced by appropriately timed mechanical loading [10, 11, 12]. For example, dynamic loading introduced after the inflammatory phase has been shown to improve callus formation and mineralization, while premature or excessive loading can destabilize the fracture site and impair angiogenesis [10]. However, most of these studies have manipulated loading in a simplified manner, such as varying fixation stiffness or using a single treadmill protocol, without addressing the more complex and multifactorial nature of clinical rehabilitation [13]. Real-world rehabilitation

involves a spectrum of variables, including not only load magnitude and timing but also exercise frequency, rest intervals, and recovery duration. These variables can interact in nonlinear ways, making it difficult to generalize results from controlled models to heterogeneous clinical populations.

As a result, this often complicates researchers' ability to draw mechanistic conclusions. In many studies, variables such as load intensity, duration, frequency, and timing are adjusted together, making it difficult to disentangle their individual effects on bone healing. This lack of control reduces the translational clarity of the findings, as clinicians cannot confidently identify which aspect of a rehabilitation regimen is driving observed outcomes. To advance the field, there is a growing need for carefully designed studies that isolate specific parameters, such as rest intervals or incline progression, to determine their independent and interactive effects on regeneration.

In parallel, most immunological studies of bone healing in animal models have been conducted in sedentary conditions to eliminate confounding mechanical variables [14, 15]. While this approach has been useful for establishing baseline immune responses to injury, it fails to account for how rehabilitation itself might modulate systemic immune profiles. Given that mechanical loading can influence inflammation and immune cell dynamics, preclinical models that combine physical activity with immune profiling are urgently needed. Such models would more accurately reflect the post-injury environment encountered by patients and could offer critical insight into the mechanisms by which rehabilitation protocols influence both local and systemic healing trajectories.

Together, these findings point to a fundamental mismatch between our growing understanding of how mechanical signals shape bone healing and the static, nonspecific

rehabilitation strategies currently used in clinical settings. The failure to individualize rehabilitation based on the biological status of the injury and the mechanical environment of the fixation construct likely contributes to the high variability in patient outcomes. There remains a critical need for rehabilitation frameworks that integrate time-sensitive mechanical principles and are tailored to the unique physiological demands of healing bone.

2.3 Mechanobiology of Bone Healing

Bone is a highly mechanosensitive tissue, and its capacity to regenerate after injury is closely tied to mechanical cues [8]. During healing, cells within the fracture environment—such as osteoblasts, osteocytes, and mesenchymal progenitors—respond to physical forces by activating signaling pathways that regulate inflammation, differentiation, angiogenesis, and matrix deposition [16]. These mechanotransductive processes are especially responsive to strain magnitude, timing, and rest.

Moderate levels of strain have been shown to enhance callus formation and ossification, while excessive loading can disrupt revascularization and destabilize the healing tissue, resulting in fibrous nonunion [10]. Conversely, insufficient strain, often due to over-rigid fixation or prolonged immobilization, can suppress osteogenic signaling [13]. The timing of load introduction is also critical: early loading during the inflammatory phase can be detrimental, whereas loading during the soft callus phase tends to promote repair.

Rest between loading bouts has emerged as an important yet often overlooked factor [17, 18, 19]. Evidence suggests that rest may enhance the responsiveness of osteogenic cells and improves outcomes by preventing desensitization to mechanical signals. However, most rehabilitation protocols, both clinical and preclinical, focus on total loading duration without accounting for rest intervals or activity patterning.

These mechanical parameters also influence key biological processes underlying regeneration. Mechanical stimulation promotes angiogenesis, supports progenitor cell recruitment, and guides the organization of the callus [4]. Together, these effects determine whether a fracture successfully bridges or progresses to nonunion. Despite decades of research, most rehabilitation protocols fail to leverage this knowledge in a systematic, individualized way. To address this critical gap, Aim 1 systematically isolates key mechanical variables—including load timing, intensity, and rest intervals—to determine their independent and interactive effects on bone regeneration.

2.4 Systemic Immune Response to Musculoskeletal Injury

Bone healing is not only a mechanical process but also requires a tightly regulated immune response that progresses from early inflammation and pathogen clearance to anti-inflammatory and pro-regenerative signaling programs that promote tissue repair [14, 15, 20]. Following injury, the body initiates an acute immune response characterized by rapid recruitment of innate immune cells, including neutrophils and monocytes, which clear debris, eliminate potential pathogens, and secrete pro-inflammatory cytokines such as interleukin-6 (IL-6) that amplify early inflammatory signaling [21]. As healing progresses, adaptive immune cells, including T cells and B cells, contribute to resolution of inflammation and support tissue regeneration through anti-inflammatory and pro-regenerative signaling pathways mediated in part by cytokines such as interleukin-10 (IL-10) and members of the transforming growth factor- β (TGF- β) and bone morphogenetic protein (BMP) superfamily [22, 23, 24]. These signaling molecules regulate the transition from inflammation to regeneration by promoting angiogenesis, matrix deposition, and coordinated tissue remodeling, ultimately guiding successful bone repair.

Disruption of this immune sequence can have profound consequences. Dysregulated or prolonged inflammation has been linked to impaired angiogenesis, altered progenitor cell recruitment, and ultimately nonunion [14]. Recent preclinical studies have shown that systemic immune profiles reflect these local disruptions: elevated levels of immunosuppressive cells such as myeloid-derived suppressor cells (MDSCs) and reduced populations of T cells have been observed in animals and patients with poor healing outcomes [14, 15]. These circulating markers may not only reflect local healing status but also actively influence regenerative processes through systemic signaling.

Despite this emerging evidence, our understanding of how specific immune cell populations evolve during healing, and how they differ between successful and failed repair, is limited. Most studies characterizing immune responses to fracture have been conducted in sedentary animal models, which do not capture the effects of rehabilitation or mechanical stimulation on immune dynamics. Additionally, although prior studies have characterized immune responses at multiple early timepoints following fracture [25, 26], many rely on limited immune panels, focus primarily on local tissue environments, or do not capture longitudinal systemic immune dynamics in the context of rehabilitation or healing outcomes. This lack of early, mechanistically relevant biomarkers represents a major gap in the field. If immune signatures could be identified during the early post-injury period, they could serve as prognostic indicators to identify patients at risk of nonunion long before clinical symptoms appear. Such insights would open new avenues for intervention, whether by modifying rehabilitation protocols, administering targeted therapies, or intensifying monitoring in high-risk cases.

Aim 2 directly addresses this need by characterizing the systemic immune landscape in a controlled preclinical model of bone injury. By focusing on early and longitudinal immune

dynamics in response to trauma, this work lays the foundation for identifying the cellular profiles that distinguish successful healing from failure—an essential step toward building immune-informed strategies for musculoskeletal recovery.

2.5 Barriers to Early Detection of Fracture-Related Complications

While the clinical course of bone healing is routinely monitored through radiographic imaging and basic bloodwork, current diagnostic tools remain inadequate for detecting complications such as nonunion and infection in their earliest stages. Standard laboratory markers—such as C-reactive protein (CRP), white blood cell (WBC) count, and erythrocyte sedimentation rate (ESR)—are widely used to monitor systemic inflammation [7]. However, these markers lack both specificity and sensitivity for fracture-related complications. For example, elevated CRP and WBC levels may occur due to surgical trauma, sterile inflammation, or other comorbid conditions, while normal values do not reliably exclude infection or delayed healing. As a result, clinicians most commonly do not confirm infection until 6 to 12 weeks postoperatively, by which point opportunities for early intervention may have been lost. Nonunion detection is delayed even further, as nonunion is defined clinically at 6-9 months post-injury [2].

Imaging modalities such as X-ray, CT, or MRI offer important structural insights but are similarly limited in their temporal resolution. Radiographic evidence of impaired healing or infection typically becomes apparent only after significant tissue degradation or lack of callus progression, making imaging more reflective of past events than predictive of future outcomes [27]. More invasive diagnostics, such as deep tissue sampling or biopsy, are rarely performed in the early post-injury period due to concerns about procedural risk, cost, and patient discomfort.

This diagnostic lag is especially concerning given the burden of fracture-related infection, which is present in up to 38% of nonunion cases [7]. Without early biomarkers capable of distinguishing high-risk patients, many infections go undetected until they require surgical revision or hardware removal. These delays not only complicate treatment but can further impair healing, leading to permanent functional deficits or, in severe cases, limb loss. Clearly, there is a pressing need for noninvasive, high-resolution tools that can detect molecular signatures of poor healing before irreversible damage occurs.

Preclinical studies, including those conducted in models of segmental bone defects, have begun to show that systemic immune profiles, including changes in circulating MDSCs, monocytes, and T cells, may serve as early indicators of healing trajectory [28]. These changes have been observed even before structural evidence of nonunion is detectable, suggesting that the immune system may encode predictive information about bone regeneration status. While promising, this line of research is still in its infancy. Most studies to date have used small cohorts, limited time points, or sedentary models that do not account for the impact of rehabilitation and the mechanical environment.

Aim 3 builds directly on these insights by applying comprehensive immune cell profiling in a clinically relevant patient population. This integrative approach is designed to identify systemic immune signatures associated with infection risk and healing outcomes. By leveraging machine learning and multivariate modeling techniques, this work evaluates the feasibility of using immune profiles to distinguish infection risk and establish a foundation for future development of clinically actionable diagnostic tools, helping to bridge the gap between immune biology and clinical decision-making in trauma care.

2.6 Rationale for an Integrated, Translational Approach

This thesis builds a unified framework to address these challenges through three interconnected aims. Aim 1 investigates how distinct rehabilitation parameters influence bone regeneration in a preclinical model, providing foundational data on the mechanical conditions that support healing. Aim 2 characterizes the immune landscape of bone injury under controlled loading conditions, establishing a mechanistic link between physical activity and systemic immune responses. Aim 3 translates these insights into a human clinical context, using high-dimensional immune and proteomic profiling to develop predictive tools for fracture-related infection and nonunion. Together, these aims converge on a singular goal: to integrate mechanical and immune perspectives into a translational strategy that informs both rehabilitation protocols and diagnostic innovation in musculoskeletal care.

CHAPTER 3: REHABILITATION PARAMETERS AND THEIR IMPACT ON BONE HEALING

3.1 Introduction

As established in Chapter 2, bone regeneration is governed by tightly regulated interactions between mechanical and biological signals. While mechanical loading is recognized as a critical determinant of healing, clinical rehabilitation protocols remain largely empirical, with limited guidance on how specific features of loading, such as intensity, duration of exercise, and rest, should be structured to optimize regeneration [10, 29]. As a result, rehabilitation prescriptions are often generalized rather than biologically informed, potentially contributing to variability in healing outcomes [30].

Although mechanobiological studies have demonstrated that both insufficient and excessive loading can impair repair, the specific parameters that define optimal rehabilitation remain poorly understood [31, 32]. This gap stems in part from the multifactorial nature of mechanical loading experienced throughout clinical physical therapy. Rehabilitation-based studies often either isolate a single loading parameter, which limits the translational relevance of their findings, or manipulate multiple variables simultaneously—including load magnitude, frequency, and duration—making it difficult to isolate the independent contribution of individual factors to healing outcomes. Of the potential variables to investigate, rest—the temporal structure separating mechanical stimuli—has received comparatively little attention, despite evidence suggesting it may play a critical role in modulating cellular responsiveness to mechanical signals [33, 34].

To address this knowledge gap, this chapter integrates findings from two complementary investigations: the *Multivariate Modeling Study* and the *Intermittent Rest Study*. The *Multivariate*

Modeling Study systematically evaluated how multiple rehabilitation parameters—including activity distance, exercise duration, velocity, bout structure, and rest time—relate to bone regeneration in a rat model of segmental femoral defect. By leveraging continuous activity tracking and nonlinear modeling, this study identified which features of mechanical loading profiles were most strongly associated with regenerative outcomes. Notably, rest time emerged as one of the most consistent predictors of bone volume, suggesting that temporal structure, rather than cumulative load alone, may play a critical role in determining healing trajectory.

Building on these findings, the *Intermittent Rest Study* was designed to test whether introducing intermittent rest within a controlled treadmill rehabilitation protocol improves bone regeneration compared to continuous running and sedentary conditions. In this investigation, animals were exposed to rehabilitation protocols with matched mechanical workloads but differing temporal structures, allowing isolation of the effect of intermittent rest from total loading volume. By experimentally controlling activity patterns while holding cumulative load constant, this study evaluated whether structured recovery intervals enhance healing outcomes. Together, these two studies provide both data-driven identification and validation of key rehabilitation parameters, establishing a framework for optimizing rehabilitation to support bone repair.

3.2 Methods

3.2.1 Surgical Procedures

Female Wistar rats (Envigo, Indianapolis, IN, USA) were used to model post-traumatic bone healing. In the *Multivariate Modeling Study*, 17-week-old rats weighing 275-325 g (N = 28) underwent bilateral mid-diaphyseal femoral segmental defect surgery, resulting in 56 defects, with a 2 mm defect created in the right femur and a 3 mm defect created in the left femur. In the

Intermittent Rest Study, 16-week-old rats weighing 250-313 g (N = 21) underwent bilateral segmental defect surgery, with a 2 mm defect created in the right femur and a 3 mm defect created in the left femur, resulting in 42 defects.

All surgical procedures were performed under isoflurane (VetOne Fluriso, Boise, ID, USA) anesthesia using aseptic technique. The surgical site was shaved and sterilized with alternating applications of 70% isopropyl alcohol and chlorhexidine (VetOne, Boise, ID, USA). An anterolateral incision was made, and surrounding musculature was bluntly separated to expose the femoral shaft. An internal fixation plate composed of ultra-high molecular weight polyethylene (UHMWPE) was positioned along the dorsolateral surface of the femur and secured using stainless steel screws. A mid-diaphyseal segmental defect measuring either 2 mm or 3 mm was created using an oscillating saw, and defect size was confirmed using a precision measuring device. Defects were left empty. The incision was closed in layers using absorbable sutures and wound clips.

Immediately prior to surgery, animals received a subcutaneous injection of sustained-release buprenorphine at 1 mg per 1 kg of body weight and 3 mL of Lactated Ringer's solution (VetOne, Boise, ID, USA). An additional 2 mL Lactated Ringer's solution was administered subcutaneously during the early postoperative recovery period. Animals were singly housed during the initial 7-day postoperative recovery period and monitored daily. Animals were euthanized at 8 weeks post-injury by CO₂ asphyxiation and cervical dyslocation, and femora were harvested for downstream analysis. All procedures were approved by the University of Oregon Institutional Animal Care and Use Committee (Protocol 20-32).

3.2.2 Exercise Rehabilitation Protocols

Animals underwent rehabilitation protocols specific to each study following a 7-day postoperative recovery period without mechanical loading interventions.

In the *Multivariate Modeling Study*, voluntary wheel running was used to generate a range of rehabilitation activity profiles. Prior to surgery, for two weeks, animals were acclimated to in-cage running wheels equipped with revolution counters. One week following surgery, animals were randomly assigned to one of four rehabilitation conditions with varying access to voluntary wheel running: sedentary, low-distance control, restricted rehabilitation, and unrestricted rehabilitation (Figure 2). Sedentary animals were housed in standard cages without wheel access. Low-distance control animals were housed in cages containing wheels with braking mechanisms that were released for 2 hours per day. Restricted rehabilitation animals were housed in standard cages and transferred to cages with running wheels for 2 hours per day. Unrestricted rehabilitation animals were housed in cages with continuous access to running wheels. Wheel activity was recorded continuously using revolution counters. Running activity parameters were computed using custom Python scripts and included total daily distance, activity duration, velocity, number of activity bouts, bout duration, and daily rest time.

