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The local and systemic effects of immune function on fracture healing

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Abstract The immune system plays an integral role in the regulation of cellular processes responsible for fracture healing. Local and systemic influences on fracture healing correlate in many ways with fracture-related outcomes, including soft tissue healing quality and fracture union rates. Impaired soft tissue healing, restricted perfusion of a fracture site, and infection also in turn affect the immune response to fracture injury. Modern techniques used to investigate the relationship between immune system function and fracture healing include precision medicine, using vast quantities of data to interpret broad patterns of inflammatory response. Early data from the PRECISE trial have demonstrated distinct patterns of inflammatory response in polytrauma patients, which thereby directly and indirectly regulate the fracture healing response. The clearly demonstrated linkage between immune function and fracture healing suggests that modulation of immune function has significant potential as a therapeutic target that can be used to enhance fracture healing.

Keywords: immune modulation, fracture healing, precision medicine

1. Immune Cellular and Molecular Contributions to Fracture Healing

Fracture healing remains one of the hottest topics in the field of trauma surgery. Over the years, our knowledge has vastly expanded because of the advances in molecular medicine, molecular biology, diagnostics, and immunology. Stages of fracture healing have been described including hematoma formation, inflammation, angiogenesis, cartilage formation (calcification, cartilage removal, bone formation), and remodeling (Fig. 1). Each of these stages involves the interaction of mediators (signaling molecules) with different cell types and cell populations acting within the local tissue environment.[1]

Interestingly, while the focus of research was for some time on the behavior and activity of progenitor cells, recently it became clear that the innate immune system participates in this process of fracture repair with the involvement of macrophages, monocytes, neutrophils, natural killer (NK) cells, and a variety of cytokines. In addition, the adaptive immune system also contributes to this process, particularly the T and B lymphocytes. This interplay between progenitor cells, mediators, and innate adaptive immune systems is known as "osteoimmunology."[2]

Experimental studies have provided evidence in relation to the involvement of both the innate and the adaptive immune system in each of the different phases of bone repair. During the initial inflammatory phase, the formation of hematoma facilitates release of platelet-derived mediators (IL-6, IL-1, TNF-α) and within the first few days trapping of lymphocytes (T & B cells), monocytes, and macrophages takes place, which are capable of secreting further proinflammatory cytokines and growth factors.[3]

The process of cleaning of the damaged bone edges and the necrotic surrounding soft tissue follows, which is undertaken by osteoclasts and polymorphonuclear leucocytes (PMNLs). PMNLs are attracted by debris and secrete chemokines (CCL2, IL-6), which then leads to the attraction of macrophages. Noteworthy, the receptor activator of nuclear factor kappa-B ligand (RANKL) produced by activated T lymphocytes and NK cells induces the differentiation of the osteoclasts from monocytes.

In parallel to this process, migration of musculoskeletal stem cells (MSC) is supported by stromal derived factor-1 (SDF-1), TNF-α, macrophage-derived chemokines, MCP-1, and monocyte inflammatory protein 1 alpha (MIP-1a) as well as the chemokine CXCL7 released by NK cells. Interestingly, MSC also have the

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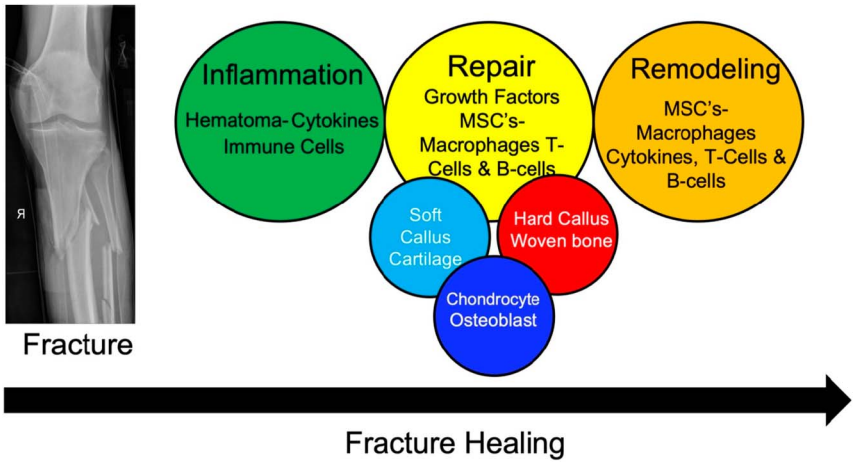

