Phenotyping the Immune Response to Trauma: A Multiparametric Systems Immunology Approach*

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Objective: Trauma induces a complex immune response that requires a systems biology research approach. Here, we used a novel technology, mass cytometry by time-of-flight, to comprehensively characterize the multicellular response to trauma.

Design: Peripheral blood mononuclear cells samples were stained with a 38-marker immunophenotyping cytometry by time-of-flight panel. Separately, matched peripheral blood mononuclear cells were stimulated in vitro with heat-killed *Streptococcus pneumoniae* or CD3/CD28 antibodies and stained with a 38-marker cytokine panel. Monocytes were studied for phagocytosis and oxidative burst.

Setting: Single-institution level 1 trauma center.

Patients or Subjects: Trauma patients with injury severity scores greater than 20 ($n = 10$) at days 1, 3, and 5 after injury, and age- and gender-matched controls.

Interventions: None.

Measurements and Main Results: Trauma-induced expansion of Th17-type CD4+ T cells was seen with increased expression of interleukin-17 and interleukin-22 by day 5 after injury. Natural killer cells showed reduced T-bet expression at day 1 with an associated decrease in tumor necrosis factor-β, interferon-γ, and monocyte chemoattractant protein-1. Monocytes showed robust expansion following trauma but displayed decreased stimulated proinflammatory cytokine production and significantly reduced human leukocyte antigen - antigen D related expression. Further analysis of trauma-induced monocytes indicated that phagocytosis was no different from controls. However, monocyte oxidative burst after stimulation increased significantly after injury.

Conclusions: Using cytometry by time-of-flight, we were able to identify several major time-dependent phenotypic changes in blood immune cell subsets that occur following trauma, including induction of Th17-type CD4+ T cells, reduced T-bet expression by natural killer cells, and expansion of blood monocytes with less proinflammatory cytokine response to bacterial stimulation and less human leukocyte antigen - antigen D related. We hypothesized that monocyte function might be suppressed after injury. However, monocyte phagocytosis was normal and oxidative burst was augmented, suggesting that their innate antimicrobial functions were preserved. Future studies will better characterize the cell subsets identified as being significantly altered by trauma using cytometry by time-of-flight, RNAseq technology, and functional studies. (*Crit Care Med* 2017; 45:1523–1530)

Key Words: cytometry by time-of-flight; immune phenotype; immunology; mass cytometry; trauma

Clinical management of traumatic injury has significantly improved over the past several decades, reducing mortality (1, 2). However, these incredible strides are somewhat mitigated when the trauma patient succumbs to postinjury sepsis, which increases mortality from 7.6% to 23.1% (3). Therefore, it is critically important to better understand the changes in the immune system that contribute to loss of immune function, in order to discover treatable cellular and molecular targets to reduce postinjury infection rates and reduce trauma-induced complications.

It has been known for some time that the immune system is altered by trauma (4–7), but the complexity of trauma has made it difficult to perform a comprehensive and in-depth analysis of the multiple immune cell types affected by injury in humans. In this study, we used mass cytometry by time-of-flight (CyTOF) as a systems biology tool to collect single cell phenotyping data on circulating peripheral blood mononuclear cells (PBMCs) from severely injured trauma patients. The major advantage of CyTOF over conventional flow cytometry is that CyTOF uses antibodies that are conjugated to rare earth metal isotopes rather than to fluorescent tags. This allows for improved discrimination between markers with reduced cross talk and expands the number of markers that can be used simultaneously in a cell staining panel (8, 9).

*See also p. 1577.

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Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's website (http://journals.lww.com/ccmjournal).

Supported by National Institutes of Health 5R01 AI092905-05.

Dr. Keegan and Mr. Lederer received support for article research from the National Institutes of Health. The remaining authors have disclosed that they do not have any potential conflicts of interest.

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DOI: 10.1097/CCM.0000000000002577

METHODS

Patient Selection

Patients were enrolled from May to October 2015 from Brigham and Women's Hospital who met the following inclusion criteria: over the age of 18 years, not pregnant, with Injury Severity Score greater than 20 (average ISS = 34), no medical history, or medications predisposing immune dysregulation (e.g., chemotherapy or steroid use) (10). ISS was estimated at admission, and final calculation was performed after discharge. Blood was drawn at days 1, 3, and 5 after injury. Data collected included demographics, mechanism of injury, and development of culture-proven infection. Blood samples were also collected from healthy, uninjured, and age- and gender-matched volunteers. This study protocol was approved by the Brigham and Women's Hospital Institutional Review Board.

Blood Sample Processing

Six milliliters of blood was collected in EDTA tubes. PBMCs were purified using a Ficoll density gradient (GE Healthcare Ficoll-Paque PLUS, Marlborough, MA) and then frozen using Cryostor CS10 (BioLife Solutions, Bothell, WA) by the manufacturer's protocol.