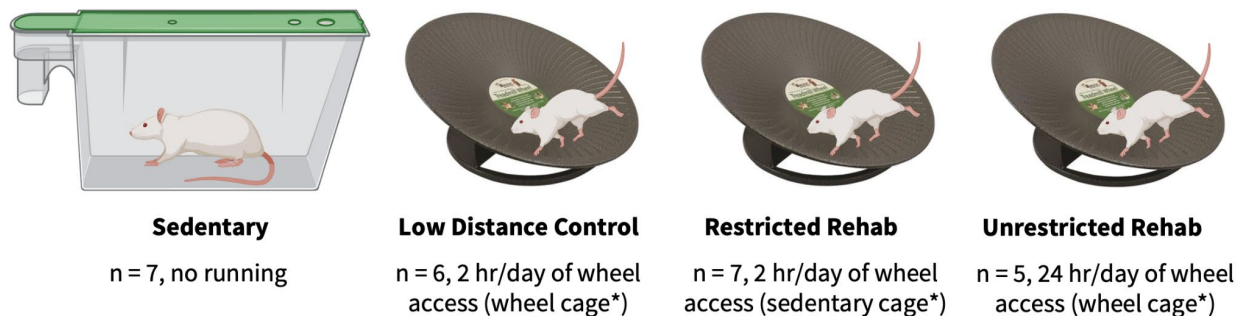


Figure 2. Rehabilitation group structure in the *Multivariate Modeling Study*.

Animals were acclimated to in-cage running wheels prior to surgery and subsequently assigned to one of four rehabilitation conditions that produced distinct voluntary activity profiles: sedentary (no wheel access), low-distance control (2

h/day wheel access with braking mechanisms released), restricted rehabilitation (2 h/day access to running wheels following transfer from sedentary housing), and unrestricted rehabilitation (continuous, 24 h/day wheel access).

In the *Intermittent Rest Study*, treadmill-based rehabilitation protocols were implemented. Animals underwent treadmill acclimation prior to surgery. Following postoperative recovery, animals were assigned to one of three rehabilitation conditions with a treadmill: sedentary, intermittent rest rehabilitation, or continuous rehabilitation (Figure 3). Rehabilitation was performed twice weekly on a motorized treadmill at a constant speed of 13 m/min. Sedentary animals did not undergo treadmill rehabilitation. Animals in the intermittent rest group completed alternating 1-minute running intervals and 1-minute rest intervals until a total of 12 minutes of running was achieved. Animals in the continuous rehabilitation group ran continuously for 12 minutes without rest intervals.

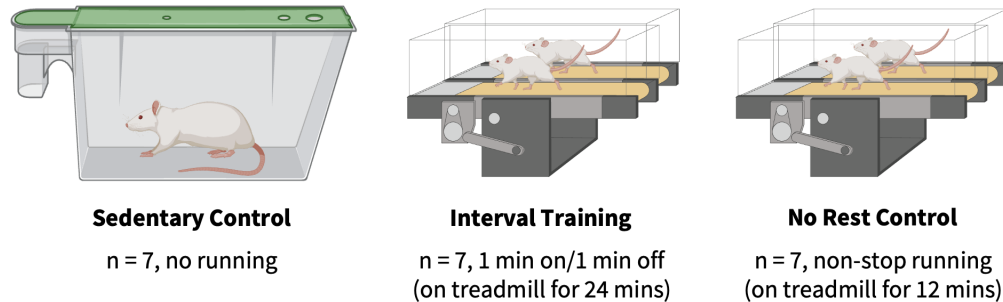


Figure 3. Experimental design and treadmill rehabilitation protocols in the *Intermittent Rest Study*. Following postoperative recovery, animals were assigned to one of three rehabilitation conditions: sedentary control (no treadmill exposure), intermittent rest rehabilitation (alternating 1-minute running and 1-minute rest intervals until 12 minutes of cumulative running was achieved), or continuous rehabilitation (12 minutes of uninterrupted running). All treadmill rehabilitation was performed at a constant speed of 13 m/min.

3.2.3 In vivo Radiographs and Micro-CT Scans

Bone regeneration was evaluated using high-resolution micro-computed tomography (micro-CT) at 2, 4, and 8 weeks post-injury. Digital radiographs were captured at 40 kV with a 7

s exposure using a Faxitron MX-20 system (Faxitron Bioptics LLC, Tucson, AZ, USA). Micro-CT scans were performed using an isotropic voxel size of 48 μm , 55 kVp, 145 μA , and a 750 ms integration time using a VivaCT 80 micro-computed tomography system (Scanco Medical, Brüttisellen, Switzerland). Bone volume was quantified within a standardized volume of interest centered on the defect in the micro-CT scans.

3.2.4 *Statistical Analysis*

Significance was determined at $p < 0.05$. Two-way ANOVA with Tukey's multiple comparisons test was used to analyze *in vivo* bone formation as a function of rehabilitation conditions and time. All data are reported as mean $\pm$ standard deviation (SD). All statistical analyses and graph generation were performed using GraphPad Prism version 10.4.1 (GraphPad Software, San Diego, CA, USA).

3.2.5 *Multivariate Modeling*

Multivariate modeling was performed exclusively on data from the *Multivariate Modeling Study*. Genetic programming was performed using DataModeler software (Evolved Analytics, Rancho Santa Fe, CA, USA) to generate mathematical models relating rehabilitation parameters to bone volume. The algorithm iteratively generated candidate equations and evaluated model performance based on predictive accuracy and complexity (Figure 4). The best-performing models were identified within the Pareto knee, representing optimal trade-offs between model accuracy and simplicity.

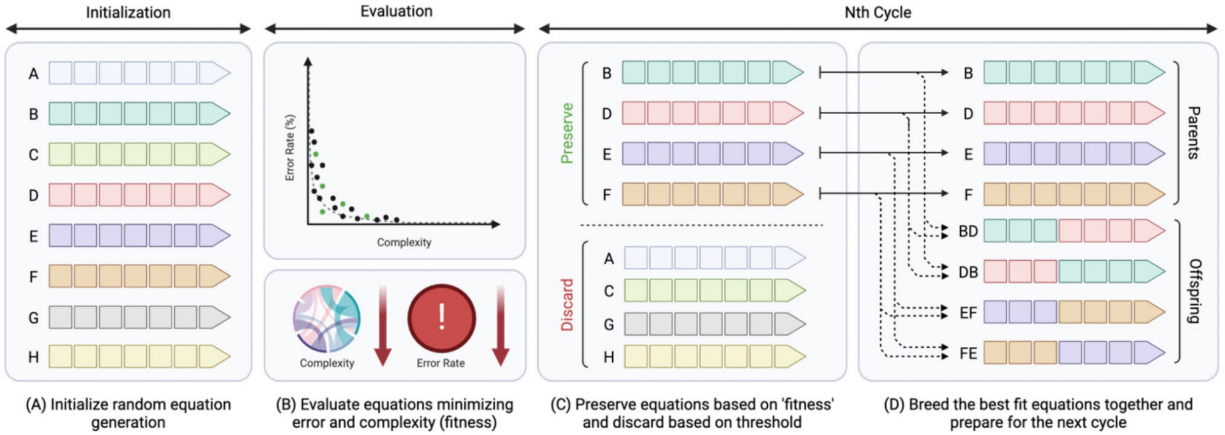


Figure 4. Outline of the workflow of genetic programming. This workflow iteratively identifies simple yet accurate models relating rehabilitation parameters to bone healing outcomes. Figure created by Ethan Dinh, University of Oregon. Methodology adapted from *Romanowicz et al. (2024) [35]*.

3.3 Results

3.3.1 Voluntary Activity Patterns Diverge by Access Schedule

Animals across the voluntary rehabilitation cohorts exhibited distinct mechanical loading behaviors based on their access to running wheels. As previously mentioned, daily wheel activity was quantified by six parameters: total distance, duration, average velocity, number of bouts, bout duration, and rest time (Table 1). In the *Multivariate Modeling Study*, animals in the unrestricted rehabilitation group (continuous voluntary wheel access, 24 h/day) exhibited the highest average running duration and bout frequency, along with the shortest daily rest times, reflecting frequent and fragmented activity patterns. Despite continuous wheel access, the unrestricted group ran daily distances that were not significantly different from the restricted rehabilitation group (voluntary wheel access limited to 2 h/day, 8-10 pm each day). The restricted group demonstrated similarly high running distances but with fewer bouts and longer daily rest periods, reflecting more temporally constrained activity. In contrast, the low-distance control group (2 h/day access to braked running wheels, 8-10 pm each day) exhibited

significantly less running distance, duration, and bout frequency than the other two rehabilitation conditions, consistent with minimal voluntary activity. Running velocity was the only rehabilitation parameter that did not differ significantly across rehabilitation conditions, likely reflecting high inter-individual variability.

These wheel access schedules produced distinct voluntary activity profiles, resulting in systematic differences in running distance, duration, bout frequency, and rest time across rehabilitation groups. This variation in activity-derived mechanical loading provided the basis for subsequent multivariate analysis examining relationships between loading parameters and bone healing outcomes.

Table 1. Rehabilitation conditions produced distinct exercise parameter profiles across experimental groups in the *Multivariate Modeling Study*. Sample sizes were n = 6 (low-distance control), n = 7 (restricted rehabilitation), and n = 5 (unrestricted rehabilitation). Values are presented as mean $\pm$ SD. Figure adapted from *Williams et al. (2025)* [11].

	Low Distance Control	Restricted Rehabilitation	Unrestricted Rehabilitation
Distance (m/day)	65.08 $\pm$ 13	197.3 $\pm$ 33	180.8 $\pm$ 16
Duration (min/day)	3.722 $\pm$ 13	11.40 $\pm$ 1.6	24.78 $\pm$ 2.1
Daily Bouts (bouts/day)	23 $\pm$ 3	66.65 $\pm$ 9	158.6 $\pm$ 14
Bout Duration (s/bout)	6.616 $\pm$ 0.12	10.2 $\pm$ 0.05	9.326 $\pm$ 0.02
Velocity (m/s)	0.22 $\pm$ 0.002	0.25 $\pm$ 0.003	0.11 $\pm$ 0.0001
Rest Time (hrs)	23.51 $\pm$ 5.6	22.65 $\pm$ 0.5	10.22 $\pm$ 2.9

3.3.2 Healing Outcomes Reflect Load Dose and Injury Severity

Longitudinal bone volume quantification using *in vivo* micro-CT revealed that rehabilitation condition significantly influenced healing outcomes in 3 mm defects (Figure 5A). Unrestricted rehabilitation resulted in significantly reduced bone formation compared to

restricted rehabilitation and sedentary conditions. Consistent with these findings, the unrestricted rehabilitation group exhibited a 0% bridging rate, with all defects remaining unbridged at 8 weeks post-operatively (Figure 5C). Representative micro-CT reconstructions confirmed persistent nonunion across animals in this group (Figure 5D). In comparison, the restricted rehabilitation group demonstrated increased bone formation and achieved bridging in 57% of defects (Figure 5C). The low-distance control group exhibited intermediate bone formation and bridging outcomes relative to unrestricted and restricted rehabilitation groups.

In contrast, rehabilitation condition did not significantly influence average bone formation in 2 mm femoral segmental defects (Figure 5B). All groups exhibited substantial bone regeneration, including an 80% bridging rate in the unrestricted rehabilitation group (Figure 5C).

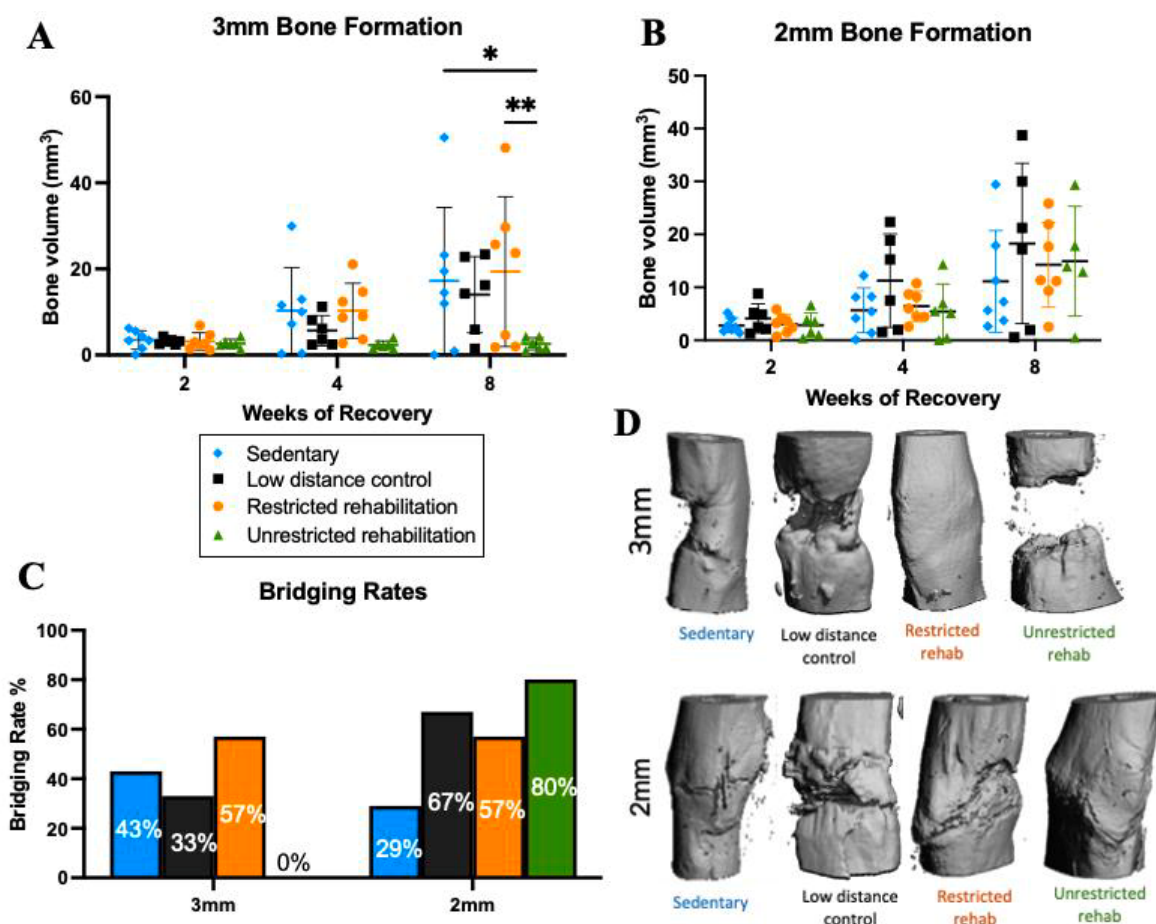


Figure 5. Rehabilitation effects on bone healing are dependent on defect size. (A) Bone volume within a centered 2.5 mm region of 3 mm defects measured longitudinally over 8 weeks of recovery. Unrestricted rehabilitation significantly reduced bone formation compared to sedentary (* $p = 0.0203$) and restricted rehabilitation (** $p = 0.0053$) at week 8 (two-way ANOVA with Šídák's multiple comparisons test). (B) Bone volume within a centered 1.5 mm region of 2 mm defects measured over 8 weeks of recovery. (C) Endpoint bridging rates determined by radiographic and micro-CT assessment demonstrate defect size-dependent rehabilitation effects. (D) Representative *in vivo* micro-CT reconstructions. Data are presented as mean $\pm$ SD. Figure reprinted from *Williams et al.* (2025) [11].

3.3.3 Nonlinear Modeling Reveals an Optimal “Goldilocks Zone” for Healing

AI-based genetic programming was used to perform multivariate regression analysis and identify mathematical relationships between rehabilitation parameters and endpoint bone healing (Figure 6A). A total of 1315 candidate models were generated and evaluated based on predictive accuracy and equation complexity. To focus on models that balanced simplicity and performance, 108 models from the knee of the Pareto front were selected for further analysis, representing equations with low complexity and high predictive accuracy.