Figure 1. Stages of fracture healing.

capacity to contribute to the clearing process of dead tissues by phagocytosis of apoptotic cells.[4]

This background collaborative work between the interaction of the different cell types and MSC provides the foundation for the next phase, the reparative phase of fracture healing. However, for the repair phase to initiate, the inflammatory phase must be completed. This is supported by the so-called "licensing of MSC," which refers to the activation of MSCs to perform immunosuppressive functions. During this stage, molecules including IFN-γ, TNF-α, and IL-17 contribute to this process. NK cells and T lymphocytes are believed to be the major sources of TNF-α availability. Secretion of other mediators follows the wave of MSC licensing that further augments the osteogenic potential of MSC. Toll-like receptors (TLRs) support the osteogenic differentiation of MSC using NFκ-b and PI3 kinase signaling pathways. In addition, macrophages release bone morphogenetic proteins and oncostatin M while activated monocytes induce the expression level of *Cbfa1/Runx2* and alkaline phosphatase (ALP) by MSC.[5]

The desirable decline of immune cell response is supported by MSC that release soluble molecules (TGF-β, indoleamine 2,3-dioxygenase [IDO], inducible nitric oxide synthases [iNOS], PGE2, IL-1 receptor antagonist, and tumor necrosis factor-inducible gene 6 [TSG6]) and also produce IL-10 which facilitates the generation of anti-inflammatory $CD4^{+}CD25+Foxp3+$ T reg lymphocytes, thus suppressing the proliferation and functions of proinflammatory Th1 and Th17 subsets and inhibiting the proliferation, secretory, and cytotoxicity functions of cytokine-activated NK cells. In addition, the function and the migration of B lymphocytes is decreased while MSC also regulate macrophages to exhibit the anti-inflammatory M2 phenotype that suppresses innate and adaptive immune responses by the IL-10 and TGF-β pathways. Overall, it can be appreciated that MSC

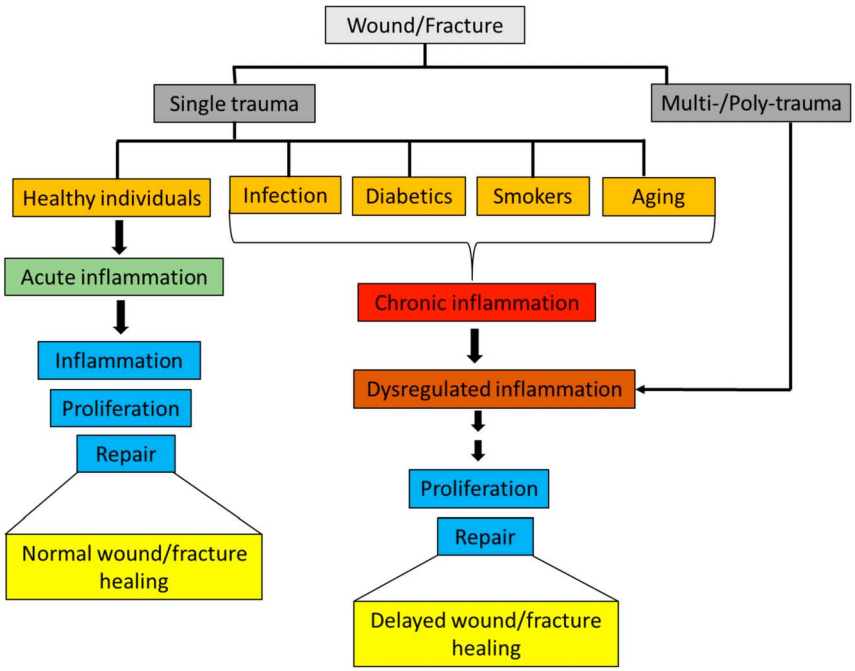

Figure 2. Classification of wounds/fracture and wound/fracture healing outcomes.