CyTOF Staining Methods

PBMCs were thawed at 37°C for 3 minutes and then mixed into 37°C thawing medium consisting of RPMI 1640 supplemented with 5% heat-inactivated fetal calf serum, 1 mM glutamine, 10 mM HEPES, 2 mM nonessential amino acids, penicillin/streptomycin/amphotericin B, 2.5×10^{-5} M 2-mercaptoethanol, 20 units/mL heparin sodium salt, and 25 units/mL benzonase nuclease (Sigma-Aldrich, St. Louis, MO). For the immunophenotyping stains, PBMCs were washed by centrifugation, plated, and immediately stained. For cytokine stains, PBMCs were plated into tissue culture plates (Corning) and stimulated with anti-CD3 (4 μg/mL)/anti-CD28 (2 μg/mL) antibodies (BioLegend, San Diego, CA), with 2×10^4 bacteria/mL heat-killed *S. pneumoniae*, or left unstimulated. Heat-killed bacteria (HKB) were prepared by exposing *S. pneumonia* to 95°C for 15 minutes. Cells were cultured with stimulants or no stimulation at 37°C for 30 minutes, after which Brefeldin A (Sigma) was added at a final concentration of 10 μg/mL to prevent cytokine release. After 4-hour incubation, cells were stained.

All CyTOF stains were performed at room temperature in 96-well round-bottom polypropylene plates (Corning). After washing cells by centrifugation at 200 × *g* for 5 minutes, 5 μM cisplatin viability staining reagent (Fluidigm, South San Francisco, CA) was added for 5 minutes. After centrifugation, TruStain FcX Fc receptor blocking reagent (BioLegend) was added for 10 minutes. CyTOF antibodies were labeled using purified antibodies from several different suppliers (BioLegend, eBioscience [Waltham, MA], and R&D Systems [Minneapolis, MN]). The CyTOF staining panels used in this study are shown in **Supplemental Table 1** (Supplemental Digital Content 1, http://links.lww.com/CCM/C747) and **Supplemental Table 2** (Supplemental Digital Content 2, http://links.lww.com/CCM/C748). The antibodies for cell surface staining were incubated with cells for 30 minutes. After fixation and permeabilization of cells using the FoxP3 Staining Buffer Set (eBioscience), cells were barcoded using palladium barcoding reagents and combined into a single sample for pooled intracellular antibody staining for 30 minutes (11). After intracellular staining, the cells were fixed with 1.6% formaldehyde. To stain DNA, 18.75 μM iridium intercalator solution (Fluidigm) was added to the cells. Cells were subsequently washed and reconstituted in Milli-Q filtered distilled water with EQ four element calibration beads (Fluidigm) and then analyzed on a Helios CyTOF Mass Cytometer (Fluidigm).

Bacteria Phagocytosis Assay

Five separate trauma patient samples and controls were selected to test monocyte phagocytosis. PBMCs were thawed and plated at 100,000 cells per well and then incubated at 37°C for 2 hours with or without the pHrodo Green *Staphylococcus aureus* Bioparticles (ThermoFisher Scientific, Waltham, MA) prepared by the manufacturer's protocol. After incubation, the cells were washed with PBS, stained with PE/Cy7-labeled CD14 antibody, and analyzed by flow cytometry. Percentage phagocytosis was calculated as $\left(\frac{\text{FITC}+\text{PE/Cy7}^+\text{cells}}{\text{PE/Cy7}^+\text{cells}}\right)*100.$

Oxidative Burst Assay

Matched vials from the same 5 patients and controls were used to measure monocyte oxidative activity. PBMCs were plated and incubated at 37°C for 5 minutes with or without DHR123 (50 μM). Cells were stimulated with phorbol 12-myristate 13-acetate (4 μM) for another 20 minutes and then centrifuged and fixed in 0.4% PFA for 10 minutes. The cells were stained with PE/Cy7-labeled CD14 antibody and analyzed by flow cytometry. The percentage of monocytes achieving oxidative burst was calculated as $\left(\frac{\text{FITC}+\text{PE/Cy7}^+\text{cells}}{\text{PE/Cy7}^+\text{cells}}\right)*100.$

Statistics

CyTOF data were analyzed using Cytobank (Mountain View, CA). Gating was performed and statistics of mean expression intensity (MEI) and percentages of cell populations was exported. A sample gating strategy for $CD4^+$ T cells, natural killer (NK) cells, and monocytes is provided in **Supplemental Figure 1** (Supplemental Digital Content 3, http://links.lww.com/CCM/C749; **legend**, Supplemental Digital Content 6, http://links.lww.com/CCM/C752). Flow cytometry data were analyzed using FlowJo (Ashland, OR). All statistical analysis was performed in GraphPad Prism (La Jolla, CA). Paired one-way analysis of variance was performed to identify statistically significant ($p < 0.05$) differences among multiple groups, with Tukey multiple comparison test where appropriate.