Analysis of variable presence across the selected models revealed that rest time, distance, and duration were consistently included in the 108 highest-performing equations, appearing in 100%, 99%, and 93% of models, respectively (Figure 6B). In contrast, bout frequency, velocity, and bout duration appeared less frequently, suggesting a reduced contribution to model performance. Because several rehabilitation variables were inherently dependent (e.g., duration and bout frequency), feature selection was performed to isolate independent predictors that were both highly represented and minimally redundant. Based on these criteria, distance and rest time were selected for nonlinear evaluation of rehabilitation parameters and bone healing.

Response plots revealed a nonlinear relationship between average daily running distance and endpoint bone volume. Bone volume increased with running distance up to an intermediate range, after which further increases in distance were associated with reduced bone formation

(Figure 6C). Three-dimensional surface plots further demonstrated a nonlinear interaction between average daily distance and rest time, with peak bone volume occurring within a specific combination of moderate running distance and moderate rest duration (Figure 6D). A two-dimensional contour plot visualizing predicted bone volume across these variables revealed a distinct region associated with maximal healing outcomes, bounded by regions of lower bone formation at lower and higher loading conditions (Figure 6E).

Overlay of individual animal data onto the surface and contour plots illustrated sparse sampling within this optimal region, particularly at intermediate combinations of running distance and rest time. Despite this limited representation, the nonlinear regression model consistently predicted increased bone volume within this region, as supported by elevated predicted values in surrounding parameter space. These findings demonstrate a nonlinear relationship between voluntary mechanical loading and bone regeneration, characterized by reduced healing at both insufficient and excessive activity levels.

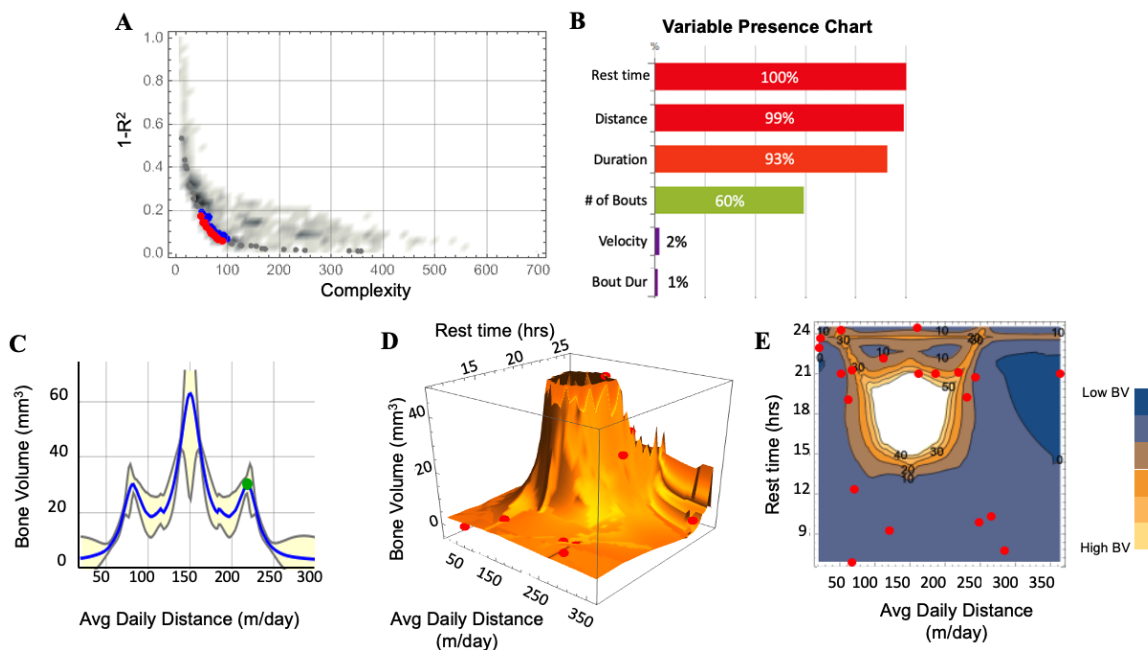


Figure 6. Nonlinear multivariate modeling reveals relationships between rehabilitation parameters and bone formation. (A) Candidate models generated through genetic programming were evaluated based on accuracy and complexity, with optimal models selected from the “knee” of the Pareto front. (B) Variable presence chart showing the frequency with which each rehabilitation parameter appeared in high-performing models, highlighting rest time, distance, and duration as dominant contributors. (C) Two-dimensional response profile illustrating the relationship between average daily running distance and bone volume. The blue line represents the ensemble model prediction, and the yellow envelope indicates model variance. (D-E) Three-dimensional response surface and corresponding two-dimensional contour plot demonstrating predicted bone volume as a function of rest time and average daily running distance. Red datapoints represent individual animals used to generate the model. Figure reprinted from *Williams et al.* (2025) [11].

3.3.4 Intermittent Rest Enhances Healing Independent of Load Volume

Micro-CT quantification revealed that incorporating intermittent rest into rehabilitation protocols increased bone formation compared to continuous rehabilitation without rest and no exercise. In 2 mm defects, intermittent rehabilitation resulted in a near-significant increase in endpoint bone volume relative to both continuous rehabilitation and sedentary conditions (Figure 7A). Correspondingly, endpoint bridging rates in 2 mm defects were approximately twofold higher in the intermittent rehabilitation group compared to both continuous rehabilitation and sedentary groups (Figure 7C).

In 3 mm defects, intermittent rehabilitation produced a significant increase in endpoint bone volume compared to continuous rehabilitation without rest (Figure 7A). Despite these increases in bone formation, overall union rates remained low in 3 mm defects, with only a single subject achieving bridging by the endpoint (Figure 7C). Continuous rehabilitation without rest did not improve bone formation relative to sedentary controls and exhibited lower average bone volume.

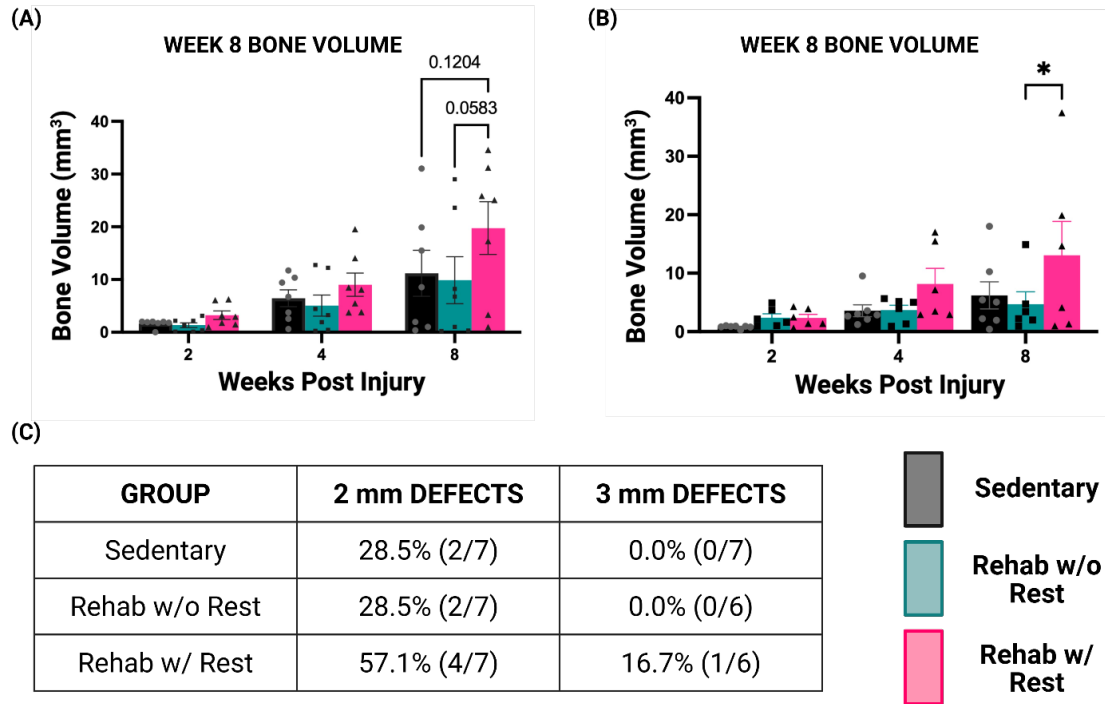


Figure 7. Rehabilitation with rest increases bone formation and endpoint bridging rates compared to rehabilitation without rest in 2 and 3 mm bone defects. (A) *In vivo* bone volume within centered 1.5 mm of 2 mm defects across 8 weeks of recovery (Two-Way ANOVA with Tukey's multiple comparisons, $n = 7$). (B) *In vivo* bone volume within centered 2.5 mm of 3 mm defects across 8 weeks of recovery (Two-Way ANOVA with Tukey's multiple comparisons: * $p = 0.0455$, $n = 6-7$). One subject was excluded in the rehabilitation with rest and rehabilitation without rest because the defect was not 3 ± 0.5 mm. (C) Endpoint bridging rates after 8 weeks of recovery determined by radiographs and 3D micro-CT reconstructions. Data are mean $\pm$ SD. Figure adapted from *Williams et al.*, submitted [12].

3.4 Discussion

The findings of this study demonstrate that bone regeneration is governed not simply by the total amount of mechanical loading applied during rehabilitation, but by the temporal structure of that loading. By integrating continuous activity tracking, nonlinear predictive modeling, and controlled treadmill rehabilitation experiments, this work identified rest as a critical determinant of bone healing and experimentally validated its influence on regenerative

outcomes. Together, these results establish that bone healing follows a nonlinear, dose-dependent relationship with mechanical loading, in which both insufficient and excessive activity impair regeneration, while moderate loading combined with sufficient recovery promotes optimal healing.

The *Multivariate Modeling Study* revealed that rehabilitation conditions producing similar total activity distances could nonetheless result in markedly different healing outcomes, highlighting the importance of activity structure rather than cumulative load alone. Notably, unrestricted rehabilitation animals exhibited activity profiles characterized by high bout frequency, prolonged running duration, and minimal daily rest, yet failed to achieve defect bridging in the more challenging 3 mm injury model. In contrast, restricted rehabilitation animals, which ran comparable daily distances but incorporated substantially longer rest periods, demonstrated significantly improved bone formation and higher rates of bridging. These findings suggest that excessive loading without adequate recovery may impair regeneration, even when total mechanical exposure is similar. This observation aligns with established mechanobiological principles indicating that excessive strain can disrupt angiogenesis, destabilize nascent tissue, and promote fibrous nonunion rather than ossification.

Nonlinear genetic programming further clarified this relationship by identifying rest time, distance, and duration as the most consistent predictors of bone volume across high-performing models. Response surface analyses revealed a distinct nonlinear relationship between activity parameters and bone regeneration, characterized by maximal bone formation within a narrow range of intermediate loading and rest conditions. Outside this range, both low and excessive activity levels were associated with reduced bone formation. This finding supports the existence of a “Goldilocks zone” for rehabilitation, in which loading must be sufficient to stimulate

osteogenesis but not so excessive that it disrupts regenerative processes. Importantly, rest emerged as one of the most consistently represented variables in predictive models, appearing in all high-performing equations, underscoring its central role in modulating healing outcomes.

To directly test the influence of rest, the *Intermittent Rest Study* isolated temporal structure while holding total mechanical workload constant. Despite identical running duration, speed, and distance, rehabilitation protocols incorporating intermittent rest significantly increased bone formation compared to continuous loading. In 2 mm defects, intermittent rehabilitation resulted in a nearly significant increase in bone volume and approximately twofold higher bridging rates relative to continuous rehabilitation. In 3 mm defects, intermittent rest significantly increased bone volume compared to continuous rehabilitation, although bridging remained limited overall due to the greater severity of the injury. These findings demonstrate that rest intervals enhance bone regeneration independent of cumulative load magnitude, confirming predictions from nonlinear modeling and validating rest as an important component of rehabilitation.

The biological basis for this effect likely reflects the fundamental mechanosensitivity of bone-forming cells. Osteocytes and osteoblast progenitors respond to mechanical strain through mechanotransductive signaling pathways that regulate proliferation, differentiation, and matrix deposition [24]. However, repeated loading without adequate recovery can lead to cellular desensitization, reducing responsiveness to subsequent mechanical stimuli [33, 34, 36]. Rest intervals allow mechanosensitive cells to reset their signaling sensitivity, restoring their ability to respond to mechanical cues and enhance osteogenic activity. This phenomenon has been observed in prior mechanobiology studies, where intermittent loading produces greater bone formation than continuous loading of equivalent magnitude [34]. The present study extends these

findings by demonstrating that rest intervals influence not only bone formation but also clinically relevant healing outcomes, including defect bridging.

The effects of rehabilitation were also strongly dependent on injury severity. In smaller 2 mm defects, healing occurred across most rehabilitation conditions, reflecting the intrinsic regenerative capacity of this injury model. In contrast, rehabilitation conditions exerted a pronounced influence on healing outcomes in 3 mm defects, where regenerative demands were greater. Excessive loading without sufficient rest was associated with impaired healing and persistent nonunion, whereas rehabilitation incorporating rest improved bone formation. This suggests that optimal rehabilitation parameters may vary depending on injury severity, with larger defects exhibiting greater sensitivity to loading. These findings have important translational implications, as they suggest that rehabilitation protocols should be tailored not only to patient activity tolerance but also to the biological demands of the injury.

Collectively, these findings challenge the prevailing clinical paradigm in which rehabilitation is prescribed primarily based on subjective measures such as pain tolerance or generalized weight-bearing guidelines. Instead, they demonstrate that rehabilitation outcomes depend on a complex interaction between loading magnitude, temporal structure, and biological responsiveness. Importantly, this study shows that more activity is not necessarily better. Excessive mechanical loading without adequate recovery may impair healing, while appropriately structured rehabilitation that incorporates rest can enhance regeneration. These results underscore the limitations of current one-size-fits-all rehabilitation strategies and highlight the need for individualized, biologically informed rehabilitation protocols.

Beyond identifying optimal rehabilitation parameters, this work also establishes a framework for integrating predictive modeling into rehabilitation design. By leveraging subject-

specific activity data and nonlinear modeling approaches, it becomes possible to identify rehabilitation profiles associated with optimal healing and predict outcomes based on activity patterns. Such approaches could eventually enable personalized rehabilitation strategies that dynamically adjust loading and rest parameters based on individual healing responses.

In summary, this study demonstrates that bone regeneration is governed by a nonlinear interaction between mechanical loading and temporal structure. Rest emerged as a critical determinant of healing, and experimental validation confirmed that intermittent rest enhances bone formation independent of total load. These findings establish the existence of an optimal rehabilitation zone characterized by moderate loading and sufficient recovery and illustrates a predictive framework for designing rehabilitation protocols that improve healing outcomes following orthopaedic trauma.

CHAPTER 4: CELLULAR DYNAMICS OF TRAUMATIC INJURY AND THEIR ASSOCIATION WITH HEALING OUTCOMES

4.1 Introduction

In Chapter 3, we demonstrated that rehabilitation parameters, including loading magnitude, timing, and rest, profoundly influence bone regeneration following traumatic injury. These findings reinforced the concept that fracture healing is not governed solely by structural or mechanical conditions, but rather by a complex interplay between biomechanical stimuli and biological processes. Notably, even under controlled surgical and rehabilitation conditions, substantial variability in healing outcomes was observed between subjects. This heterogeneity suggests that intrinsic biological factors, beyond mechanical loading alone, play a critical role in determining regenerative success or failure.

Among these biological factors, the systemic immune response has emerged as a key regulator of musculoskeletal repair. Fracture healing initiates with a robust inflammatory phase characterized by recruitment and activation of innate immune cells, including monocytes and myeloid-derived suppressor cells (MDSCs), which orchestrate downstream processes such as angiogenesis, progenitor cell recruitment, and extracellular matrix remodeling (Figure 8) [14, 15, 21]. This inflammatory response must be tightly regulated; while early immune activation is essential for initiating repair, prolonged or dysregulated inflammation has been strongly associated with impaired regeneration and nonunion [12, 15]. Importantly, immune responses are highly sensitive to mechanical cues, and rehabilitation-induced loading has been shown to modulate both local and systemic inflammatory signaling [12, 47]. These observations suggest that rehabilitation may influence healing outcomes not only through mechanical effects on tissue strain, but also through its ability to shape the systemic immune landscape.

