# Single Cell RNA Sequencing in a Preclinical Composite Trauma Model Reveals Systemic Immune Dysregulation and Altered Immune Cell Populations in Peripheral Blood

Tyler Guyer[1†], Drishti Maniar[2†], Casey E. Vantucci[2,3], Paramita Chatterjee[2], Kelly Leguineche[1], Krishnendu Roy[2,3,4], and Robert E. Guldberg[1]
[1]University of Oregon, Eugene, OR [2]Georgia Institute of Technology, Atlanta, GA [3]Emory University, Atlanta, GA [4]Vanderbilt University, Nashville, TN
† These authors have contributed equally to this work and share first authorship
TGuyer@UOregon.edu

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**INTRODUCTION:** Severe musculoskeletal trauma is highly prevalent and frequently presents challenging clinical scenarios such as bone non-unions, delayed healing, and infections. Many of the biological factors underlying such complications are still poorly understood. Recently, a dysregulated systemic immune response has been implicated as one such factor in late emerging complications and deaths in trauma patients. Furthermore, we have previously identified correlations between bone regeneration and various circulating immune cell populations including T cells, monocytes, and myeloid-derived suppressor cells in a preclinical model of musculoskeletal trauma.[1,2] The specific cellular mechanisms underlying the development of systemic immune dysregulation and subsequent poor healing outcomes remain unclear, however. As a result, there is an urgent need for deeper characterization of the post-trauma systemic immune response including more expansive phenotyping of cells correlated to regenerative outcomes. Here, in our preclinical trauma model, we investigated the altered gene expression and frequency of systemic immune cells post-injury using single cell RNA sequencing. We hypothesized that a composite traumatic injury would decrease effector cells (i.e., T cells, natural killer cells), suppress their activity, and lead to global dysregulation of critical biological pathways.

**METHODS:** All animal care and experimental procedures were approved by the IACUC at Georgia Institute of Technology and University of Oregon. Surgical methods have been described in detail previously.[3] Briefly, composite injuries were created in fourteen-week-old Sprague Dawley rats consisting of unilateral eight-millimeter segmental femur defects and eight-millimeter volumetric muscle defects in the adjacent quadriceps. The bone defects were stabilized using rigid internal fixation plates. Five days later, blood was collected from three surgerized rats and pooled for single cell RNA sequencing. Blood was also collected from three age-matched naïve rats and pooled for sequencing. Sequencing libraries were prepared using the 10X Genomics Single Cell 3' Solution (version 3.1), sequencing was conducted with an Illumina NovaSeq 6000 system, and data were de-multiplexed, aligned, and counted using Cell Ranger version 6.0.0 before analysis using Seurat.[4] Cells with a mitochondrial gene percentage exceeding 15%, RNA counts below 500, and feature counts under 250 were removed for quality control. We next normalized 3000 variable genes with the SCTransform method, regressed out effects due to cell-cycle heterogeneity, and integrated the datasets. The clustering and cell type identification of the combined naïve and trauma datasets were performed using the canonical correlation analysis (CCA) method. Finally, the differential gene expression (DEG) was determined using the Wilcoxon Rank Sum test for each cell type to identify the differences between the trauma and naïve groups. Gene set enrichment analysis (GSEA) was performed (Reactome, KEGG, and Hallmark databases) on DEGs to determine immune pathways that were differentially regulated between the two conditions.

**RESULTS:** The scRNA-seq integrated naïve and trauma blood data revealed an abundance of differences in myeloid and lymphocyte cell population frequencies [Figure 1A]. B cells increased in frequency post-trauma (*Cd19, Ighm, Cd79b)*, and in-depth analysis of the B cell population revealed high expression of genes *Nfkbia, Lyn* and *Syk* which are associated with the RANK/RANKL pathway. Several sub-types of T cells (*Cd3e, Cd3d, Cd3g*) decreased significantly in frequency in the trauma condition. These sub-populations included Activated-T cells cluster 1 (AT1) and cluster 2 (AT2), Cd8+T cells, and regulatory T cells (TRegs). Further investigation of the DEGs of the different sub-populations of T-cells was conducted with Reactome to analyze immune-related pathways. Specifically, Activated-T cells cluster 1 revealed a downregulation of effector pathways associated with mitogen-activated protein kinase signaling (MAPK), Toll-like receptors (TLRs) and NF-kB signaling [Figure 1B]. Investigating the DEGs of other T cell populations and effector cells such as natural killer (NK) cells with GSEA revealed a downregulation of effector pathways such as TCR signaling and NK cell-mediated cytotoxicity.