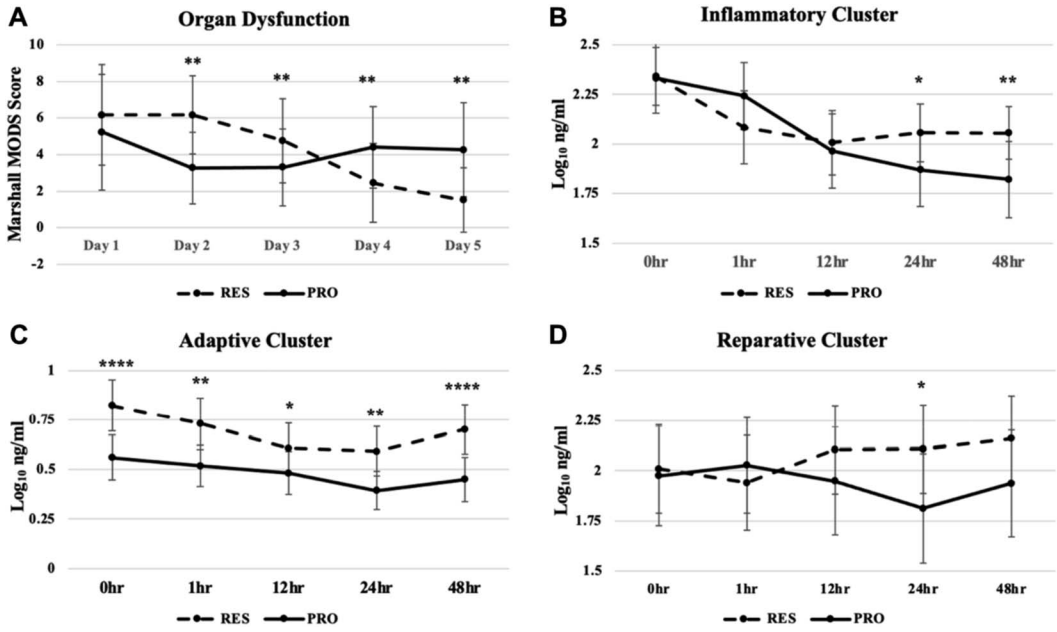

Figure 3. Organ dysfunction and immunologic profiles in RES and PRO patient groups. A: RES patients (dashed lines) have decreases in MODS scores beginning on day 2 through day 5. By contrast, MODS scores increase in PRO patients (solid lines) in the same time frame. B: Inflammatory cluster scores diverge in the 12 hour–24 hour interval with increases in RES patients compared with PRO patients. C: Adaptive cluster scores are significantly higher in RES patients at all 5 time points including immediately at the time of admission. D: Reparative cluster scores showed increases at the 24-hour time point in RES patients and trended higher at 48 hours. Results are using standard t tests. *P, 0.05; **P , 0.01; ***P , 0.001; ****P , 0.0001.

being licensed by inflammatory signals perform the necessary activities to suppress the inflammatory responses of immune cells as part of a negative feedback mechanism.[6]

The reparative phase of fracture healing is supported by the stimulatory signals on cells induced by PDGF, TGF-b, IGF, FGF-1, and bone morphogenetic protein. The hallmark event is the differentiation of MSC into osteoblasts or chondroblasts forming soft callus, providing support for new blood vessel formation with metalloproteinase-dependent mechanisms. Other cell types also have important roles to play. For instance, macrophages promote induction of angiogenesis, collagen type I deposition, produce matrix metalloproteinases (MMPs) to degrade the cartilage matrix, and regulate MSC differentiation to osteoblasts. B and T cells release TNF-α that leads to the death of mature chondrocytes helping the transition from cartilage into bone. Finally, regulation of soft into hard callus is supported by the function of macrophages, T and B cells, various cytokines, and growth factors (GFs).

Subsequently, the remodeling phase follows during which the functions of both osteoblasts and osteoclasts is regulated by the activities of MSC, macrophages, T cells, and cytokines (TNF-α and IL-17). Interestingly, osteoblasts release RANKL and macrophage-colony stimulating factor, thus improving the survival/function of osteoclasts. MSC have an inhibitory effect on monocyte differentiation into osteoclasts through the production of osteoprotegerin (OPG).