RESULTS

Blood samples were collected from 10 patients within the inclusion criteria as well as 10 age- and gender-matched uninjured controls. The final demographics of the trauma patient and control groups are described in **Table 1**, with additional clinical data on the trauma patients in **Supplemental Table 3**

TABLE 1. Demographics of Patient Cohort, Injury Severity, and Infectious Complications

Demographic Factor	Patient	Control
Mean age (yr ± SEM)	49.4 ± 7.37	50 ± 6.47
Male, *n* (%)	5 (50)	5 (50)
Mean injury severity score (score ± SEM)	38.3 ± 2.43	NA
Mean Abbreviated Injury Scale (score ± SEM)		NA
Head and neck	4.75 ± 0.25	
Face	2 ± 1	
Chest	3.6 ± 0.4	
Abdomen	3.75 ± 0.48	
Extremity	3.14 ± 0.26	
External	1.5 ± 0.5	
Head injury present, *n* (%)	7 (70)	NA
Development of culture-proven infection during index hospitalization, *n* (%)	4 (40)	NA
Types of infection developed, *n* (%)		
Urinary tract infection	1 (10)	NA
Wound infection	1 (10)	NA
Ventilator-associated pneumonia	1 (10)	NA
Bacteremia	1 (10)	NA

NA = not applicable.

(Supplemental Digital Content 4, http://links.lww.com/CCM/C750). Of note, four of the patients developed subsequent infections after their injuries, ranging from postinjury day 2 to postinjury day 19. PBMCs from patients and controls were stained with CyTOF staining panels to measure immune cell subset phenotypes or to measure cytokine expression following stimulation with HKB or a mixture of anti-CD3 and CD28 antibodies. Initial analysis of the trauma patient PBMC populations indicated that one patient had to be excluded from subsequent analysis due to a skewed monocytic condition.

The results from PBMC stains revealed multiple time-dependent changes in immune cell subset percentages occurring in trauma patients versus uninjured controls (**Supplemental Fig. 2**, Supplemental Digital Content 5, http://links.lww.com/CCM/C751; legend, Supplemental Digital Content 6, http://links.lww.com/CCM/C752). This report focuses primarily on the trauma-induced immune cell subset changes that were most significant.

Trauma Induces an Increase in Th17 CD4+ T Cells

$CD4^+$ T cells expressing RAR-related orphan receptor γ (RORγT) a transcription factor that identifies Th17-type $CD4^+$ T cells, were found to expand in the blood of trauma patients at all time points after injury. The expansion of RORγT-expressing $CD4^+$ T cells was observed visually in the CyTOF data by biaxial gating (**Fig. 1*A***) as well as statistically by plotting MEI values for uninjured versus trauma patients (**Fig. 1*B***). We also measured the constitutive and anti-CD3/CD28 stimulated levels of cytokines associated with the Th17 phenotype, interleukin (IL)-17F and IL-22. We found that IL-17F and IL-22 expression in stimulated $CD4^+$ T cells was increased, but did not demonstrate significance until day 5 after trauma at which time the production of these cytokines was constitutive (**Fig. 1*C***).

Changes in NK Cell Phenotype in Response to Trauma

CyTOF staining data showed that T-bet transcription factor expression was significantly reduced in NK cells at day 1 after trauma. By day 3, we found that T-bet expression in NK cells returned to levels observed in uninjured controls, but by day 5 after trauma, T-bet expression was significantly higher than controls (**Fig. 2*A***). When PBMCs were stimulated with heat-killed *S. pneumoniae* and stained with the cytokine CyTOF panel, we observed that tumor necrosis factor (TNF)-β, interferon (IFN)-γ, and monocyte chemoattractant protein-1 cytokine expression in NK cells mirrored the expression pattern of T-bet (**Fig. 2*B***). Stratifying the CyTOF staining data based on the development of infection, we identified that patients with posttrauma infection ($n = 4$) had a delayed increase in NK cell T-bet expression (**Fig. 2*C***).

Monocyte Response to Trauma

Monocyte percentages as a subset of all circulating PBMCs significantly increased after injury (**Fig. 3*A***). This increase in monocytes was likely due to proliferation, as evidenced by increased staining for the nuclear cell proliferation marker, Ki-67 (**Fig. 3*B***). This increase in expression of Ki-67 in monocytes was easily visualized in viSNE plots, which clusters groups