**DISCUSSION:** The scRNA-seq data demonstrated an increase in B cells and a decrease in several T cell subpopulations post-trauma. B cells play a significant role in bone homeostasis especially through the RANK/RANKL signaling pathway. Increased RANKL production by activated B cells induces osteoclast differentiation and bone resorption.[5] The B cell population in the trauma condition was found to highly express genes *Nfkbia, Lyn* and *Syk* which are associated with RANK/RANKL signaling. Therefore, increased B cells in the trauma condition likely promote osteoclastogenesis, favoring bone resorption over bone formation. Additionally, our previous studies using flow cytometry have shown that trauma decreases T cell frequency, especially within one week of injury, and that circulating T cells positively correlate with bone regeneration. Considering the decline in T cell counts, evidence of their diminished function, and downregulation of multiple T cell effector pathways [Figure 1A,B], these findings suggest broad alterations to systemic T cell populations post-trauma. Collectively, our results demonstrate myriad evidence of systemic immune dysregulation following trauma and highlight key cells and pathways affected.

**SIGNIFICANCE/CLINICAL RELEVANCE:** Despite countless advancements in trauma care, many patients still experience poor outcomes, likely in part due to the poorly understood post-trauma immune response. This work further elucidates the changes to the immune environment at the single cell transcriptomic level in both systemic and local tissues following traumatic injury. A better understanding of the relationship between musculoskeletal trauma and the immune system may inform new immunomodulatory therapeutic interventions to improve healing and other patient outcomes.

**REFERENCES:** [1] Cheng, A. and Vantucci, C.E. et al., PNAS, 2021, 118. [2] Vantucci, C.E. and Guyer, T. et al., Frontiers in Surgery, 2022, 9. [3] Willett, N.J. et al., Tissue Eng Part C Methods, 2013, 19. [4] Hao, Y. et al., Cell, 2021, 184. [5] Horowitz, M.C. et al., Bone, 2010, 47.

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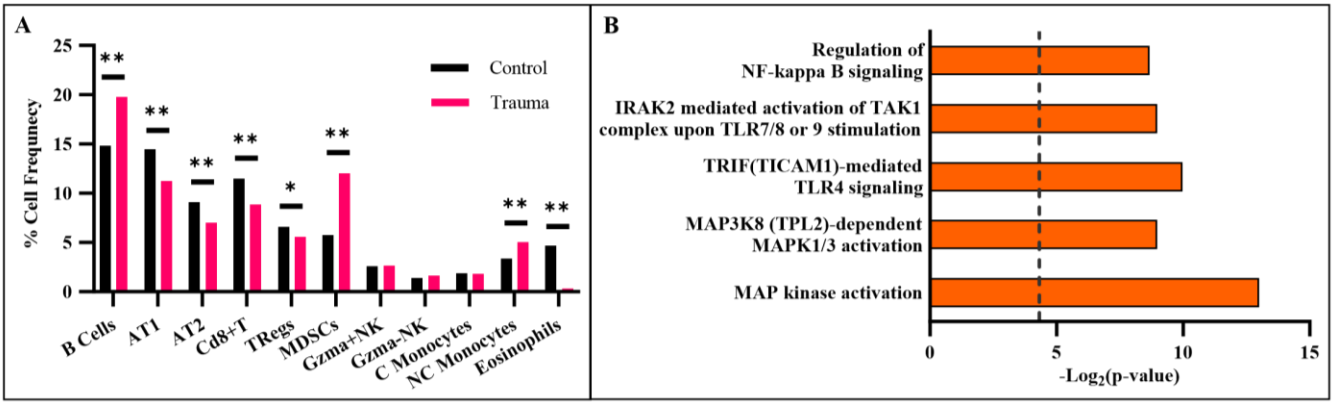


**Figure 1.** (A) Frequencies of blood immune cell populations in a naïve control sample and trauma rat sample five days post-injury. *P < 0.05, **P < 0.0001 as indicated. (B) Differential gene expression of immune-related pathways in activated T cells cluster 1 as measured by GSEA using Reactome. The dashed line is located at $-Log_2(0.05)$ indicating the threshold for significance with P<0.05. All pathways shown were downregulated in the trauma condition.