Overall, the remodeling phase denotes the equilibrium that must prevail between the function of osteoblasts and osteoclasts.

2. Immune Dysregulation Causes Impaired Wound and Fracture Healing

It is recognized that there are many risk factors for fracture nonunions.[7] Many are modifiable, such as smoking, while others are relatively nonmodifiable such as age, osteoporosis, steroids, male sex, and renal insufficiency. To add to the challenges, risks of impaired healing increase the most with various patient independent factors such as open fractures with substantial bone loss and/or soft tissue injury with periosteal stripping and compromised perfusion, as well as infection. Most of these risk factors affect the immune response to the injury (Fig. 2). Recent work has demonstrated not just an association between dysregulated immune response and impaired healing, but that restoring a more normal immune response resulted in better fracture healing. A recently published review highlights how normal healing of different wound types is affected by various comorbidities and risk factors and how that healing is affected by the temporal immune response.[8] Here we will review the impacts of infection, an impaired or absent soft tissue envelope, and polytrauma on the immune response to fracture.

Infection is a relatively common complication of open fractures and is almost always associated with delayed or nonunions.[9] It is fairly well established that the different bacterial phenotypes illicit distinct immune response.[10] The planktonic phenotype generally causes a robust inflammatory reaction whereas the biofilm phenotype seems to skew the reaction toward more of a resolving environment. An example of this is how *staphylococcus aureus* (S. aureas) biofilms promote the M2 macrophage phenotype, which may contribute to the inability of the host to clear biofilm infections.[11] Interestingly, this has implications on fracture healing. In a rodent model with a noncritical size defects, uncontaminated fractures demonstrate healing, but when contaminated with *Staphylococcus aureus*, the bone did not regenerate.[12] Two different antibiotic powders were placed topically in bone defects to evaluate their ability to eradicate established infection. Rifampin, which works against both planktonic and biofilm phenotypes, eradicated the infection and allowed bone formation within the defect. Conversely,

vancomycin is primarily effective against only planktonic bacteria. In the wounds treated with vancomycin, there were robust quantities of bacteria in the bone and on the implants. Remarkably, there was also robust bone formation. Vancomycin killed the immunogenic planktonic bacteria but not the organisms within the biofilm. This explains why hardware can often be retained while administering a suppressive systemic antibiotic after early detection of infection. Most of these fractures are able to heal, and the implants can be removed after union.[13]

The Gustilo–Anderson open fracture classification has demonstrated that open fractures with more extensive concomitant soft tissue injury to the surrounding envelope have the highest complication rates.[14] Type IIIB fractures typically require soft tissue transfer to cover the exposed bone defect, and it is general consensus that this muscle flap promotes healing by restoring blood flow and providing multipotent stem cells to these severe wounds. Recently, a series of preclinical studies have suggested that immune dysregulation may be a driving factor of poor healing in Type 3B open fractures. In a rat open fracture model (tibial osteotomy with tibialis anterior muscle loss), it was observed that muscle loss impaired bone healing.[15] Specifically, there was diminished mechanical strength and lower quantity of mineralized healing bone. The injured muscle surrounding the fracture site had both innate and adaptive immune responses that were not typical of canonical muscle injury healing. The muscle injury perturbed the inflammatory phase of fracture healing, with increased CD3(+) lymphocytes and $CD68^{+}$ macrophages in the fracture callus early after injury. As a result, it was believed that repairing the volumetric muscle loss (VML) injury would improve fracture healing. However, when a decellularized extracellular matrix that has been shown to improve force production in muscles that have irrecoverable frank loss of tissue, but does not regenerate appreciable amount of skeletal muscle, is inserted at a fracture site with VML, it does not improve fracture healing.[16] Recent work demonstrated that the use of autologous minced muscle grafts to treat VML defects in mice improves function and regenerates new fibers. Remarkably, minced muscle grafts restored the immune response and rescued the fracture healing, but because it provided cells and promoted vascularization, biological not immunological intervention may have caused the healing.[17] Subsequently, it was shown that a 14-day systemic dose of FK506 to mice with an open tibial fracture, which suppresses the immune response by inhibiting calcineurin, thereby preventing growth and differentiation of the T-cell response, rescued the recovery of tibial mechanical properties in the presence of concomitant muscle trauma.[18] However, it did not augment mechanical recovery of an isolated osteotomy, which did not have muscle trauma. FK506 attenuated the immune response because T lymphocytes and macrophage presence within the traumatized musculature was heightened by trauma. In comparison, the T-lymphocyte presence was reduced, but macrophage presence was maintained within fracture callus. FK506 did not improve the function of the injured muscle. This suggests that one potential mechanism of action of FK506 may be that it reduces local T-lymphocyte presence within the injured musculoskeletal tissue. This drug has fairly broad actions besides being an immunosuppressant, so further investigation is needed to fully elucidate the mechanism.