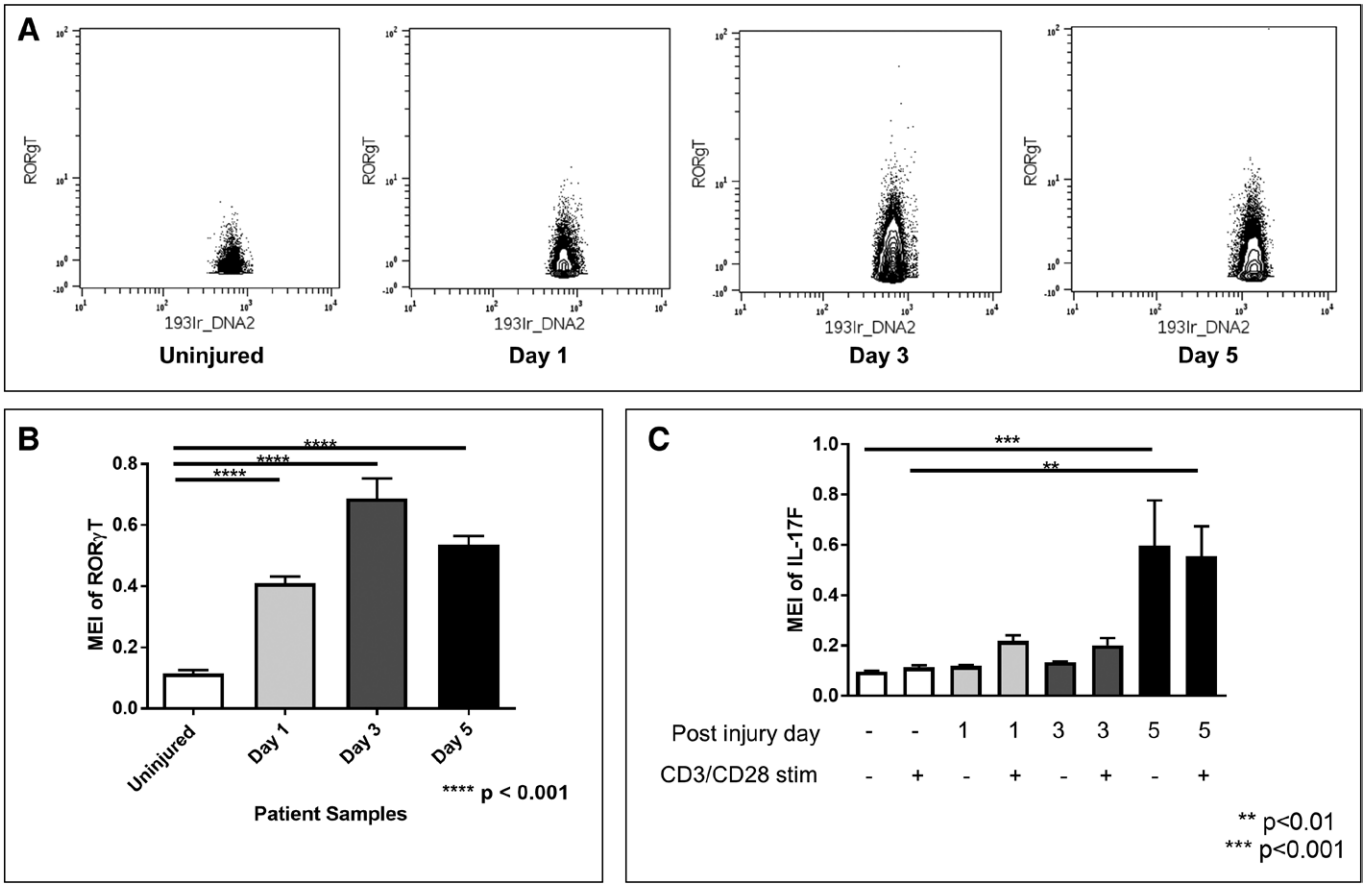

Figure 1. Trauma causes expansion of CD4+ Th17 cells all time points after injury, with constitutive activation at day 5. **A**, Biaxial plots of representative samples from each experimental group showing increased RAR-related orphan receptor γ (RORγT) expression in $CD3^+CD4^+$ gated T cells after injury, plotted as DNA+ versus RORγT+. **B**, RORγT expression in CD4+ T cells increases significantly at all time points after injury, with a peak at day 3. **C**, Interleukin (IL)-17F expression in CD4+ T cells remains low after injury until day 5, at which time there is a high level of expression in both unstimulated samples and samples stimulated with anti CD3/CD28 antibodies. Statistics were performed using one-way analysis of variance; results were considered significant if $p < 0.05$. MEI = mean expression intensity.

of cells based on similar staining patterns (12). As shown in **Figure 3*C***, Ki-67 staining colocalized with $CD14^+$ monocytes, which are also shown to increase in density after trauma.

After stimulation with heat-killed *S. pneumoniae*, there was a marked difference in proinflammatory cytokine production between controls and trauma patients. Specifically, TNFα production, which was high in monocytes from controls, showed significantly decreased expression in monocytes from trauma patients at all time points (**Fig. 3*D***). Similarly, IL-1β was produced at high levels in monocytes after stimulation in controls but was significantly lower in monocytes from trauma patients (**Fig. 3*E***). We also evaluated changes in human leukocyte antigen - antigen D related (HLA-DR). HLA-DR was robustly expressed on the cell surface of monocytes from controls. In contrast, the percentage of circulating $HLA\text{-}DR^+$ monocytes decreased significantly after injury at all time points (**Fig. 3*F***). Furthermore, among $HLA\text{-}DR^+$ monocytes, the MEI for HLA-DR significantly decreased (**Fig. 3*G***).

