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MACROPHAGE SWITCHING: POLARIZATION AND MOBILIZATION AFTER TRAUMA

Lara Hoteit,*† Patricia Loughran,* Shannon Haldeman,*† Danielle Reiser,* Nijmeh Alsaadi,*† Elizabeth Andraska,*† Jillian Bonaroti,*† Amudan Srinivasan,*† Kelly M. Williamson,*† Jurgis Alvikas,*† Richard Steinman,*† Joshua Keegan,‡ James A. Lederer,‡ Melanie Scott,*† Matthew D. Neal,*† and Anupamaa Seshadri*§

**Department of Surgery, University of Pittsburgh Medical Center, Pittsburgh, PA; †Pittsburgh Trauma & Transfusion Medicine Research Center, University of Pittsburgh, Pittsburgh, PA; ‡Department of Surgery, Brigham and Women's Hospital/Harvard Medical School, Boston, MA; and §Department of Surgery, Beth Israel Deaconess Medical Center, Boston, MA*

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ABSTRACT—**Introduction:** Trauma alters the immune response in numerous ways, affecting both the innate and adaptive responses. Macrophages play an important role in inflammation and wound healing following injury. We hypothesize that macrophages mobilize from the circulation to the site of injury and secondary sites after trauma, with a transition from proinflammatory (M1) shortly after trauma to anti-inflammatory (M2) at later time points. **Methods:** C57Bl6 mice (n = 6/group) underwent a polytrauma model using cardiac puncture/hemorrhage, pseudofemoral fracture, and liver crush injury. The animals were killed at several time points: uninjured, 24 h, and 7 days. Peripheral blood mononuclear cells, spleen, liver nonparenchymal cells, and lung were harvested, processed, and stained for flow cytometry. Macrophages were identified as $CD68^+$; M1 macrophages were identified as $iNOS^+$; M2 macrophages as arginase 1^+. **Results:** We saw a slight presence of M1 macrophages at baseline in peripheral blood mononuclear cells (6.6%), with no significant change at 24 h and 7 days after polytrauma. In contrast, the spleen has a larger population of M1 macrophages at baseline (27.7%), with levels decreasing at 24 h and 7 days after trauma (20.6% and 12.6%, respectively). A similar trend is seen in the lung where at baseline 14.9% of $CD68^+$ macrophages are M1, with subsequent continual decrease reaching 8.7% at 24 h and 4.4% at 7 days after polytrauma. M1 macrophages in the liver represent 14.3% of $CD68^+$ population in the liver nonparenchymal cells at baseline. This percentage increases to 20.8% after trauma and decreases at 7 days after polytrauma (13.4%). There are few M2 macrophages in circulating peripheral blood mononuclear cells and in spleen at baseline and after trauma. The percentage of M2 macrophages in the lungs remains constant after trauma (7.2% at 24 h and 9.2% at 7 days). In contrast, a large proportion of M2 macrophages are seen in the liver at baseline (36.0%). This percentage trends upward and reaches 45.6% acutely after trauma and drops to 21.4% at 7 days. The phenotypic changes in macrophages seen in the lungs did not correlate with a functional change in the ability of the macrophages to perform oxidative burst, with an increase from 2.0% at baseline to 22.1% at 7 days after polytrauma ($P = 0.0258$). **Conclusion:** Macrophage phenotypic changes after polytrauma are noted, especially with a decrease in the lung M1 phenotype and a short-term increase in the M2 phenotype in the liver. However, macrophage function as measured by oxidative burst increased over the time course of trauma, which may signify a change in subset polarization after injury not captured by the typical macrophage phenotypes.