Fractures in polytrauma patients also can heal poorly. To illustrate the relationship between polytrauma and delayed healing, a rat polytrauma model which encompassed a 3 mm osteotomy, blunt chest trauma, and full-thickness scald burn was used. Significant differences in the bone volume fraction between polytrauma and animals that only underwent osteotomy were observed. Polytrauma animals also exhibited significantly altered gene expression in osteogenic pathways as well as the innate and adaptive immune response. Significantly elevated plasma levels of high-mobility group box 1 protein (HMGB1) at 6 and 24 hours post-trauma were seen.[19] Extracellular HMGB1, a chromosome-binding protein, is passively released by necrotic tissue or stressed cells and is actively secreted by monocytes. When this proinflammatory upstream cytokine is blocked by an HBGB1 antibody, healing is restored.[20] Importantly, blocking inflammation upstream restores RAGE and TLR4 surface expression on circulating T cells and increases the number of γδTCR + T cells at the fracture site. The observations provide rich potential targets for future interventions.[21]

3. Immune Modulation of Fracture Healing—A Precision Medicine Approach

Precision medicine has entered the clinical arena in cancer treatment, but orthopaedic care is still relying on the same methods and techniques we used decades ago. We still use radiographs and clinical examination to define union of a long bone and have not made any significant strides toward predicting the development of a nonunion rather than diagnosing it when it has already happened. For the affected patients, this decades-old diagnostic approach means waiting for up to 9 months before a decision is made to proceed with revision surgery or, in the most severe cases, amputation. The socioeconomic and psychological burden of this delay for the individual patient is enormous, and recent studies have shown a similar psychological burden of a nonunion and cancer.[22–25] These shortcomings have motivated many orthopaedic surgeon–scientists to identify predictive biomarker profiles as a critical step toward clinical use of a validated point-of-care diagnostic test applied at the time of injury or surgical fixation to predict bony union. In addition, the identified predictive biomarkers could provide opportunities for targeted immunotherapies and pharmacotherapies designed to correct systemic immune dysregulation responses after severe trauma.

Several studies over the last decade have identified putative relationships between massive early inflammation, long-term persistence of inflammation, and delayed bone union or nonunion. These studies have pointed to a massive early proinflammatory response or the persistence of a proinflammatory phase longer than typical for uncomplicated bone union as being associated with poor outcomes.[15,26–31] For example, interleukin 6 (IL-6) gained recognition as a screening tool after Pape et al[32] showed that multiple organ failure was observed more often in patients with IL-6 concentrations >500 pg/dL. In response to this immediate proinflammatory response, a systemic compensatory anti-inflammatory response is activated almost concurrently, resulting in a suppression of immune effector cells and an increase in immunosuppressive mediators. The balance of this proinflammatory and anti-inflammatory response restores immune homeostasis and is essential for successful tissue regeneration. Immunomodulation to restore immune homeostasis has been shown in animal models to improve bone healing after traumatic bone and bone-muscle injury.[18,33,34] However, in cases where the compensatory anti-inflammatory response overwhelms the initial proinflammatory environment, systemic immune dysregulation and immunosuppression ensues, rendering patients susceptible to infections and poor regeneration, requiring secondary procedures that further impair healing/regenerative potential. Clinically, this immune imbalance during the early stages of fracture repair can

have deleterious long-term effects by prolonging hospitalization due to postsurgical complications and impairing functional recovery due to delayed bony union.