Functional Assessment of Trauma-Induced Monocyte Phagocytosis and Oxidative Burst Responses

The findings above suggested that monocytes were significantly altered by trauma and may have suppressed antimicrobial function. Thus, we performed bacteria phagocytosis and oxidative burst assays on five separate patients to test for potential changes in antimicrobial immune reactivity by monocytes. Phagocytosis assays were performed using fluorescence-labeled *S. aureus*. Surprisingly, we found that there was no difference in phagocytic activity, as all monocytes showed nearly 90% uptake of fluorescence-labeled *S. aureus* (**Fig. 4*A***). Next, we tested monocytes for oxidative burst activity by measuring nicotinamide adenine dinucleotide phosphate oxidase–dependent fluorescence of DHR123 rhodamine dye in cells. Rather than decreased oxidative burst activity by monocytes from trauma patients, we found increased oxidative burst over the entire time course as compared with controls (**Fig. 4*B***).

DISCUSSION

Although trauma patients are now surviving traumatic injuries that would have been fatal decades ago, this leaves critically injured people vulnerable to postinjury infections (1, 2), due in part to disruption of immune system homeostasis. Therefore, it is critical to map cellular changes and interactions that could predict which patients are more susceptible to posttrauma

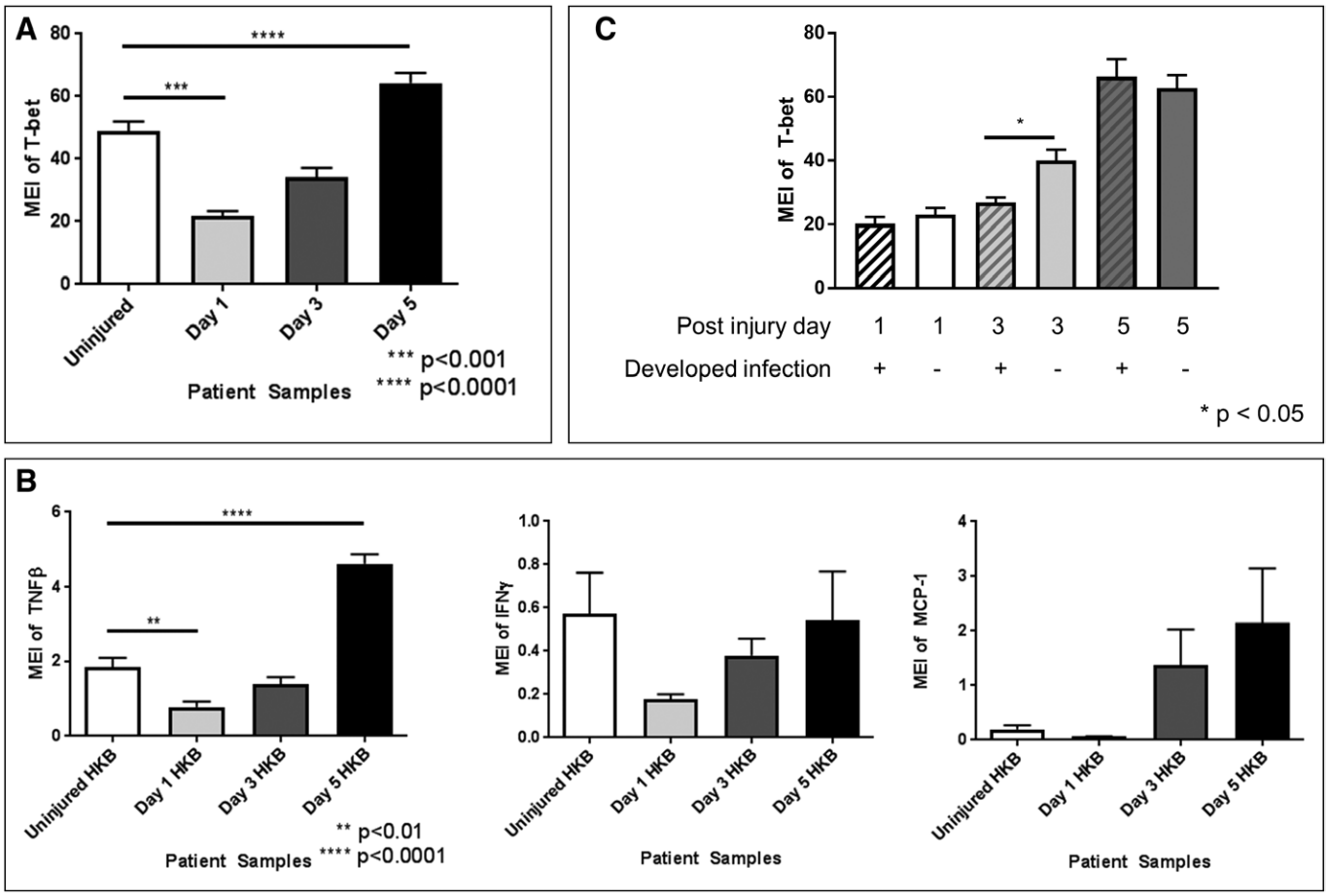

Figure 2. Changes in T-bet expression in blood natural killer (NK) cells in trauma patients. **A**, NK cells demonstrate decreased expression of the transcription factor T-bet on day 1 after injury, which returns to normal by day 3 and becomes significantly increased compared to baseline by day 5. **B**, Expression of tumor necrosis factor (TNF)-β, interferon (IFN)-γ, and monocyte chemoattractant protein-1 (MCP-1) showing similar expression patterns; IFNγ and MCP-1 show a similar trend as TNFβ although not statistically significant. **C**, Reduced T-bet expression in patients that subsequently develop an infection. Statistics were performed using one-way analysis of variance; results were considered significant if $p < 0.05$. HKB = heat-killed bacteria, MEI = mean expression intensity.