KEYWORDS—Inflammation, M1 macrophages, M2 macrophages, macrophage polarization, polytrauma

INTRODUCTION

Trauma is one of the leading causes of death worldwide (1). Although death within the first 24 h of injury is typically related to hemorrhagic shock and the injury itself, in the subsequent days and weeks, the main cause of death after traumatic injury becomes infectious complications, affecting approximately 10% of trauma patients (1). The incidence of sepsis increases with the severity of the trauma and is related to the dysregulation of the immune response caused by injury (2). Numerous changes occur to both the innate and adaptive arms of the immune response, leading to the clinical presentation of the systemic inflammatory response syndrome, as well as a compensatory anti-inflammatory response syndrome (1). These changes can lead to the findings that Moore and colleagues (3) described as a two-hit response in survivors of trauma, where the initial insult of traumatic injury causes a maladaptive immune response to a secondary insult such as an infection. This maladaptive response may present as a dangerous hyperinflammation leading to multiple organ failure or, alternatively, a diminished response to microbial threat leading to overwhelming infection. Better delineation of the specific effects that trauma has on immune cell phenotypes may provide insight into which subgroup of patients will have impaired responses to subsequent infection and may also provide targets for therapies to avoid infection after trauma.

Macrophages play an important role in innate immunity (4). Macrophages exist in a spectrum of phenotypes and can change their polarization, depending on a number of factors such as their microenvironment (5). The M1 or "classical" proinflammatory phenotype is stimulated by microbial products or proinflammatory

Address reprint requests to: Anupamaa Seshadri, MD, Beth Israel Deaconess Medical Center, 110 Francis St, Lowry 2G, Boston, MA 02215. E-mail: aseshadr@bidmc.harvard.edu

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cytokines such as TNF-α and interferon γ (5). M1 macrophages typically produce nitrous oxide and reactive oxygen intermediates, as well as proinflammatory cytokines, promote a T helper 1 response, and have microbicidal and tumoricidal activity (5). The M2 anti-inflammatory phenotype is observed in healing tissue and is induced by anti-inflammatory cytokines such as IL-4, IL-10, and IL-13 (5). The M2 phenotype is involved in the promotion of a T helper 2 response, tissue remodeling, immune tolerance, and tumor progression (5). These phenotypes are malleable, existing on a spectrum, and it is known that macrophages can "repolarize" (6).

In previous work, we found that monocytes harvested from the peripheral blood of human trauma patients had increased ability for innate functions such as oxidative burst, but had decreased ability to produce proinflammatory cytokines after stimulation with heat-killed bacteria (7). This work led to the hypothesis that macrophage polarization may be affected by traumatic injury, leading to this mixed phenotype. Macrophage polarization has not been widely studied after trauma. The bulk of this literature is focused on traumatic brain injury, with studies showing the presence of a chronic and persistent M1 polarization lasting months after traumatic brain injury within the lesion microenvironment (8). In this study, we attempted to fill this knowledge gap by comprehensively phenotyping macrophage polarization in the blood, spleen, liver, and lung over several time points in a mouse polytrauma (PT) model. We then set out to examine the functional changes in macrophages corresponding to these time points by evaluating oxidative burst in end organs. We hypothesized that in a process of inflammation and subsequent wound healing, we would observe an increase in M1 inflammatory macrophages early after trauma with a conversion to an M2 anti-inflammatory phenotype at later time points and that the functional change would match any phenotypic change seen.

MATERIALS AND METHODS

Animals

Wild-type adult male mice (C57BL; age, 8–12 weeks; weight, 26–28 g) were obtained from Jackson Laboratory, Bar Harbor, ME. All animals were housed in a controlled environment and provided with standard rodent nutrition and water *ad libitum*, under a 12-h light-dark cycle. All surgical procedures were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh Medical Center.

Polytrauma model

Mice were weighed and anesthetized with isoflurane (3% on induction and 2% for maintenance). Mice were placed on heated pads with temperature maintained at 37°C. A blind cardiac puncture was performed where 25% of the total blood volume (determined by the weight of the mice) was taken. A bilateral pseudofemoral fracture was then performed during which 0.15 mL of ground bone marrow matrix from a matched-age donor uninjured mouse was injected around the femur of each leg (9). A laparotomy was then performed, and 10 liver crush injuries were completed by placing the lobes of the liver between the tips of a forceps. The abdomen was then closed with 4-0 PDS (polydioxanone), anesthesia was terminated, and the mice were placed into a cage with access to a heated pad. They were given buprenorphine at a dose of 0.03 mg/mL every 12 h by subcutaneous injection for pain management postoperatively.