Immune diagnostics tools designed to predict, measure, and then guide treatment currently lack reproducibility and accuracy. This weakness is mostly because of the fact that our attention has been focused on cytokines and their levels within blood. However, cytokine levels fluctuate rather dramatically; cytokines themselves are not especially stable, and therefore, their measured levels often do not reflect the true systemic involvement, and cytokine levels may not mean much because their activity is dependent on cells that are responsive to them. Therefore, it is not surprising to see a shift to using immune cell analysis rather than cytokines as biomarkers. Immune cells, such as T cells, monocytes, and myeloid-derived suppressor cells, are easily collected through phlebotomy. They are relatively stable after collection and can be analyzed using standard laboratory practice. T cells, for example, have been shown to be essential in the activation and maintenance of the post-traumatic immune response. T_{reg} cells play a crucial role in controlling the level of the immune response by regulating the activation of the innate immune cell response.[35] T cells release cytokines that stimulate inactive or polarize macrophages. Through these mechanisms, the T_{reg} cell is pivotal to the local immune response to fracture. Thus, measuring systemic or local levels of T_{reg} activation could not only serve as a readout of fracture union but could also offer a therapeutic angle to modulate the early immune reaction in an effort to reprogram the healing response from nonunion to union. Future research will undoubtedly uncover other immune cell types that play essential roles during fracture repair and will allow us to finally come closer to a precision medicine approach in orthopaedic surgery, similar to those already in practice in other medical specialties.

4. Trauma Immunotypes and Fracture-Related Outcomes After Polytrauma Using a Precision Medicine Approach—The PRECISE Study

Fracture treatment in multiply injured patients (MIPs) is based on injury magnitude and response to injury. The immunologic response to injury affects outcomes in MIPs. Accordingly, understanding individualized immunologic response in MIPs would offer a precision approach to optimize initial and subsequent orthopaedic surgical titration in MIPs.

The PRECISE study is a prospective observational study of MIPs who have sustained operative injuries including pelvic ring disruptions, acetabular fractures, femur fractures, diaphyseal tibia fractures, and traumatic amputations. Patients have to be admitted to an intensive care (ICU) or a monitored bed. The overall goal of the PRECISE study is to formulate patient-specific indices of injury, responses to injury, and subsequently measure correspondence with outcomes. Ultimately, the study is designed to optimize orthopaedic surgical magnitude titration in patients sustaining multiple injuries. In this interim analysis (224 of 325 patients), we sought to interrogate relationships between progression of acute individual immunologic profiles in the initial 48 hours after injury with resolution (RES) or progression (PRO) of trauma-associated organ dysfunction.

Clinical and organ dysfunction data were collected. Research protocols were approved by the Institutional Review Boards (IRBs) of all participating institutions. Injury magnitude was measured using indices injury severity score (ISS) and admission serum lactate. Duration of ICU admission and mechanical ventilation was quantified. Organ dysfunction was quantified using the Marshal Organ Dysfunction Score (MODS), which assigns an integer score from 0 (normal function) to 4 (complete organ failure) for the pulmonary, cardiac, hematologic, renal, hepatic, and central nervous systems. MODS scores were calculated daily by summing individual organ scores for all 6 organs for 5 days after injury.

The goal of this analysis was to explore immunologic differences in patients who resolved organ dysfunction compared with patients who had progression of organ dysfunction. Organ dysfunction resolution or progression was determined by calculating differences between the mean MODS score from postinjury days 4 and 5 ($aMODS_{D45}$) with the mean MODS score on postinjury days 2 and 3 ($aMODS_{D23}$). We observed 3 distinct groups that demarcated patients who rapidly resolved their organ dysfunction, patients with slow improvements in organ dysfunction, and patients who had progression of organ dysfunction. Patients who rapidly resolved (n = 58; RES) organ dysfunction were defined by decrease in $aMODS_{D45}$ -$aMODS_{D23}$ of at least 2.5 points. By contrast, patients who had progressive organ dysfunction (n = 30; PRO) were defined by either an increase in $aMODS_{D45}$ -$aMODS_{D23}$ of at least 1 point or patients with an MODS score on Day 2 of at least 2 and $aMODS_{D45}$ -$aMODS_{D23} \geq 0$.