infections and to identify targets for immunotherapy. This challenging task is now more feasible with the development of systems biology tools like CyTOF, Luminex multiplex cytokine assays, and RNA sequencing. For example, Gaudillière et al (13) used CyTOF to study blood samples in patients undergoing hip arthroplasty, identifying signaling changes in circulating $CD14^+$ monocytes that correlated with functional impairment during recovery. Here, we used CyTOF to generate unbiased, blood immune cell phenotyping data to identify cells and markers that are significantly affected by traumatic injury (14–17).

In our trauma patient cohort, we found changes in cell populations and cytokine production that were specific to injury and dynamic over time. Furthermore, these changes were common among the patients who were analyzed, despite their varied age, gender, and specific types of injuries. A prior report that measured gene expression profiles of blood cells from trauma and burn patients also found that there is high similarity between these different types of traumatic injuries (18).

In this study, the cell subsets showing the most significant changes were $CD4^+$ T cells, NK cells, and monocytes. In $CD4^+$ T cells, we identified an increase in Th17 cells, which are proinflammatory T cells that have detrimental activity in autoimmune diseases and beneficial activity for antimicrobial immune function by stimulating mobilization of neutrophils (19–21). Our data demonstrated increased $CD4^+$ T cells expressing RORγT by day 1 after injury. By day 5, we found that the RORγT-expressing $CD4^+$ T cells were producing detectable IL-17F or IL-22 even without stimulation. This suggests that trauma-induced Th17 cells take several days before being able to produce Th17-type cytokines. Furthermore, the fact that these cells are constitutively producing IL-17F and IL-22 demonstrates that injury alone is a sufficient stimulus to activate these cells. A recent report by Abboud et al (22) used computational immunology methods on circulating cytokine results and patient outcome to identify a possible pathologic role for IL-17 or Th17 cells in trauma patients. Our findings by CyTOF concur that trauma induces an increase in circulating Th17 cells. The observed expansion of Th17 cells after injury compels further investigation to determine if immunotherapies to modulate Th17 cells or IL-17 protect trauma patients from opportunistic infections or organ injury.

NK cells are group 1 innate lymphoid cells that play critical roles in immune function by detecting tumor cells, virus-infected cells, and stressed cells (23). NK cells express the