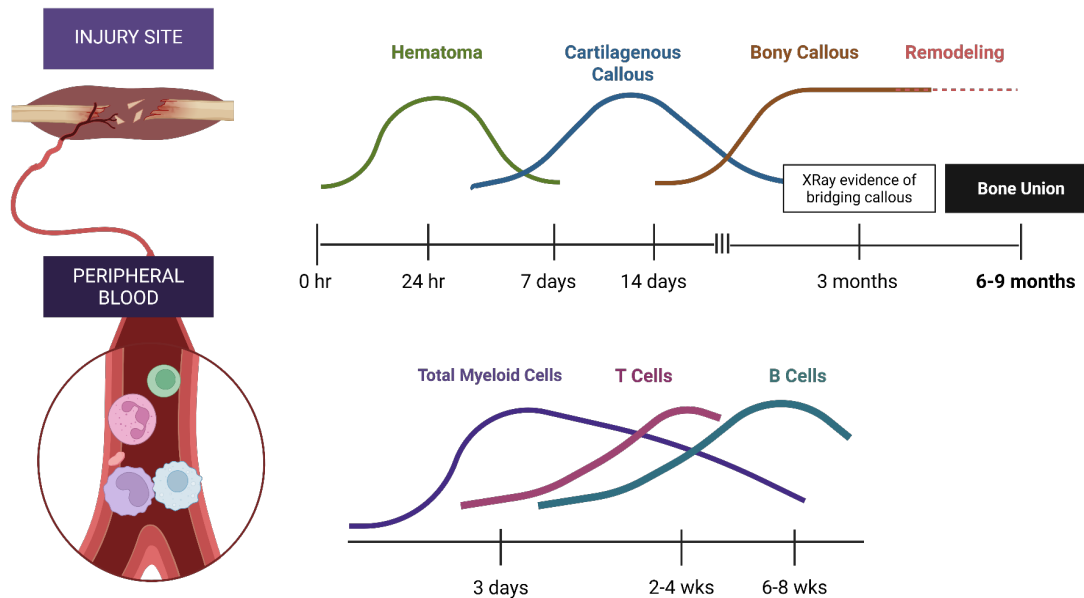


Figure 8. Changes in systemic immune populations during different phases of regeneration following traumatic bone injuries. Figure originally created by Kelly Leguineche, University of Oregon.

Despite growing recognition of the immune system's role in bone regeneration, the temporal dynamics of systemic immune responses during rehabilitation, and their relationship to eventual healing outcomes, remain incompletely understood. In particular, it is unclear whether divergence in immune cell trajectories occurs early in the healing process, prior to the appearance of radiographic or structural differences. Identifying such early immune signatures could provide valuable insight into the biological mechanisms underlying regenerative success and failure and may ultimately enable the development of prognostic biomarkers capable of guiding personalized rehabilitation strategies.

To address this gap, we performed longitudinal immune profiling in a validated rat model of segmental femoral defect injury using flow cytometry to quantify circulating immune cell populations over time. These analyses were conducted in the context of two rehabilitation-focused studies: the *Incline Ramp-Up Study*, which investigated the effects of progressively

increasing mechanical loading, and the *Intermittent Rest Study*, which was introduced in Chapter 3 in the context of healing outcomes and examined the influence of structured rest between loading bouts. These studies provided a unique opportunity to evaluate systemic immune responses across distinct rehabilitation conditions and correlate immune cell dynamics with eventual healing outcomes.

The objective of this chapter was to characterize the systemic immune response following traumatic bone injury and determine whether early immune cell population dynamics are associated with regenerative success or failure. By integrating longitudinal immune profiling with rehabilitation and healing outcome data, this work aims to establish a cellular framework linking rehabilitation mechanics to biological repair processes. These findings provide critical mechanistic insight into how rehabilitation modulates systemic physiology and lay the foundation for subsequent work investigating immune signatures as predictive biomarkers in clinical populations.

4.2 Methods

4.2.1 Animal Models and Surgical Procedures

All procedures were approved by the University of Oregon Institutional Animal Care and Use Committee (IACUC) and performed using aseptic technique. Female Wistar rats (16 weeks old, Envigo, Indianapolis, IN, USA) were used for both the *Incline Ramp-Up Study* and the *Intermittent Rest Study*.

Segmental femoral defects were created using the surgical approach described in Chapter 3. Briefly, animals were anesthetized with isoflurane (VetOne Fluriso, Boise, ID, USA), and a lateral approach was used to expose the femoral diaphysis. Internal fixation plates were secured using stainless steel screws, and a mid-diaphyseal segmental defect was created using an

oscillating saw. Defects were left empty to isolate the effects of rehabilitation on healing. The surgical site was closed in layers, and animals received postoperative analgesia and supportive care.

The *Incline Ramp-Up Study* utilized unilateral 3 mm defects in the left femur (N = 15). In contrast, the *Intermittent Rest Study* utilized bilateral defects, consisting of a 2 mm defect in right femurs and a 3 mm defect in left femurs (N = 21), as described in Chapter 3. Because the surgical technique and fixation approach were identical between studies, any observed differences in immune response or healing outcomes reflect differences in rehabilitation protocol rather than surgical variation.

4.2.2 Rehabilitation Protocols

All animals underwent two weeks of treadmill acclimation prior to surgery to ensure compliance with postoperative rehabilitation. Following a one-week recovery period postoperatively, animals underwent twice-weekly treadmill rehabilitation at a speed of 13 m/min for 12 minutes per session.

In the *Incline Ramp-Up Study*, animals were randomly assigned to one of three rehabilitation conditions: non-adaptive control, delayed adaptive, and gradual adaptive (Figure 9). The non-adaptive control group ran on a flat treadmill (0% incline) throughout the recovery period. In the delayed adaptive group, animals ran on a flat treadmill for the first three weeks of rehabilitation, after which incline was increased to 1.5% at week 4 and further increased to 3.0% at week 6 for the remainder of the study. In the gradual adaptive group, incline increases began at week 2 post-injury, with treadmill incline increasing incrementally by 0.5% per week until reaching 3.0% by week 8. These rehabilitation conditions were designed to isolate the effects of both timing and magnitude of progressive mechanical loading on healing outcomes.

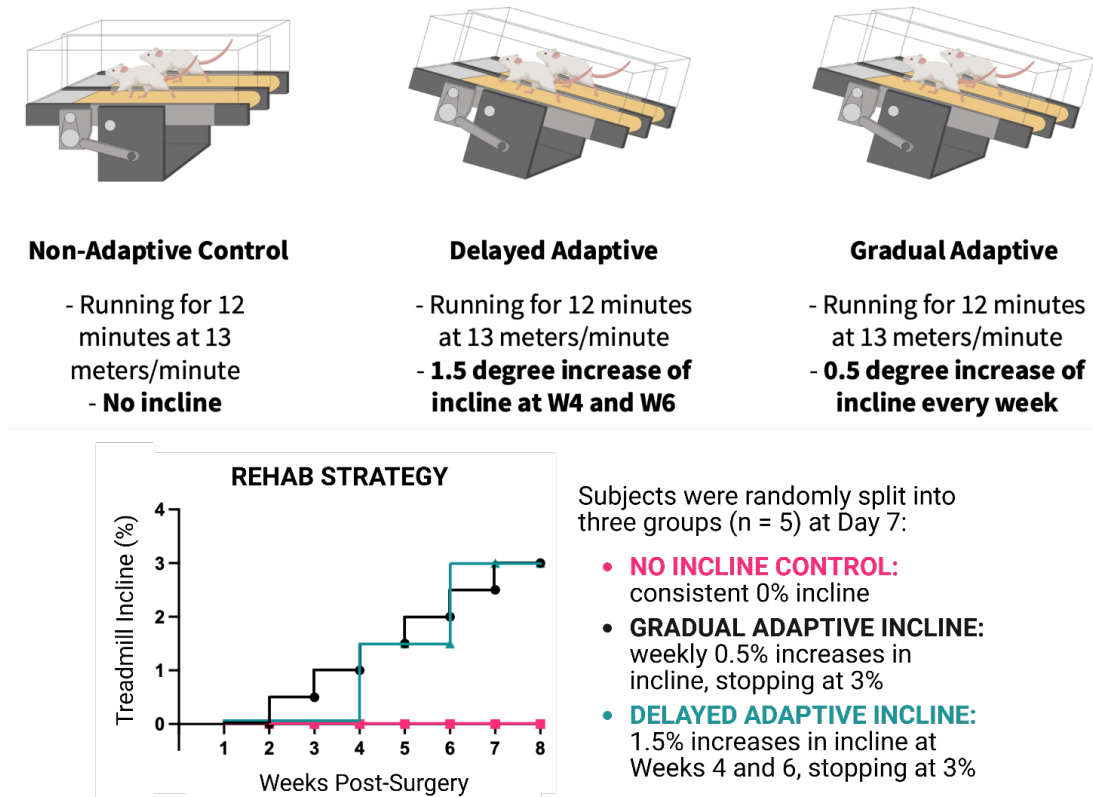


Figure 9. Experimental design and treadmill rehabilitation protocols in the *Incline Ramp-Up Study*.

As previously discussed, in the *Intermittent Rest Study*, animals were assigned to one of three groups: sedentary, intermittent rest rehabilitation (1 minute running alternated with 1 minute rest, repeated until 12 minutes of total running was completed), or continuous rehabilitation (12 minutes uninterrupted running) (Figure 3). These protocols allowed evaluation of how rest between loading bouts influences immune response and healing outcomes.

4.2.3 Blood Collection

Longitudinal blood samples were collected to characterize systemic immune responses over time. Blood was collected longitudinally at baseline (pre-surgery), an early post-injury timepoint (post-injury day 5 in the *Incline Ramp-Up Study* and post-injury day 4 in the *Intermittent Rest Study*), day 7, and weeks 2, 4, and 8.

Animals were anesthetized with isoflurane (VetOne Fluriso, Boise, ID, USA) and positioned supine. The ventral tail surface was disinfected using alcohol, and blood was collected from the tail artery using a sterile needle inserted at approximately a 35° angle. Approximately 300-350 µL of blood was collected into 1.3 mL lithium-heparin coated tubes (Sarstedt AG & Co. KG, Nümbrecht, Germany) to prevent clotting and preserve leukocyte populations for flow cytometric analysis. To minimize immune perturbation caused by repeated sampling, no more than four collection attempts were performed per timepoint. Following collection, hemostasis was achieved using gentle pressure and clotting agents, and animals were allowed to recover fully prior to returning to their home cages. Collected samples were maintained at controlled temperatures and processed promptly to preserve immune cell integrity.

4.2.4 Blood Processing and Red Blood Cell Lysis

Whole blood samples were processed immediately following collection to isolate leukocytes for flow cytometric analysis. Red blood cells were lysed by incubating blood samples in 1x RBC lysis buffer (Invitrogen, Carlsbad, CA, USA) at a ratio of approximately 2 mL buffer per 100 µL blood. Samples were incubated at room temperature and gently mixed to ensure complete erythrocyte lysis. Following incubation, samples were washed with FACS buffer consisting of phosphate-buffered saline supplemented with 2% fetal bovine serum (Gibco, Grand Island, NY, USA) and 0.1% sodium azide (Sigma-Aldrich, St. Louis, MO, USA) to preserve cell viability and prevent nonspecific binding.

Cells were centrifuged at 500 G at 4 °C, and the supernatant was discarded. This lysis and wash procedure was repeated to ensure complete removal of erythrocytes and debris. Following washing, leukocytes were fixed using flow cytometry fixation buffer (R&D Systems,

Minneapolis, MN, USA) to preserve cellular morphology and antigen integrity. Cells were then resuspended in FACS buffer and stored at 4°C until staining and acquisition.

4.2.5 Antibody Staining and Immune Cell Panels

Immune cell populations were identified using multicolor antibody panels designed to characterize both innate and adaptive immune cell subsets. Prior to staining, Fc receptors were blocked using anti-rat CD32 antibody (BD Biosciences, San Jose, CA, USA) to minimize nonspecific antibody binding and improve staining specificity. Cells were then incubated with fluorescently conjugated antibodies targeting cell surface and intracellular markers to enable identification of distinct immune populations.

Two complementary staining panels were used to characterize myeloid and lymphoid immune compartments by flow cytometry. The myeloid panel was used to identify innate immune cell populations, including myeloid-derived suppressor cells (MDSCs), monocytes, and B cells. In parallel, a T cell panel was used to characterize adaptive immune populations, including total T cells, helper T cells, cytotoxic T cells, and regulatory T cells. The specific antibody markers used to identify and gate each population are summarized in Table 2.

Table 2. Composition of conventional flow cytometry panels used to identify circulating immune cell populations in the *Incline Ramp-Up Study* and the *Intermittent Rest Study*. 7 immune cell populations were identified using 8 antibodies. All populations were first gated to exclude debris and doublets.

Myeloid Panel		T Cell Panel	
MDSCs	CD11b+/His48+	Total T Cells	CD3+
Monocytes	CD11b+/CD68+	Helper T Cells	CD3+/CD4+
Total B Cells	CD45+	Cytotoxic T Cells	CD3+/CD8+
		Regulatory T Cells	Helper T Cells/FoxP3+

Extracellular staining was performed by incubating cells with antibody cocktails on ice in the dark for 20-25 minutes to preserve fluorophore stability and prevent photobleaching. Following extracellular staining, intracellular targets, including FoxP3 and CD68, were labeled using fixation and permeabilization buffers to enable antibody access to intracellular compartments. Cells were subsequently washed and resuspended in FACS buffer prior to acquisition.

Fluorescence-minus-one (FMO) controls and unstained controls were prepared for each experiment to enable accurate gating and minimize false-positive signal detection.

4.2.6 Flow Cytometry Acquisition

Flow cytometric data were acquired using a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA). This instrument utilizes conventional photomultiplier-based fluorescence detection, distinct from the spectral flow cytometry platform used in Chapter 5. Samples were acquired using standardized acquisition settings, with a target of 100,000 events per sample to ensure sufficient statistical power for population analysis. Instrument compensation was performed using compensation beads and verified prior to analysis. Data were exported as FCS files for downstream analysis.

4.2.7 Flow Cytometry Analysis and Gating Strategy

Flow cytometric data were analyzed using FlowJo software (version 10.8.2; FlowJo LLC, Ashland, OR, USA). A standardized gating strategy was applied across all samples. First, debris was excluded using forward and side scatter parameters. Doublets were removed using forward scatter area versus height gating to isolate single-cell populations.

Following exclusion of debris and doublets, immune populations were identified using sequential gating based on marker expression. Myeloid and T cells populations were identified

using the aforementioned markers (Table 2). Gates were defined using FMO controls to establish positive and negative boundaries, ensuring that background signal remained below 1% of total events (Figure 10). Identical gating strategies were applied across all samples to ensure consistency and enable direct comparison between experimental groups (Figure 11). Population frequencies were calculated as a percentage of parent leukocyte populations and analyzed.