Immunotyping was performed using serum samples collected at 0 hour, 1 hour, 12 hours, 24 hours, and 48 hours after injury. Thirty-three mediator concentrations were measured using a multiplex platform (Luminex). Three mediator clusters that grouped Inflammatory, Adaptive, and Reparative mediators were quantified. The Inflammatory cluster included mediators that recruit inflammatory cells, amplify, or mitigate inflammation including IL-6, IL-8, IL-10, monocyte chemotactic protein-1 (MCP1), tumor necrosis factor alpha (TNF-α), monokine induced gamma interferon (MIG), and interferon induced protein 10 (IP10). The Adaptive cluster included 7 mediators that initiate and amplify the adaptive T-cell and B-cell immune responses, including IL-2, IL-4, IL-5, IL-7, IL-1β, IL-17a, and granulocyte macrophage colony stimulating factor. Finally, the Reparative cluster included 7 mediators that were concentrated in boundary organs (lung, gut, skin) and whose function is to facilitate tissue repair, including IL-9, IL-21, IL-22, IL-23, IL-17E, IL-27, and IL-33.

In our data analysis, demographics, ISS, admission lactate, ICU LOS (days), and mechanical ventilation days were compared between RES and PRO patients using standard *t*-tests. We compared overall mediator cluster scores between RES and PRO patients for the 3 clusters. Mediator concentrations within each cluster were initially normalized by performing Log_{10} transformations to scale differential magnitudes. Subsequently, Log_{10}-transformed values were summed for individual mediators and over the entire cluster at each time point. Cluster scores at each time point were compared between RES and PRO patients using standard *t* tests. Individual mediators were compared at each time point with Chi-squared analysis.

The following results were observed:

- Demographics and Injury Severity: RES and PRO demonstrated similar age (37.3 ± 11.4 years vs. 36.1 ± 11.0; $P = 0.65$), sex (76% male vs. 63% male; $P = 0.24$), ISS (29.3 ± 13.4 vs. 30.1 ± 11.8; $P = 0.78$), and admission lactate (3.6 ± 2.8 mmol/L in RES vs. 3.6 ± 2.1 mmol/L in PRO patients; $P = 0.49$).
- Clinical Outcomes: ICU LOS (RES 7.8 ± 6.5 days vs. PRO 11.4 ± 12.5 days; $P = 0.15$) and mechanical ventilation (RES 3.1 ± 1.8 days vs. PRO 3.0 ± 2.8 days; $P = 0.74$) were similar. Daily MODS scores (Fig. 3A) demonstrated divergent trajectories beginning on Day 2 with daily decreases

in RES patients in contrast to modest increases in PRO patients. On day 5, 39% of RES patients had complete resolution of organ dysfunction with an MODS score of 0 compared with 0% in PRO patients ($P < 0.0001$).

- Immunologic Progression: RES patients had significant increases in overall Inflammatory cluster concentration scores at days 4 and 5 after injury (Fig. 3B). These differences were driven primarily by significant increases in IL8, IL10, and MCP1 in RES patients. Overall Adaptive cluster concentration scores were significantly increased in RES compared with PRO patients at all time points after injury. All individual Adaptive mediators trended higher in RES patients, and increases in IL4 and IL5 were significantly increased at all time points in RES patients. Reparative mediators diverged at 24 hours and 48 hours with increases in RES patients. At 24 hours after injury, Reparative cluster concentrations were significantly increased in RES patients.

The data from this study are preliminary, and conclusions need to be notably tempered. While organ dysfunction trajectories were divergent, demographics and injury severity were similar between groups, suggesting that individual response to injury had jurisdiction over outcomes. The most striking difference in immunologic response between RES and PRO patients occurred within the Adaptive immune cluster. Individual mediators within the Adaptive cluster consistently trended higher in RES patients, and increases in IL-4 and IL-5 were significantly higher at all 5 time points.

Preliminary review of PRECISE study data demonstrates that the local (tissue-specific) and systemic inflammatory state is influenced by orthopaedic trauma and that the body responds in measurable and potentially predictable ways to trauma within specified ranges of severity. Local and systemic immune function directly influences fracture healing through modulation of molecular mediators that regulate cellular function through transcriptional and translational mechanisms. As a result, this clearly illustrates that immune function has a direct role in affecting the effectiveness of fracture healing, thereby playing a crucial role in fracture-related outcomes.

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