Harvest of organs

Animals were killed either after no injury (out-of-box control mice), at 24 h after PT, or 7 days after PT (n = 6 per group). Blood was collected into a citrated tube and peripheral blood mononuclear cells (PBMCs) were purified using a Ficoll gradient (Millipore Sigma, Burlington, MA). Harvest of the spleen, liver, and lung was also performed. The spleens were macerated, and immune cells were extracted. Lung tissues were subjected to tissue digestion using liberase TL (50 μg/mL final concentration; Millipore Sigma) and DNase I (1 μg/mL final concentration; Millipore Sigma) (10). The liver was first perfused with a Per 1 ready solution, and tissue was digested using a Per 2 ready solution. A detailed list of components for both solutions can be found in Appendix 1. For PBMCs and spleen, experiments for every timepoint were split into two with the processing of tissues from three mice at one time. For liver and lung processing, each group of six from each timepoint was processed at once. Repeat experiments were done if mice from a group did not survive the PT, to reach a total of six mice per group.

We chose PBMCs and spleen to characterize macrophages in the circulation. We then evaluated liver as a site of injury in this model and the lung because of the link between the systemic immune response to trauma and the development of localized organ damage as seen in acute respiratory distress syndrome (11).

Flow cytometry staining methods

The cells were counted and equally split into different FACS sample tubes and washed by centrifugation. Mouse Fc receptor blocking agent (BD Biosciences, San Jose, CA) was added to all tubes containing cells for 5 min at 4°C. Cells were then stained for viability (Live/Dead Stain; Thermo Fisher, Waltham, MA) and for macrophage surface markers CD68 (Biolegend, San Diego, CA) and F4/80 (BD Biosciences). Samples were kept at 4°C for 30 min. Samples were then washed by centrifugation, and cells were fixed with 2% paraformaldehyde (Thermo Fisher Scientific). The appropriate cells were permeabilized with BD Cytofix/Cytoperm (BD Biosciences) for 30 min. After washing the cells with the permeabilization buffer and centrifugation, antibodies that stained intracellular markers were added for 60 min at 4°C. Those included iNOS (Thermo Fisher Scientific) as a marker for the M1 phenotype and arginase 1 (R&D, Minneapolis, MN) as markers for the M2 phenotype (12–14). The staining panel is included in Appendix 2. The samples were then analyzed with a BD LSR II flow cytometer, and results were analyzed with FlowJo version 10.7.1 (BD Biosciences, San Jose, CA). Gating strategy is shown in Figure 1.

Oxidative burst assay

Lung and liver nonparenchymal cells were harvested from out-of-box control and PT mice. After an initial wash, cells were incubated at 37°C for 5 min with or without DHR123 (50 μM; Thermo Fisher Scientific). Mouse Fc receptor blocking agent (BD Biosciences) was added to stained tubes for 10 min at room temperature. The cells were stained for viability (Live/Dead Stain Kit; Thermo Fisher Scientific), PE/Cy7-labeled CD68 antibody (Biolegend), and BUV395-labeled F4/80 antibody (BD Biosciences). The cells were fixed with 2% paraformaldehyde (Thermo Fisher Scientific). The assay was verified using a positive and negative control. Our positive control consisted of cells stimulated with PMA (4 μM) or LPS (100 ng/mL), and our negative control consisted of cells coming from out-of-box mice, not treated with any stimulant. The samples were then analyzed with a BD LSR II flow cytometer immediately after staining, and results were analyzed with FlowJo version 10.7.1.

Statistical analysis

All data were analyzed using Prism 9 version 9.0.2 (Graphpad Software, San Diego, CA). All data are reported as mean ± SD. Statistical outliers were identified through the ROUT (robust regression and outlier removal) method and removed before analysis. A Shapiro-Wilk test was run to determine normality. Based on the results, a one-way ANOVA with Tukey *post hoc* testing or a Kruskal-Wallis test was performed for the examination of the dynamic change in phenotype or of function over time. An independent *t* test or Mann-Whitney *U* test was used to compare macrophages before and after trauma. Statistical significance was set at $P = 0.05$.