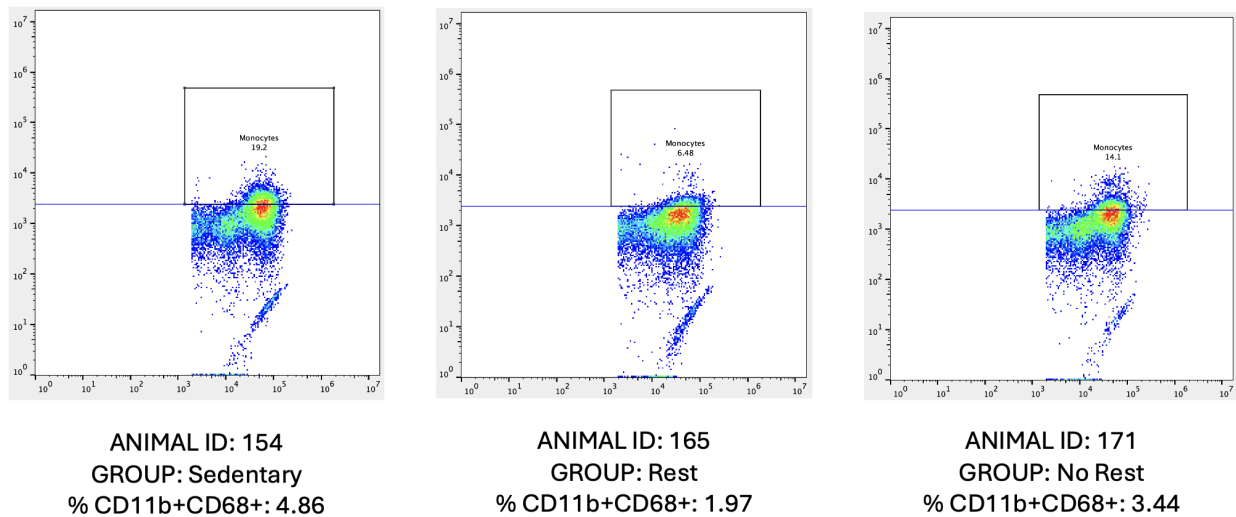


Figure 10. Representative demonstration of gating using FMO controls. FMO controls were used to define gating thresholds and ensure accurate identification of cell types while minimizing false-positive signal detection. Gates were applied consistently across all samples to maintain analytical consistency. Figure adapted from *Williams et al.*, submitted [12].

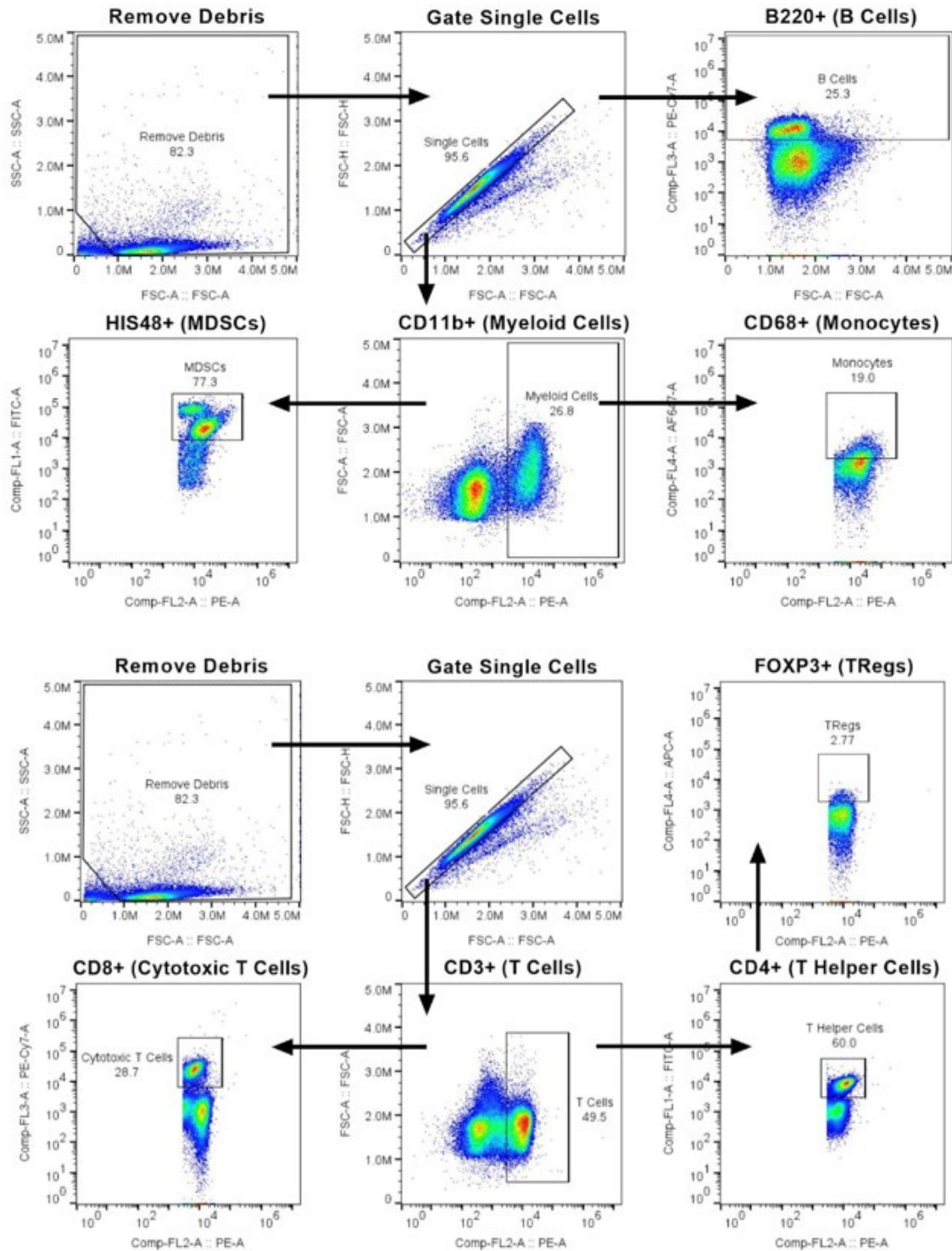


Figure 11. Flow cytometry gating strategy used to identify circulating immune cell populations. Representative plots demonstrate sequential gating to exclude debris and doublets, followed by identification of myeloid and lymphoid immune subsets based on marker expression. Gates were defined using fluorescence- minus-

one controls and applied consistently across all samples. Figure reprinted from *Vantucci and Guyer et al. (2022) [15]*.

4.2.8 Statistical Analysis

Flow cytometry data are presented as mean $\pm$ standard error of the mean (SEM), as specified in the figure captions. Significance was determined at $p < 0.05$. Two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was used to analyze longitudinal systemic immune cell populations as a function of rehabilitation condition and time. All statistical analyses and graphical outputs were generated using GraphPad Prism version 10.4.1 (GraphPad Software, San Diego, CA, USA).

4.3 Results

4.3.1 Incline Ramp-Up Rehabilitation Produced Uniform Systemic Immune Responses Independent of Incline Progression

To characterize the systemic immune response following traumatic injury and rehabilitation in the *Incline Ramp-Up Study*, longitudinal blood samples were collected and analyzed using flow cytometry in animals subjected to progressive incline rehabilitation. Across all rehabilitation groups—non-adaptive control, delayed adaptive, and gradual adaptive—a consistent immune trajectory was observed following injury. Immediately post-injury, circulating immune populations exhibited a pronounced inflammatory response characterized by suppression of T cells, helper T cells, and cytotoxic T cells, and elevation of myeloid-derived suppressor cells (MDSCs) (Figure 12). These elevations were followed by a gradual resolution phase, with immune cell frequencies returning to near baseline levels by approximately four weeks post-injury.

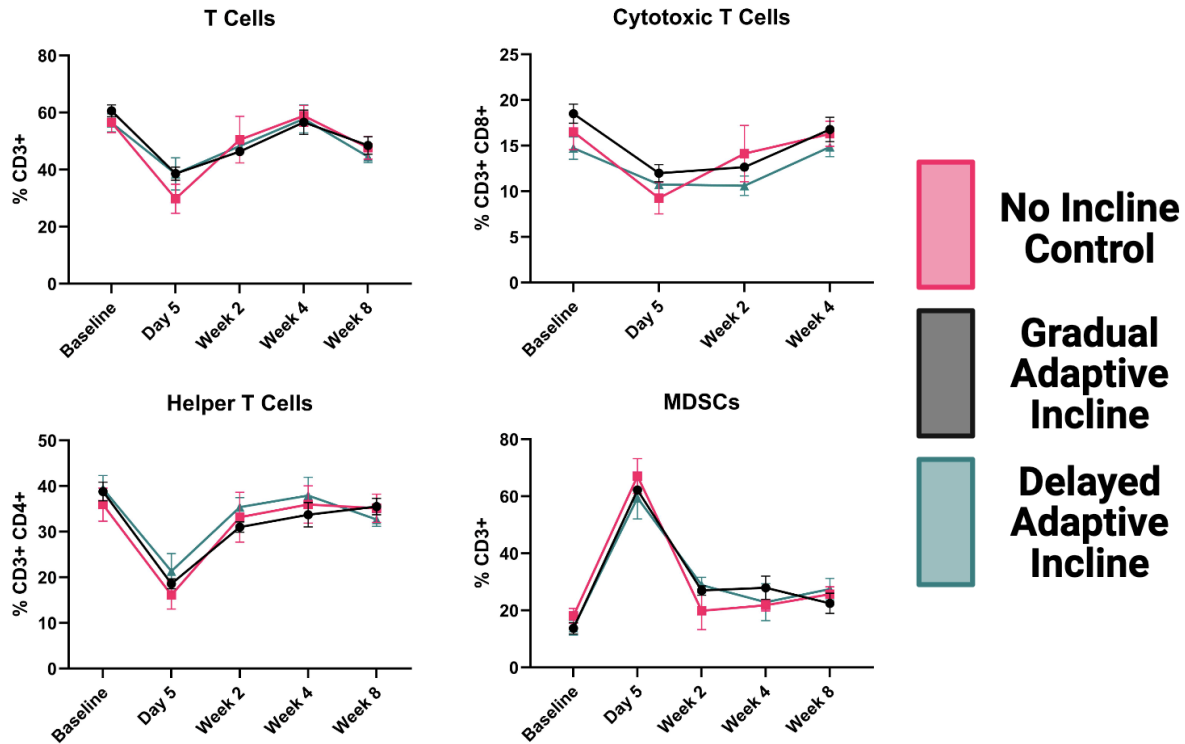


Figure 12. Longitudinal cell profiles for animals in the *Incline Ramp-Up Study*. Systemic immune cell characterization of total T cells, cytotoxic T cells, helper T cells, and MDSCs was done using flow cytometry (N = 15, n = 5 per group). No significant differences were observed. Data are mean $\pm$ SEM. Figure adapted from Williams *et al.*, submitted [12].

Importantly, no statistically significant differences in immune cell population dynamics were observed between rehabilitation groups at any timepoint. Despite differences in incline progression, immune trajectories were highly uniform across all subjects, indicating that variation in incline magnitude and timing did not meaningfully alter systemic immune responses. These findings suggest that progressive increases in treadmill incline alone were insufficient to modulate circulating immune populations in this model. Instead, all animals exhibited a shared pattern of initial immune activation followed by gradual resolution, consistent with a canonical post-traumatic immune response.

Notably, monocyte populations were not analyzed in this study due to staining inconsistencies, limiting direct comparison of this population between incline conditions. However, the uniformity observed across other major immune populations, including MDSCs and T cell subsets, indicates that incline progression did not produce detectable systemic immunomodulatory effects under continuous rehabilitation conditions.

4.3.2 Intermittent Rest During Rehabilitation Selectively Modulated Inflammatory Myeloid Cell Populations

In contrast to the uniform immune responses observed in the *Incline Ramp-Up Study*, the *Intermittent Rest Study* revealed distinct differences in systemic immune cell dynamics associated with rehabilitation protocol (Figure 13). While all animals initially exhibited a similar inflammatory response following injury, divergence in immune trajectories emerged following the initiation of rehabilitation.

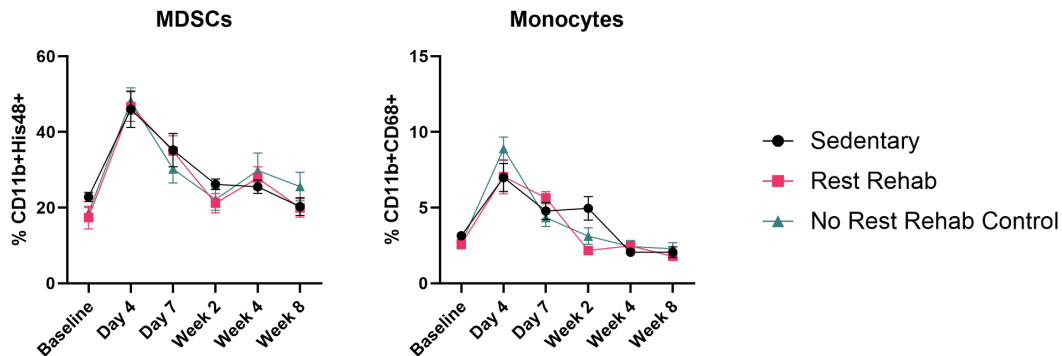


Figure 13. Longitudinal profiles of innate immune cell populations in animals from the *Intermittent Rest Study*. Systemic monocytes and MDSCs were quantified using flow cytometry. Monocyte levels differed significantly between sedentary and intermittent rest rehabilitation groups at week 2 (two-way ANOVA with Šídák's multiple comparisons, ** $p = 0.0013$, $n = 6-7$). MDSC levels in the intermittent rest rehabilitation group differed significantly at day 7 and week 2 (two-way ANOVA with Šídák's multiple comparisons, ** $p = 0.00185$, $n = 6-7$). Data are presented as mean $\pm$ SEM. Figure adapted from *Williams et al.*, submitted [12].

The most pronounced differences were observed in circulating myeloid cell populations, particularly monocytes and MDSCs. Animals undergoing rehabilitation with intermittent rest demonstrated a significant reduction in circulating monocytes by two weeks post-injury compared to sedentary animals (Figure 14). At this timepoint, monocyte levels in the intermittent rest group were 56.2% lower than sedentary controls, indicating a marked attenuation of inflammatory myeloid expansion. This reduction was transient, suggesting a temporally specific immunomodulatory effect associated with rest-modulated mechanical loading.

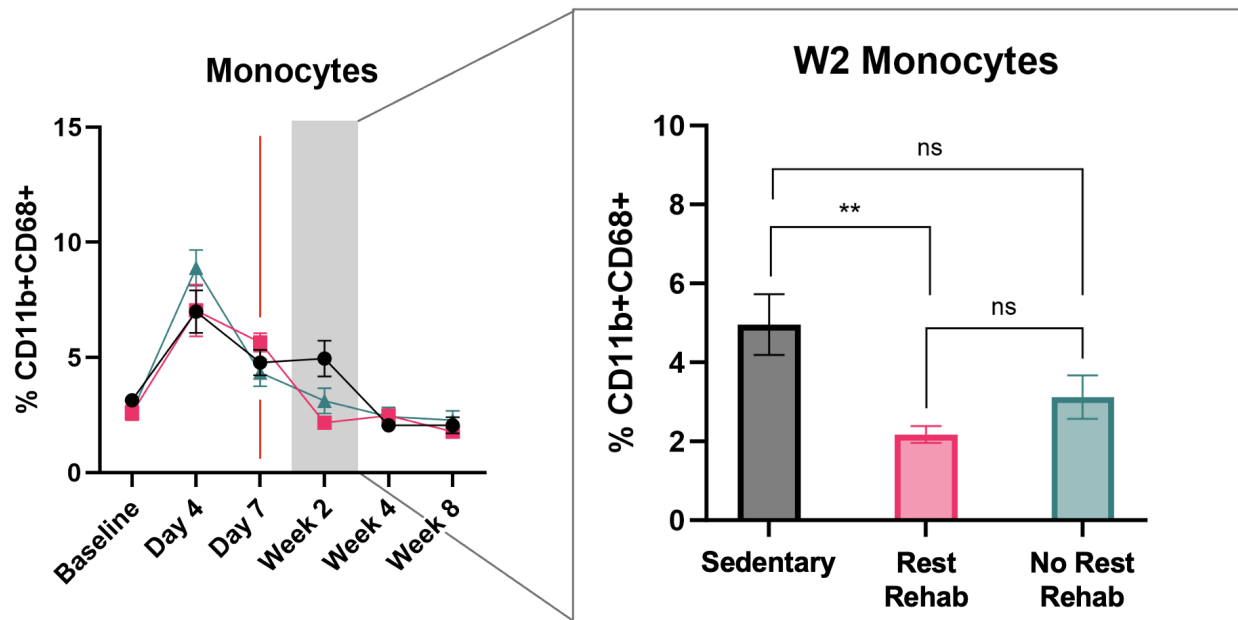


Figure 14. Characterization of circulating monocytes, highlighting week 2 populations in animals from the *Intermittent Rest Study*. Monocyte levels differed significantly between sedentary and intermittent rest rehabilitation groups at week 2 (two-way ANOVA with Šidák's multiple comparisons, ** $p = 0.0013$, $n = 6-7$). Data are presented as mean $\pm$ SEM. Figure adapted from *Williams et al.*, submitted [12].