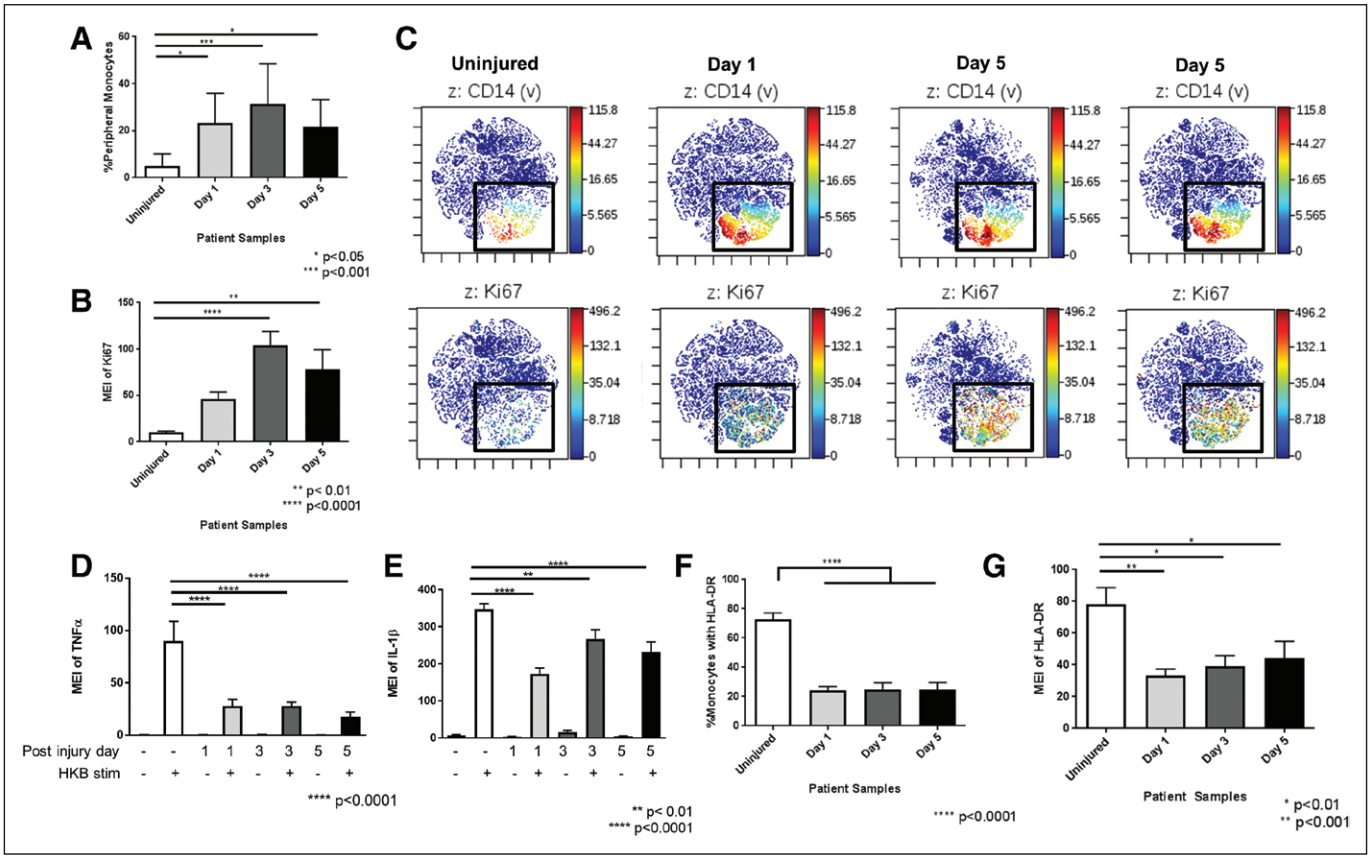

Figure 3. Circulating monocytes increase in percentage with increasing Ki-67 expression in trauma patients, but have reduced proinflammatory cytokine and human leukocyte antigen - antigen D related (HLA-DR) expression. **A**, Percentages of circulating monocytes in peripheral blood demonstrating that the percentage of circulating monocytes increases significantly at all time points after injury. **B**, Mean expression intensity (MEI) of Ki-67 expression in monocytes demonstrating a significant increase in Ki-67 expression at all time points after injury. **C**, viSNE plots illustrating the colocalization of CD14 and Ki-67 staining of proliferating monocytes. *Top*, CD14 staining and *bottom* panels show Ki-67 staining of same cell population. **D**, MEI of tumor necrosis factor (TNF)-α expression in monocytes in peripheral blood after stimulation with heat-killed bacteria (HKB). Stimulated TNFα expression in monocytes decreased at all time points after injury. **E**, MEI of interleukin (IL)-1β in HKB-stimulated monocytes, showing a significant decrease in IL-1β expression at all time points after injury. **F**, Percentages of circulating HLA-DR$^+$ monocytes from uninjured controls and trauma patients. The percentage of circulating monocytes that were HLA-DR$^+$ was significantly reduced at all time points after injury. **G**, MEI of HLA-DR on monocytes is significantly decreased at all time points after injury. Statistics were performed using one-way analysis of variance; results were considered significant if $p < 0.05$.

transcription factor T-bet and, when activated by IL-1β, IL-12, and/or IL-18, produce high levels of the Th1-type cytokines, IFNγ and TNFβ. In this study, we discovered significant time-dependent changes in T-bet and inflammatory cytokine expression patterns. T-bet expression in NK cells is thought to regulate NK-cell maturation and movement from the bone marrow and lymph nodes (24, 25). The initial decrease seen in T-bet in NK cells suggests that they may be recent emigrants from the bone marrow. Because T-bet and inflammatory cytokine expression in NK cells normalizes by day 3 and is induced beyond normal baseline levels by day 5, this observation supports that trauma induces NK-cell maturation. Finally, despite the small sample size, we discovered a delayed rebound in T-bet expression in patients who developed infections, possibly indicating that NK-cell maturation is involved in predisposition to infections after trauma. This finding requires further study with a larger cohort of patients to evaluate whether T-bet may be a prognostic marker or drug target.

Our data also demonstrated an increase in peripheral monocytes with evidence of proliferation. Though we anticipated that these monocytes would be proinflammatory, we found decreased TNFα and IL-1β expression in response to HKB. Furthermore, we found those monocytes from trauma patients expressed less cell surface HLA-DR. This diminished HLA-DR implies a decreased capacity for monocytes to interact with CD4$^+$ T cells. Several previous reports showed that a long-lived decrease in HLA-DR expression on monocytes after injury may predict poor clinical outcome and death for trauma patients (26–28). This suggests that early changes in monocytes may be a predictive measure for clinical trajectories in trauma patients.