Similarly, MDSC populations declined more rapidly in the intermittent rest group than in animals undergoing continuous rehabilitation or sedentary recovery. MDSC levels decreased significantly between day 7 and week 2 post-injury in the intermittent rest group, returning to

baseline levels earlier than in other rehabilitation conditions (Figure 15). This accelerated normalization was not observed in the continuous rehabilitation or sedentary groups, which exhibited more prolonged elevations in circulating MDSCs.

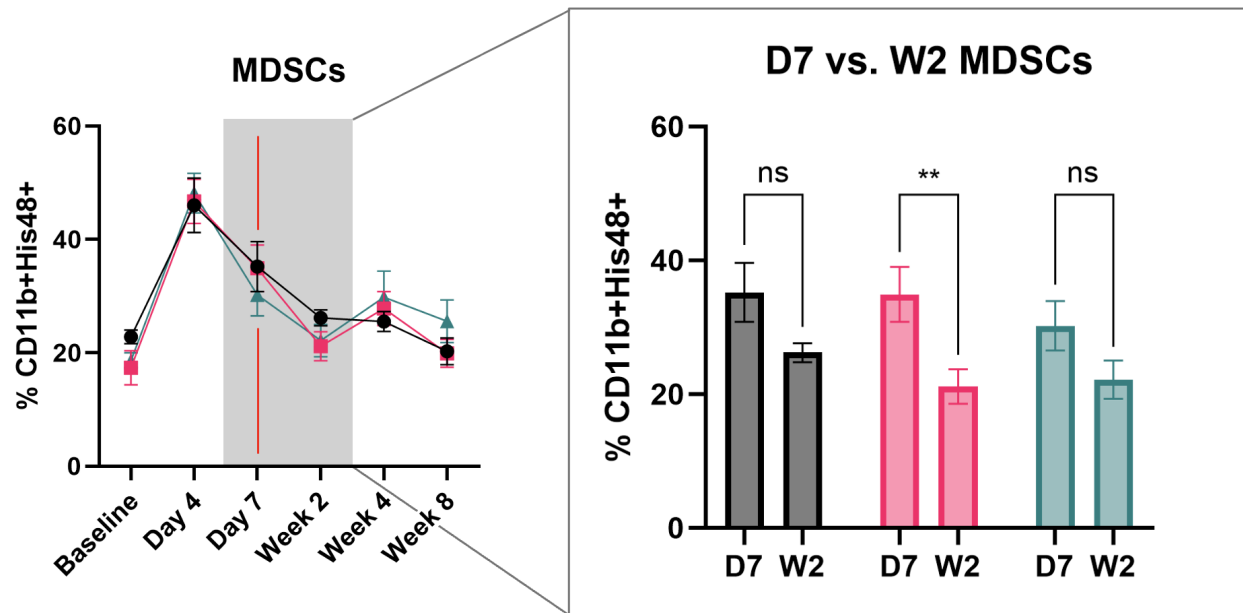


Figure 15. Characterization of circulating MDSCs, highlighting a temporal change across day 7 and week 2 populations in animals from the *Intermittent Rest Study*. MDSC levels in the intermittent rest rehabilitation group differed significantly at day 7 and week 2 (two-way ANOVA with Šídák's multiple comparisons, ** $p = 0.00185$, $n = 6-7$). Data are presented as mean $\pm$ SEM. Figure adapted from *Williams et al.*, submitted [12].

Because all animals exhibited comparable monocyte and MDSC levels prior to the initiation of rehabilitation, this finding implicates intermittent rest as the key variable responsible for the observed differences in immune trajectory. These results demonstrate that rehabilitation incorporating structured rest intervals uniquely promotes the early resolution of myeloid populations following traumatic injury.

4.3.3 *T Cell Populations Exhibited Similar Overall Trajectories but Limited Rehabilitation-Dependent Differences*

Adaptive immune populations, including total T cells, helper T cells, cytotoxic T cells, and regulatory T cells, exhibited similar temporal dynamics across rehabilitation conditions (Figure 16). Following injury, T cell populations decreased during the early inflammatory phase and gradually increased toward baseline levels during the resolution phase. However, unlike myeloid populations, T cell subsets did not exhibit statistically significant differences between rehabilitation conditions.

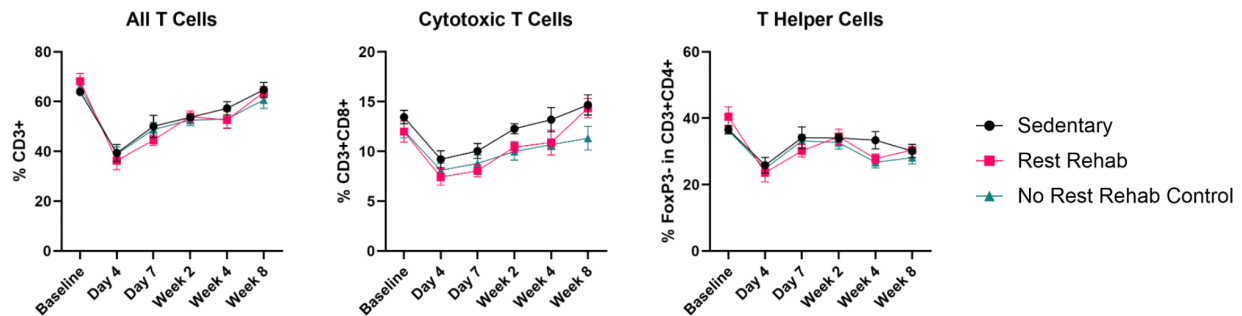


Figure 16. Longitudinal profiles of T cell populations in animals from the *Intermittent Rest Study*. Systemic immune cell characterization of total T cells, cytotoxic T cells, and helper T cells was done using flow cytometry (N = 21, n = 6-7 per group). No significant differences were observed. Data are mean $\pm$ SEM. Figure adapted from *Williams et al.*, submitted [12].

This finding suggests that rehabilitation-dependent immune modulation was primarily confined to innate immune populations, particularly myeloid-derived cells, rather than adaptive immune compartments. These results indicate that early innate immune responses may be more sensitive to rehabilitation structure than adaptive immune responses in this injury model.

4.4 Discussion

This chapter sought to characterize the systemic immune response following traumatic bone injury and determine how rehabilitation structure influences immune cell population dynamics during healing. Longitudinal immune profiling revealed that while traumatic injury

induced a predictable inflammatory response across all animals, the trajectory and resolution of specific immune populations differed depending on rehabilitation protocol. In particular, rehabilitation incorporating intermittent rest intervals uniquely accelerated normalization of inflammatory myeloid populations, whereas continuous rehabilitation—regardless of incline progression—produced uniform immune trajectories that were not significantly altered by progressive loading intensity. These findings demonstrate that systemic immune responses are sensitive not only to the presence of mechanical loading, but also to the temporal structure of rehabilitation.

Consistent with prior studies of musculoskeletal injury, all animals exhibited an early inflammatory phase characterized by elevations in circulating immune populations following surgery [14, 15]. This initial response is a well-established component of fracture healing, driven by innate immune activation and recruitment of inflammatory cells that facilitate debris clearance, angiogenesis, and progenitor cell recruitment [14, 15]. Across both studies, immune populations fluctuated during the early post-injury period and gradually returned toward baseline levels over subsequent weeks, reflecting the expected transition from inflammation to resolution. However, while this general pattern was conserved across all animals, important differences emerged in the rate and extent of immune normalization depending on rehabilitation structure.

In the *Incline Ramp-Up Study*, immune population dynamics were remarkably consistent across all rehabilitation conditions. Despite progressive increases in incline designed to modulate mechanical loading magnitude and timing, circulating immune cell populations—including MDSCs and T cell subsets—exhibited similar trajectories across all groups. This uniformity suggests that incline progression alone was insufficient to meaningfully alter systemic immune responses under continuous rehabilitation conditions. Notably, all animals in this study

underwent uninterrupted treadmill running without rest intervals, indicating that continuous loading may impose a consistent physiological stimulus that overrides subtle variations in loading magnitude. These findings suggest that, within the range tested, progressive increases in incline do not significantly influence systemic immune resolution when loading is applied continuously.

In contrast, the *Intermittent Rest Study* revealed that incorporating rest intervals within the rehabilitation protocol significantly altered immune cell dynamics, particularly within the myeloid compartment. Animals undergoing intermittent rest exhibited more rapid normalization of circulating monocytes and MDSCs compared to animals undergoing continuous rehabilitation or sedentary recovery. Because these differences emerged following initiation of rehabilitation and were not present at baseline, they directly implicate rehabilitation structure as the driving factor modulating immune resolution. These findings suggest that intermittent rest allows for more effective regulation of inflammatory responses, promoting an earlier transition from inflammatory activation toward immune homeostasis.

The observed differences in myeloid populations are particularly relevant given the known roles of monocytes and MDSCs in regulating inflammation and tissue repair. Monocytes are key mediators of early inflammatory responses and contribute to both inflammatory signaling and tissue remodeling [21, 37]. Prolonged elevation of monocyte populations has been associated with chronic inflammation and impaired healing in multiple injury models [37]. Similarly, MDSCs, often studied in cancer research, are immunosuppressive cells that expand during inflammation and modulate immune activity through suppression of T cell function and cytokine production [14, 23, 37]. While MDSCs play important roles in regulating excessive inflammation, sustained elevation of these populations has been associated with impaired

regenerative outcomes [15]. The accelerated decline of these cell populations in animals undergoing intermittent rehabilitation suggests that rest-modulated loading may facilitate more efficient resolution of the inflammatory phase.

These findings align with emerging evidence that mechanical loading influences immune function through mechanobiological signaling pathways. Mechanical stimuli are known to regulate cytokine production, immune cell trafficking, and inflammatory signaling both locally and systemically [38, 47]. However, the temporal pattern of loading appears to be a critical determinant of these effects. Continuous loading may impose sustained physiological stress, prolonging inflammatory activation and delaying immune resolution. In contrast, intermittent loading allows for recovery between loading cycles, potentially preventing overstimulation of inflammatory pathways and enabling more effective immune regulation. This concept is consistent with prior mechanobiology studies demonstrating that rest intervals enhance cellular sensitivity to mechanical stimuli and improve regenerative outcomes [33, 34].

Importantly, these immune findings complement the mechanobiological observations described in Chapter 3. While Chapter 3 demonstrated that rehabilitation structure influences bone regeneration outcomes, the present findings provide a potential biological mechanism underlying these effects. The accelerated normalization of inflammatory myeloid populations observed in intermittent rehabilitation suggests that immune regulation may be a key intermediary linking mechanical loading to regenerative outcomes. These results support the emerging concept of regenerative rehabilitation, in which mechanical stimuli influence healing not only through direct effects on tissue strain and cellular mechanotransduction, but also through modulation of systemic physiological responses.

These findings also highlight the potential utility of systemic immune profiling as a tool for evaluating rehabilitation efficacy. Because immune population dynamics differed between rehabilitation protocols prior to the completion of structural healing, immune profiling may provide early insight into the biological impact of rehabilitation. Unlike structural imaging modalities, which primarily reflect cumulative tissue formation, immune profiling captures dynamic biological responses that occur throughout the healing process. This capability may enable future work for earlier identification of favorable or unfavorable healing trajectories and could ultimately inform individualized rehabilitation strategies.

Several limitations should be considered when interpreting these findings. First, while key immune populations were successfully quantified, staining inconsistencies limited analysis of certain cell populations in specific experimental cohorts. In particular, monocyte populations were not reliably quantified in the *Incline Ramp-Up Study*, precluding direct comparison of this population across all rehabilitation conditions. Second, systemic immune profiling reflects circulating immune populations and does not directly capture local immune responses within the injury site, which also play critical roles in regeneration.

Despite these limitations, these findings demonstrate that rehabilitation structure significantly influences systemic immune dynamics following traumatic injury. Specifically, rehabilitation incorporating intermittent rest promotes more rapid normalization of inflammatory myeloid populations, while continuous rehabilitation produces uniform immune trajectories that are not substantially altered by incline progression. These results establish a mechanistic link between rehabilitation structure and systemic immune regulation and suggest that immune modulation may be an important mediator of rehabilitation-dependent healing outcomes.

Collectively, this work provides foundational evidence that systemic immune profiling can reveal biologically meaningful differences between rehabilitation strategies. These findings establish a framework for understanding how rehabilitation influences systemic physiology and support the broader hypothesis that immune signatures may serve as predictive biomarkers of healing outcomes. This concept is further explored in Chapter 5, which investigates systemic immune profiles in clinical populations and evaluates their potential utility for predicting fracture-related infection.

CHAPTER 5: SYSTEMIC IMMUNE SIGNATURES ASSOCIATED WITH FRACTURE-RELATED INFECTION

5.1 Introduction

Successful bone healing requires a coordinated interplay between mechanical stability, tissue regeneration, and immune regulation. While the preceding chapters established that rehabilitation structure and systemic immune dynamics influence regenerative outcomes, infection represents an additional and often devastating complication that can derail this process. Fracture-related infection remains one of the leading causes of delayed healing, nonunion, and limb loss, particularly in patients with high-energy or open fractures [39, 40]. Despite advances in surgical technique and antimicrobial therapy, early detection of infection remains a persistent clinical challenge. As previously emphasized, overt clinical signs often emerge only after substantial tissue damage has occurred, and commonly used systemic biomarkers—including C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and white blood cell count—lack sufficient specificity and sensitivity to reliably distinguish infection from normal postoperative inflammation [7]. As a result, diagnosis is frequently delayed, limiting opportunities for early intervention and increasing the likelihood of prolonged morbidity, revision surgery, or permanent functional impairment.

These limitations reflect a broader gap in fracture management: current diagnostic approaches primarily detect infection after structural or symptomatic manifestations, rather than identifying early biological changes that precede clinical deterioration. Mounting evidence suggests that infection is not merely a localized event but is accompanied by systemic immune dysregulation [21, 41]. Preclinical studies, including those described in earlier chapters, have demonstrated that systemic immune signatures reflect the biological state of the injury

environment and are closely associated with healing outcomes [12, 14, 15, 41]. Specifically, alterations in circulating immune cell populations have been shown to precede radiographic evidence of impaired regeneration, highlighting the immune system as a potential source of early diagnostic information [12, 14, 15, 41]. These findings raise the possibility that systemic immune profiling could provide a minimally invasive window into infection risk, enabling earlier and more accurate identification of patients at risk for complications.

The immune system plays a central role in coordinating the response to both injury and infection. Following fracture, immune cells regulate inflammation, angiogenesis, and tissue remodeling through tightly controlled signaling networks [14, 15, 21, 42]. However, infection disrupts this coordinated response, often promoting an immunosuppressive phenotype characterized by altered proportions of innate and adaptive immune cell populations [25, 28]. These systemic changes reflect the host's attempt to balance pathogen clearance with inflammation control, but they may also impair effective immune function and contribute to delayed healing. Importantly, these immune alterations can be detected in peripheral blood, making circulating immune cell profiles a promising source of noninvasive biomarkers. Advances in spectral flow cytometry now enable high-dimensional quantification of diverse immune cell subsets, allowing comprehensive characterization of systemic immune states with unprecedented resolution.

While immune profiling provides detailed characterization of cellular composition, translating these complex datasets into clinically actionable insights requires advanced analytical approaches. Multivariate modeling techniques, such as sparse partial least squares discriminant analysis (sPLS-DA), enable identification of combinations of immune variables that distinguish between biological states. Rather than relying on individual markers in isolation, these methods

leverage coordinated patterns across multiple immune populations to improve classification accuracy and capture the multidimensional nature of immune dysregulation. By identifying immune signatures associated with infection, these approaches offer a pathway toward developing predictive diagnostic tools capable of distinguishing patients at risk before overt clinical symptoms emerge.

To address this critical need, this chapter characterizes systemic immune alterations in a clinically relevant cohort of orthopaedic trauma patients with open tibia fractures. Peripheral blood samples were collected longitudinally from patients who developed fracture-related infection. Using spectral flow cytometry, circulating immune cell populations were quantified to assess systemic immune composition across a number of post-injury timepoints. Multivariate modeling was then applied to identify immune signatures capable of distinguishing healed from infected patients. By integrating high-dimensional immune profiling with multivariate analysis, this work demonstrates the feasibility of using systemic immune signatures to distinguish patients with fracture-related infection from those who heal successfully.

Together, this chapter builds directly on the mechanistic insights established in earlier aims by translating systemic immune profiling into a clinical context. Whereas previous chapters demonstrated that immune dynamics reflect regenerative outcomes in controlled preclinical models, this work investigates whether similar systemic immune signatures can be utilized in a clinical population. By characterizing immune dysregulation associated with fracture-related infection and evaluating its ability to distinguish healed from infected patients, this study provides foundational evidence supporting the potential utility of systemic immune signatures as indicators of healing outcome following orthopaedic trauma.

5.2 Methods

5.2.1 *Clinical Cohort and Study Design*

Peripheral blood samples were obtained from orthopaedic trauma patients presenting to our clinical collaborators at Indiana Methodist and NYU Langone hospitals with Type I-III A open tibia fractures as part of an IRB-approved longitudinal clinical study. Patients were retrospectively classified based on healing outcome as either infection (surgical site infection) or healed controls. Infection cases were defined based on clinical diagnosis requiring intervention, while control patients demonstrated successful radiographic healing without evidence of infection.

Peripheral blood samples were collected for a cohort of 11 patients (6 male, 5 female; ages 23-66). Peripheral blood samples were collected at multiple clinical timepoints, including pre-operation (Draw 1), point of discharge (Draw 2), and follow-up visits at approximately 2-4 weeks (Draw 3), 6-8 weeks (Draw 4), and 12-14 weeks (Draw 5). For cellular profiling, whole blood was collected into K₂EDTA tubes, diluted 1:9 in cryopreservation medium (CryoStor CS10, STEMCELL Technologies, Vancouver, BC, Canada), and stored at -80 °C until analysis.

5.2.2 *Whole Blood Processing and Cell Preparation*

Cryopreserved whole blood samples were rapidly thawed under warm running water and immediately transferred into cold red blood cell (RBC) lysis buffer (Invitrogen, Carlsbad, CA, USA) to remove erythrocytes while preserving leukocytes. Specifically, thawed blood was diluted into cold 1x RBC lysis buffer and incubated for 10 minutes on a rocker to facilitate complete lysis while minimizing cellular aggregation. Following lysis, samples were washed with cold FACS buffer consisting of 2% fetal bovine serum (Gibco, Grand Island, NY, USA) in phosphate-buffered saline and centrifuged at 500 G for 5 minutes at 4 °C.

If residual erythrocytes remained, additional RBC lysis steps were performed, with one additional cycle to prevent excessive viability loss. Following lysis and washing, leukocyte pellets were resuspended in FACS buffer and consolidated into a single suspension per sample. Final cell concentrations were adjusted to approximately 1×10^6 cells/mL to enable accurate cell counting and staining.

Cell number and viability were quantified using an NC-200 automated cell viability analyzer (ChemoMetec, Allerød, Denmark). Viability measurements were used to assess sample quality prior to staining, and samples with insufficient viability were excluded from analysis to ensure data quality and accuracy of immune population quantification.

5.2.3 Viability Staining and Fixation

To distinguish viable from nonviable cells, samples were stained using a fixable amine-reactive viability dye (Zombie UV, BioLegend, San Diego, CA, USA), which selectively labels cells with compromised membrane integrity. Cells were washed in cold phosphate-buffered saline and incubated with diluted Zombie UV viability dye at a concentration proportional to cell number. Samples were incubated for 15 minutes in the dark at 4 °C to ensure uniform labeling.

Following viability staining, cells were washed with FACS buffer and fixed using 1x flow cytometry fixation buffer (R&D Systems, Minneapolis, MN, USA) to preserve cellular structure and antigen integrity. Fixed cells were washed and resuspended at standardized concentrations to ensure consistent staining efficiency across samples. This viability staining step enabled exclusion of nonviable cells during downstream flow cytometric analysis, ensuring accurate quantification of immune cell populations. The use of amine-reactive viability dyes allows precise discrimination of viable leukocytes and reduces artifacts caused by cryopreservation or processing-induced cell damage.

5.2.4 Spectral Flow Cytometry Staining and Immune Cell Profiling

Systemic immune cell populations were characterized using a 15-marker spectral flow cytometry panel designed to quantify major innate and adaptive immune subsets. The antibody panel included markers targeting leukocyte lineage and functional subsets, including CD45, CD3, CD4, CD8, CD19, CD14, CD11b, CD15, CD16, CD33, CD56, CD66b, CD123, MHCII, and FoxP3, along with a viability dye for exclusion of nonviable cells. These markers enabled identification and quantification of key immune cell populations, including T cells, regulatory T cells, B cells, monocytes, neutrophils, natural killer cells, and dendritic cell subsets (Table 3).

Table 3. Composition of spectral flow cytometry panel. The panel comprises 15 immune cell types identified with 16 antibodies and a viability dye. All populations were first gated on No Debris, single cells, Zombie UV- and CD45+.

Myeloid Cells		Lymphocytes	
Myeloid Cells	CD11b+	Total T Cells	CD3+
Monocytes	Myeloid Cells/HLA-DR+/CD15-	Total B Cells	CD3-/CD19+
Classical Monocytes	Monocytes/CD16+/CD16-	Helper T Cells	Total T Cells/CD4+/CD8-
Nonclassical Monocytes	Monocytes/CD16+/CD14-	Cytotoxic T Cells	Total T Cells/CD8+/CD4-
PMN-MDSCs	Myeloid Cells/HLA-DR+/CD15+/CD14-	Regulatory T Cells	Helper T Cells/FoxP3+
M-MDSCs	Myeloid Cells/HLA-DR+/CD14+/CD15-	NK Cells	CD3-/CD56+
Neutrophils	Myeloid Cells/CD15+/CD16+	NKT Cells	Total T Cells/CD56+
Basophils	Myeloid Cells/HLA-DR-/CD123+		

Following fixation and viability staining, cells underwent extracellular staining using fluorophore-conjugated antibodies (BD Biosciences, San Jose, CA, USA; BioLegend, San Diego, CA, USA; Invitrogen, Carlsbad, CA, USA; Cytex Biosciences, Fremont, CA, USA) targeting surface markers. Prior to antibody staining, cells were incubated with Fc receptor blocking solution (CD32, Invitrogen, Carlsbad, CA, USA) to minimize nonspecific antibody binding. Cells were then incubated with antibody cocktails containing fluorophore-conjugated antibodies targeting extracellular markers for 25-45 minutes in the dark at room temperature.

Following extracellular staining, cells were fixed and permeabilized to allow intracellular staining of transcription factors, including FoxP3, which enables identification of regulatory T cell populations. Intracellular staining was performed using permeabilization buffer (Invitrogen, Carlsbad, CA, USA) and fluorophore-conjugated antibodies targeting intracellular markers.

Following staining, cells were washed and resuspended in FACS buffer for acquisition. Compensation controls were prepared using antibody capture beads (BD Biosciences, San Jose, CA, USA) stained with individual fluorophore-conjugated antibodies to enable accurate spectral unmixing during analysis. Samples were acquired on a spectral flow cytometer (Cytex Aurora, Cytex Biosciences, Fremont, CA, USA), which enables high-dimensional analysis of immune cell populations using full-spectrum fluorescence detection.

5.2.5 Flow Cytometry Data Processing and Immune Cell Quantification

Flow cytometry data were analyzed using FlowJo software (version 10.8.2; FlowJo LLC, Ashland, OR, USA) to identify and quantify immune cell populations. Following acquisition, data underwent compensation and spectral unmixing using compensation controls and instrument reference controls. Viable leukocytes were first identified by gating on CD45-positive cells while excluding debris and nonviable cells using viability dye staining. Sequential gating strategies

were used to identify the aforementioned major immune cell populations based on marker expression profiles (Table 3).

5.2.6 Multivariate Modeling and Statistical Analysis

To identify immune signatures associated with infection, multivariate modeling was performed using partial least squares discriminant analysis (sPLS-DA). This supervised dimensionality reduction technique identifies combinations of immune variables that best distinguish between predefined outcome groups, enabling classification based on coordinated patterns of immune cell composition.

Immune cell frequency data were normalized and analyzed using statistical computing software in R using the mixOmics package [43]. sPLS-DA models were constructed to evaluate separation between infection and healed groups based on immune profiles measured at early clinical timepoints. Model performance was evaluated based on group separation and variable contributions to discriminant components.

Variable loadings were constructed to identify immune cell populations contributing most strongly to infection classification. These analyses enabled identification of systemic immune signatures associated with infection and evaluated their potential as predictive biomarkers.

5.3 Results

5.3.1 Systemic Immune Profiling Reveals Distinct Alterations Associated with Infection

To characterize systemic immune dynamics associated with fracture-related infection, we quantified the frequencies of 16 circulating immune cell populations at two early, clinically relevant timepoints: Draw 1 (D1), collected pre-operation, and Draw 3 (D3), collected during a 2-4 week postoperative follow-up.

Comparison of immune cell frequencies between healed and infected patients revealed distinct shifts in circulating immune composition. Volcano plot analysis demonstrated significant differences in multiple immune populations between outcome groups (Figure 17). Notably, plasmacytoid dendritic cells (pDCs) were significantly elevated at D3 in patients who developed infection relative to healed controls. Regulatory T cells (Tregs) also exhibited moderate but significantly increased frequencies at D3 in infected patients. In contrast, myeloid cell populations measured at D1 were reduced in patients who later developed infection. Total T cell frequencies at D1 showed moderate increases in infected patients relative to healed controls. Total T cell frequencies at D1 showed moderate increases in infected patients relative to healed controls.

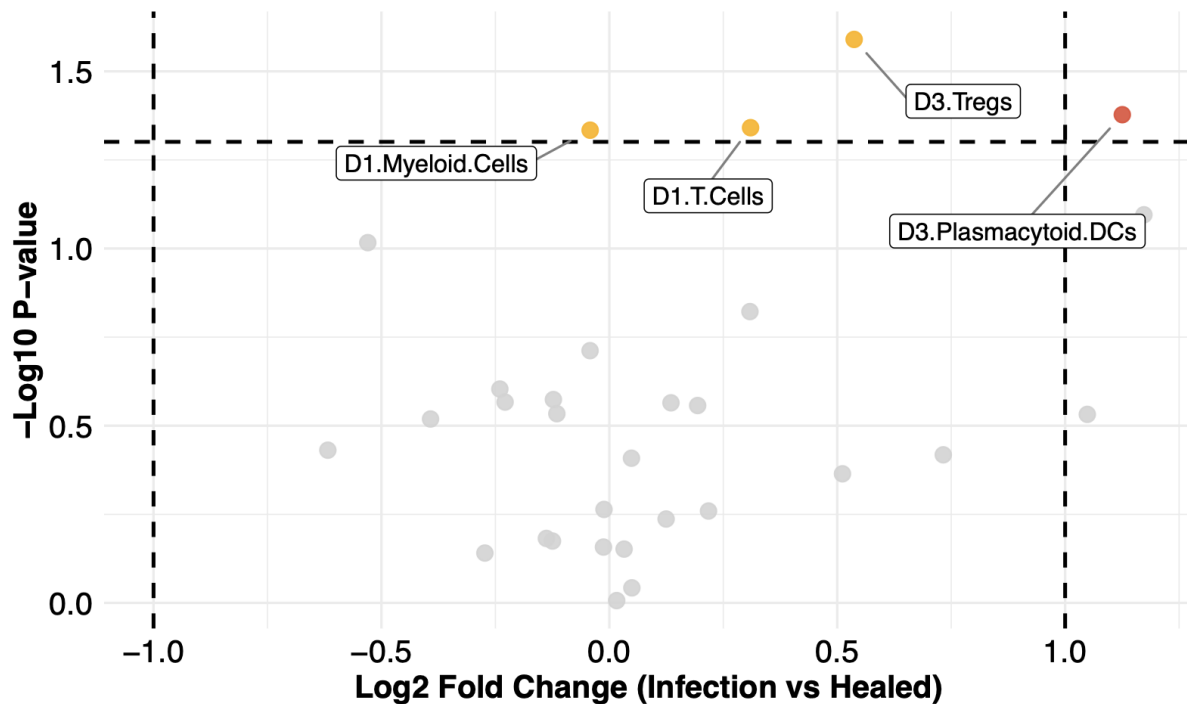


Figure 17. Volcano plot showing differences in circulating immune cell populations between infected and healed patients. Dashed lines mark $\log_2$ fold change (± 1) and $-\log_{10}$ p-value (~ 1.3) thresholds.

These coordinated changes indicate that fracture-related infection is associated with measurable alterations across both innate and adaptive immune compartments, with increases in regulatory immune populations and reductions in early myeloid cell frequencies.

5.3.2 *Multivariate Modeling Distinguishes Infection and Healed Cohorts Based on Immune Composition*

To determine whether systemic immune profiles could distinguish infection outcomes, sparse partial least squares discriminant analysis (sPLS-DA) was performed using immune cell frequency data from D1 and D3 (Figure 18). The first two discriminant components accounted for 17.5% and 13.0% of the total variance in immune composition, respectively.

Projection of individual patient samples onto these components revealed clear separation between infection and healed cohorts along Component 1. Healed patients clustered toward negative values of Component 1, while infected patients clustered toward positive values, indicating that systemic immune composition differed between outcome groups. This separation demonstrates that immune cell frequency patterns contain sufficient information to discriminate infection status.