Based on our phenotypic and cytokine production findings, we hypothesized that monocytes might also have decreased innate antimicrobial function. Although there is a paucity of data discussing monocyte oxidative burst after injury, it is known that monocyte oxidative burst is impaired in septic shock and that this could potentially be involved in the spread of infection during sepsis (29). Although a brief comparative delay in phagocytosis or oxidative burst during the short experimental incubations cannot be excluded, our findings support that trauma-induced monocytes retain their innate antimicrobial functions.

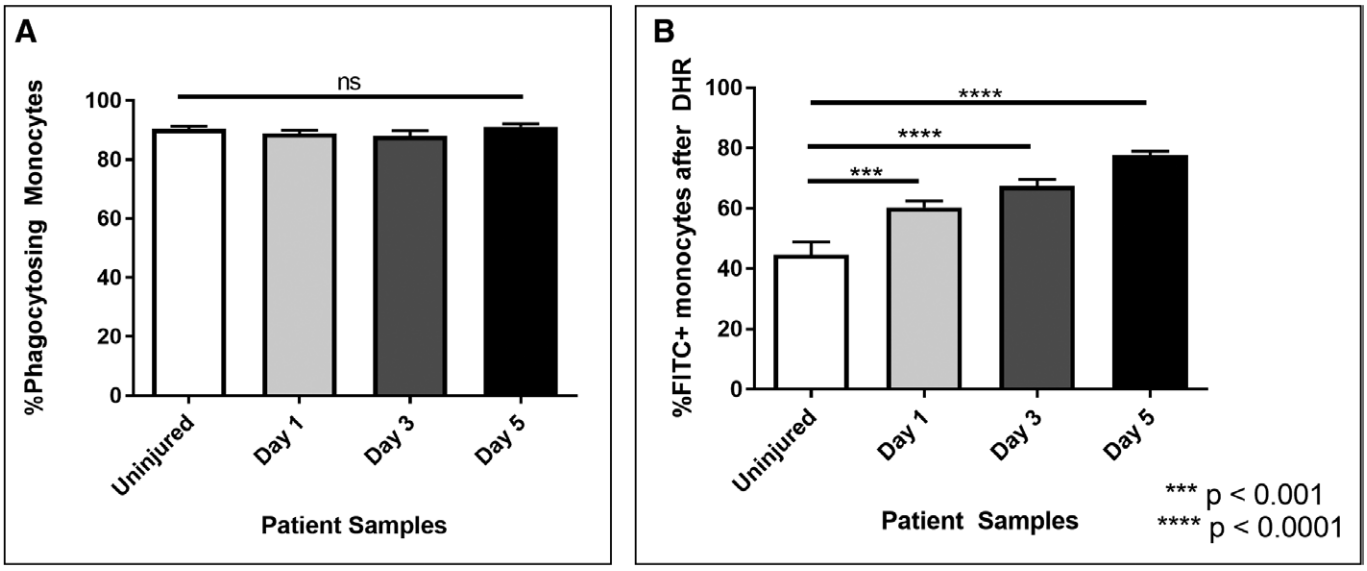

Figure 4. Phagocytosis and oxidative burst assays to assess antimicrobial function of monocytes from trauma patients after injury. **A**, Cells were incubated with fluorescein isothiocyanate (FITC)-labeled *Staphylococcus aureus* and opsonizing reagent to evaluate phagocytosis. No change is seen in monocyte phagocytosis after injury. **B**, Cells were incubated with DHR and then stimulated with phorbol 12-myristate 13-acetate and evaluated for oxidative burst. Monocyte oxidative burst in response to stimulation is augmented after injury. Statistics were performed using one-way analysis of variance; results were considered significant if $p < 0.05$.

Our study has several limitations to note. Although we were able to comprehensively phenotype multiple immune cell subsets after injury, these results do not identify the mechanisms responsible. Nevertheless, this information lays the groundwork for functional studies. Another limitation was that we were only able to evaluate blood. However, we will now pursue parallel blood and immune tissue sampling studies in a mouse trauma model. It should also be stated that although patients clinically developed an infection over a wide range of time, it is unclear at which time those patients were inoculated. Thus, there could be a component of underlying infection to the immune phenotypes identified. However, the commonality seen between these immune profiles despite the significant difference in time to infection leads us to believe that the changes identified are trauma induced. Additionally, this report includes only 10 patients. Sample collection is ongoing, and further studies including more patients would be able to validate and expand our findings. Finally, this cohort was severely injured and not all findings may be generalizable to less severely injured patients.

In conclusion, using CyTOF, we identified several novel time-dependent phenotypic changes in blood immune cell subsets following major trauma. Future directions will include in-depth study of specific immune cell subsets showing trauma-induced changes in a larger cohort of trauma patients. Relevant immune cell markers to include will be immunomodulatory receptors associated with strong T-cell activation or exhaustion, NK cell maturation and functional markers, and markers to identify immature monocytes and M1 or M2 phenotypes. We will also profile more patients to allow us to stratify gender, types of injury, and clinical features. Future studies will use these data to create new insights and hypotheses about how to restore immune homeostasis in trauma patients and prevent the development of life-threatening infections.

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