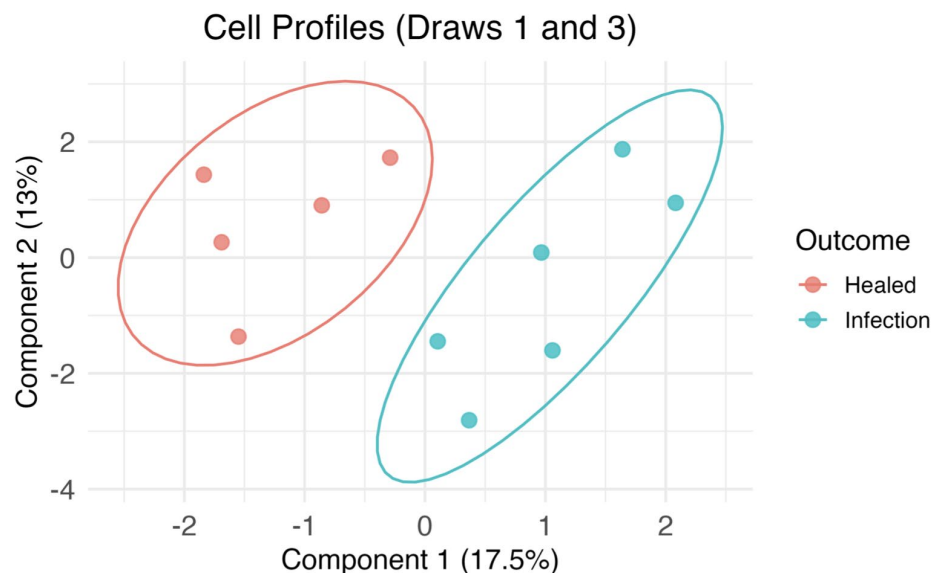


Figure 18. sPLS-DA plot showing separation of healed and infected patient samples from Draws 1 and 3 along Components 1 and 2. Analysis was able to separate infection and healed groups along Component 1 (17.5% variance) driven by immune composition.

5.3.3 *Specific Immune Cell Populations Drive Separation Between Infection and Healed Patients*

To identify immune cell populations contributing most strongly to cohort separation, variable loadings from the sPLS-DA model were examined (Figure 19). The top five contributors to Component 1 included pDCs at D3, Tregs at D3, total T cells at D1, and myeloid cells at D1.

pDCs and Tregs exhibited strong positive loadings, indicating higher frequencies in infected patients relative to healed controls. In contrast, myeloid cells at D1 exhibited negative loadings, indicating reduced frequencies in infected patients. These loading patterns demonstrate that infection status is associated with coordinated shifts across multiple immune cell populations.

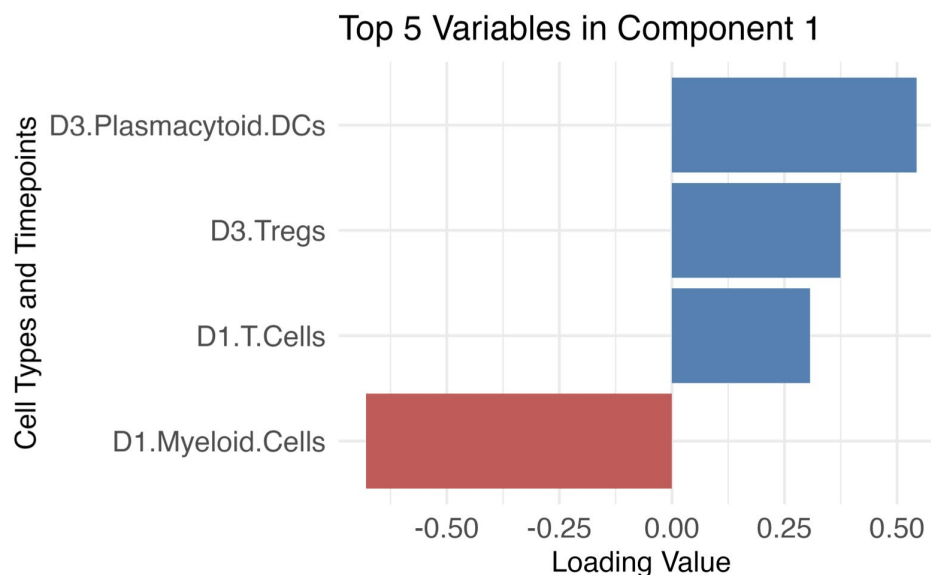


Figure 19. Top five contributors to Component 1 of sPLS-DA plot showing separation of healed and infected patient samples from Draws 1 and 3. Late pDCs and Tregs showing strong positive loadings and early myeloid cells negative loadings.

Collectively, these findings show that fracture-related infection is associated with distinct systemic immune alterations detectable in peripheral blood and that multivariate modeling of immune cell composition enables discrimination between infected and healed patients.

5.4 Discussion

The findings of this study demonstrate that fracture-related infection may be associated with distinct systemic immune alterations that are detectable in peripheral blood and distinguish infected patients from those who heal successfully. By quantifying circulating immune cell populations longitudinally and applying multivariate modeling, this work identified coordinated shifts across innate and adaptive immune compartments that characterize infection-associated immune dysregulation. Specifically, infection was associated with increased frequencies of pDCs and Tregs, along with reductions in early myeloid cell populations. These alterations contributed strongly to multivariate model separation between infected and healed patients, demonstrating that systemic immune composition contains predictive information regarding infection status.

These findings support the concept that infection is not solely a localized complication but is accompanied by systemic immune dysregulation that emerges during early stages of the clinical course. Elevated Treg populations observed in infected patients suggest a shift toward an immunosuppressive immune phenotype. Tregs play a critical role in controlling excessive inflammation, but their expansion may also impair effective immune responses to pathogens, potentially contributing to persistent infection [44]. Similarly, increased pDC frequencies may reflect altered innate immune signaling during infection. pDCs are key regulators of immune activation through cytokine production and antigen presentation, and their expansion may indicate dysregulated immune coordination in response to infection [45].

In contrast, reductions in circulating myeloid cell populations in patients who developed infection suggest impaired innate immune activation. Myeloid cells, including monocytes and neutrophils, serve as primary responders to tissue injury and microbial invasion [21, 37]. Decreased early myeloid cell frequencies may reflect altered immune recruitment dynamics or impaired innate immune responsiveness, which could compromise pathogen clearance and contribute to infection susceptibility. Importantly, these alterations were detectable at early clinical timepoints, supporting the hypothesis that systemic immune dysregulation precedes overt clinical diagnosis of infection.

The ability of sPLS-DA to distinguish infection and healed cohorts further demonstrates the feasibility of using multivariate immune profiling to identify systemic immune signatures associated with healing outcome and supports this approach as a hypothesis-generating framework for future predictive studies. Rather than relying on individual biomarkers, this multivariate framework captures coordinated patterns across immune cell populations that reflect underlying biological states. The clear separation between outcome groups along the primary discriminant component indicates that systemic immune profiles encode information relevant to infection status. Notably, specific immune populations emerged as major contributors to classification, further supporting the biological relevance of these immune alterations. Despite variability inherent to human clinical samples, systemic immune signatures were sufficient to distinguish healing outcomes, validating the technical and analytical pipeline used in this study and supporting its application in larger cohorts to refine classification performance and further evaluate its clinical utility.

These clinical findings build directly on the mechanistic framework established in earlier aims of this thesis. In Aim 2, systemic immune profiling in a controlled preclinical model

demonstrated that immune cell population dynamics reflect healing trajectories and can distinguish successful regeneration from impaired healing. The present findings extend this concept into a clinical context, demonstrating that systemic immune signatures also distinguish infection outcomes in human patients. Together, these results support a unified framework in which systemic immune state serves as a sensitive indicator of biological processes occurring at the injury site. This relationship highlights the potential of peripheral blood immune profiling as a noninvasive tool for monitoring fracture healing and detecting complications.

Several limitations should be considered when interpreting these results. The sample size was relatively small, reflecting the challenges of longitudinal clinical sample collection and maintaining cell viability in cryopreserved specimens. Additionally, as samples were collected and processed over an extended enrollment period, batch effects related to specimen handling, cryopreservation, and cytometry acquisition may also have contributed to variability in measured immune profiles. While multivariate modeling demonstrated separation between outcome groups, further validation in larger independent cohorts is necessary to establish robust and generalizable immune signatures. Importantly, patient enrollment and sample collection are ongoing, which will enable future analyses with expanded cohorts and additional longitudinal timepoints to refine classification performance and further evaluate the clinical relevance of these findings.

In summary, this study demonstrates that fracture-related infection is associated with distinct systemic immune alterations that are detectable in peripheral blood and distinguish infected patients from healed controls. Multivariate modeling identified immune signatures driven by coordinated changes in dendritic, regulatory, and myeloid cell populations, supporting the concept that infection is accompanied by early systemic immune dysregulation.

These findings extend prior mechanistic work by demonstrating the clinical relevance of systemic immune profiling and establish a foundation for developing immune-based diagnostic tools to enable earlier detection of infection and improve outcomes following orthopaedic trauma.

CHAPTER 6: SUPPLEMENTARY RESEARCH CONTRIBUTIONS TO ORTHOPAEDIC TRAUMA AND REGENERATIVE REHABILITATION

6.1 Overview of Supplementary Research Contributions

Throughout my undergraduate training, I contributed to several additional research studies that, while not forming the central narrative of this thesis, were instrumental in shaping my development as a researcher and informing my understanding of biomaterials, immune responses, and regenerative medicine. These projects provided valuable experience in biomaterial fabrication, animal surgery, immune profiling, and experimental design, and exposed me to the broader translational context of orthopaedic tissue engineering. Participation in these studies strengthened my technical skills, scientific reasoning, and appreciation for the interdisciplinary nature of regenerative rehabilitation research. Brief summaries of two such studies and my contributions are provided below.

6.2 Automated Fabrication of Electrospun Tubular Meshes for Bone Regeneration

Electrospun poly(ϵ -caprolactone) (PCL) meshes are widely used in bone tissue engineering as structural scaffolds that support localized delivery of osteoinductive biologics such as bone morphogenetic protein-2 (BMP-2). However, the original fabrication process required producing flat electrospun sheets, manually cutting and perforating them, and rolling and gluing them into tubular structures. This workflow was time-intensive, technically demanding, and often resulted in inconsistencies between meshes. These limitations reduced manufacturing efficiency and created variability that could impact both experimental reproducibility and clinical translation. This study introduced an automated method for producing seamless tubular meshes directly on cylindrical mandrels, followed by controlled

perforation using a programmed soldering system. This improved fabrication efficiency dramatically, reducing production time by approximately 67% while maintaining equivalent mechanical properties and in vivo performance in a rat segmental defect model.

My primary contribution to this study involved fabricating meshes using the original manual method to serve as a comparison against the newly developed automated workflow. Because I was trained extensively in the legacy fabrication process, I was responsible for producing meshes used in experimental validation, ensuring that comparisons between old and new manufacturing approaches were accurate and reliable. This work provided critical baseline data that helped demonstrate the advantages of the new method. I also contributed to manuscript preparation, including figure creation and editorial revisions.

This work represents an important step toward improving the scalability and reproducibility of biomaterial scaffold manufacturing. By simplifying mesh fabrication and eliminating failure-prone manual assembly steps, this method improves the feasibility of using electrospun mesh systems in both research and clinical settings. My involvement in this project strengthened my understanding of biomaterial fabrication, experimental controls, and the importance of reproducibility in translational biomedical research.

This work resulted in a published methods manuscript in *Tissue Engineering Part C*, on which I am a co-author [46].

6.3 Immune Modulation Following Local BMP-2 Delivery in Segmental Bone Defects

Bone morphogenetic protein-2 (BMP-2) is a powerful osteoinductive growth factor widely used to promote bone regeneration, but its clinical application is limited by dose-dependent inflammatory responses and adverse effects. This study investigated how varying BMP-2 doses influence both local and systemic immune responses following bone defect injury.

Using a sustained-release alginate-mesh delivery system in a rat segmental defect model, we performed longitudinal immune profiling and imaging to evaluate the biological consequences of BMP-2 administration. The study demonstrated that while supraphysiologic BMP-2 doses induced stronger systemic immune activation and inflammatory signaling, lower doses were capable of supporting bone regeneration without provoking excessive inflammatory responses.

My contributions to this study spanned multiple stages of the experimental workflow. I assisted with surgical procedures, including preparation and implantation of biomaterial constructs in the segmental defect model. I was also involved in extensive data collection, including peripheral blood collection, preparation and staining of samples for flow cytometric immune profiling, and acquisition and analysis of longitudinal micro-CT scans to assess bone regeneration. In addition, I contributed to reviewing and editing the manuscript during its preparation.

This study was particularly impactful because it provided important insights into the interaction between biologic therapies and the immune system during bone regeneration. My involvement gave me hands-on experience with animal surgery, immune profiling, and imaging-based outcome assessment. These experiences significantly strengthened my technical skills and deepened my understanding of the immune system's role in tissue regeneration, which became a central theme of my subsequent research.

This work resulted in a manuscript currently in preparation for submission to the *Journal of Bone and Mineral Research*, on which I am a contributing author [47].

CHAPTER 7: CONCLUSIONS AND FUTURE CLINICAL IMPACT

This thesis establishes an integrated, translational framework demonstrating that bone healing outcomes following orthopaedic trauma are governed by a dynamic interaction between mechanical loading and systemic immune responses. By combining controlled preclinical experimentation, longitudinal immune profiling, and multivariate predictive modeling, this work identifies mechanistic determinants of healing and lays the foundation for future prognostic tools capable of improving patient care.

The findings presented in Chapter 3 demonstrate that rehabilitation outcomes are governed not simply by cumulative mechanical load, but by the temporal structure and magnitude of that loading. Nonlinear predictive modeling revealed that rest is a critical determinant of healing, and experimental validation confirmed that rehabilitation protocols incorporating intermittent rest significantly enhance bone regeneration compared to continuous loading. These results establish the existence of an optimal rehabilitation “Goldilocks zone,” characterized by moderate activity paired with sufficient recovery. Importantly, these findings challenge the prevailing clinical paradigm that more activity uniformly improves healing and instead support a shift toward structured, biologically informed rehabilitation strategies.

Chapter 4 expands this framework by demonstrating that bone healing is accompanied by coordinated systemic immune responses that evolve over time and differ across rehabilitation conditions. Longitudinal immune profiling revealed distinct temporal patterns in circulating immune cell populations, including monocytes, MDSCs, and T cells, which were associated with regenerative outcomes. These findings suggest that systemic immune responses not only reflect local healing status but may also serve as early indicators of regenerative trajectory. This work

provides critical mechanistic insight into the biological processes underlying fracture repair and establishes a foundation for integrating immune biomarkers into prognostic models.

Chapter 5 translates these mechanistic insights to a clinical context, demonstrating that systemic immune signatures can distinguish patients who develop fracture-related infection from those who heal successfully. Multivariate modeling approaches, including sPLS-DA, identified coordinated immune signatures that separated healed and infected patients. These findings demonstrate the feasibility of using systemic immune profiling as a predictive diagnostic tool and highlight the potential of immune-informed modeling approaches to enable earlier detection of complications than current clinical methods allow.

Together, these findings converge on a central conclusion: bone healing is governed by a coupled mechanobiological and immunological system, and predictive modeling provides a powerful framework for understanding and forecasting healing outcomes. Rather than relying solely on static imaging or generalized rehabilitation guidelines, future clinical strategies may incorporate longitudinal biological data and subject-specific mechanical profiles to guide personalized treatment.

The translational implications of this work are substantial. First, these findings support the development of individualized rehabilitation protocols that account for the temporal structure of loading and recovery rather than prescribing uniform activity restrictions. Second, systemic immune profiling may serve as a minimally invasive tool to identify patients at risk for nonunion or infection before structural complications become radiographically apparent. Finally, the integration of predictive modeling and immune biomarkers provides a pathway toward clinically deployable prognostic tools capable of informing treatment decisions in real time.

Future work should focus on validating these predictive frameworks in larger clinical cohorts and integrating multimodal datasets, including immune, mechanical, imaging, and proteomic measurements. Advances in wearable technology and high-dimensional immune profiling offer opportunities to continuously monitor both mechanical loading and systemic physiology, enabling dynamic, adaptive rehabilitation strategies tailored to individual patients. Ultimately, such approaches could transform fracture care from a reactive process based on delayed structural assessment to a proactive, predictive model guided by early biological indicators.

In conclusion, this thesis demonstrates that predictive modeling and systemic immune profiling provide a powerful and translational framework for understanding bone regeneration and predicting healing outcomes. By bridging mechanobiology, immunology, and computational modeling, this work establishes a foundation for future diagnostic and rehabilitation strategies aimed at improving recovery and reducing complications in orthopaedic trauma patients.

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