Sample size

This study is novel in that it evaluates dynamic changes in macrophage populations over time. Because this was an exploratory study with no previously characterized or defined change between time points, we set out to use a sample size of six animals per group.

RESULTS

Macrophage phenotyping

Macrophages in PBMCs, spleen, lung, and liver were isolated at three timepoints: before trauma, 24 h after injury, and late after trauma (7 days). We specifically looked at circulating macrophages ($CD68^+$ cells) and characterized them as M1 ($iNOS^+$) or M2 (arginase 1^+). Values presented are percentages of all $CD68^+$ cells within the organ of interest and are presented as mean ± SD.

a. M1 macrophages in different tissues over time (Fig. 2):

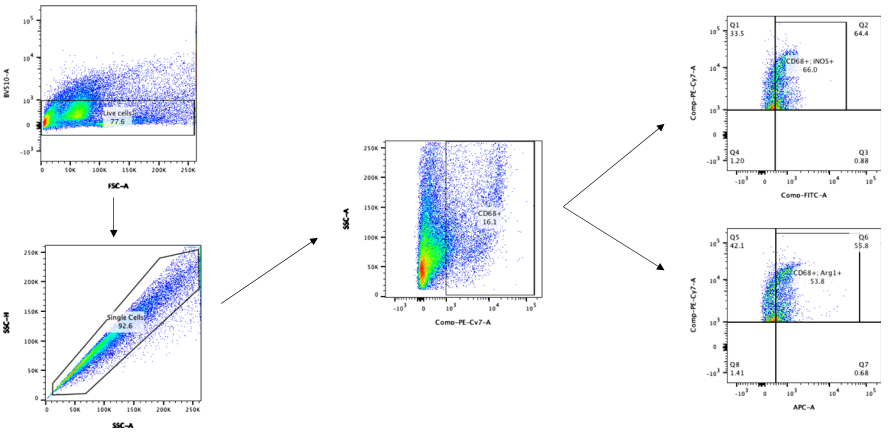

Fig. 1. **Gating strategy.** Gate 1: live cell determination. Gate 2: determination of single cell population. Gate 3: determination of macrophage CD68$^+$ population. Gate 4.a (upper right): determining M1 macrophages (CD68$^+$, iNOS$^+$ cells). Gate 4.b (lower right): determining M2 macrophages (CD68$^+$, Arg1$^+$ cells).

In PBMCs, at baseline, 6.6% (±3.9%) CD68$^+$ cells are of the M1 phenotype. That number slightly increases acutely after trauma, reaching 8.8% (±11.0%) at 24 h, and decreases again at 7 days after PT (4.9% ± 4.2%). These differences in average did not reach significance.

The spleen has a larger population of M1 macrophages at baseline (27.7% ± 7.5%), with that level decreasing after trauma to 20.6% (±13.2%) at 24 h and 12.6% (±5.6%, $P = 0.0027$ compared with out-of-box control) at 7 days.

A similar trend is seen in the lung. At baseline, 14.9% (±8.7%) of CD68$^+$ macrophages are M1, with a subsequent continual decrease reaching 8.7% (±7.4%) at 24 h and 4.4% (±0.8%, $P = 0.0265$ compared with out-of-box control) at 7 days after PT.

M1 macrophages in the liver are 14.3% (±7.5%) of the CD68$^+$ population in the liver nonparenchymal cells at baseline, increase at 24 h after trauma (20.8% ± 9.7%), and then decrease at 7 days after PT (13.4% ± 5.7%). These changes were not statistically significant.

b. M2 macrophages in different tissues over time (Fig. 3):

There is minimal presence of M2 macrophages in circulating PBMCs and in spleen at baseline (<5%), and there is minimal variation after trauma. The M2 macrophages are present in larger proportion in the lung at baseline (7.2% ± 3.9%). The percentage of M2 macrophages in the lung remains relatively constant after trauma (7.2% ± 6.3% at 24 h and 9.2% ± 8.6% at 7 days). A large proportion of M2 macrophages are also seen in the liver (36.0% ± 12.7%). This percentage trends upward and reaches 45.6% (±11.5%) acutely after trauma and

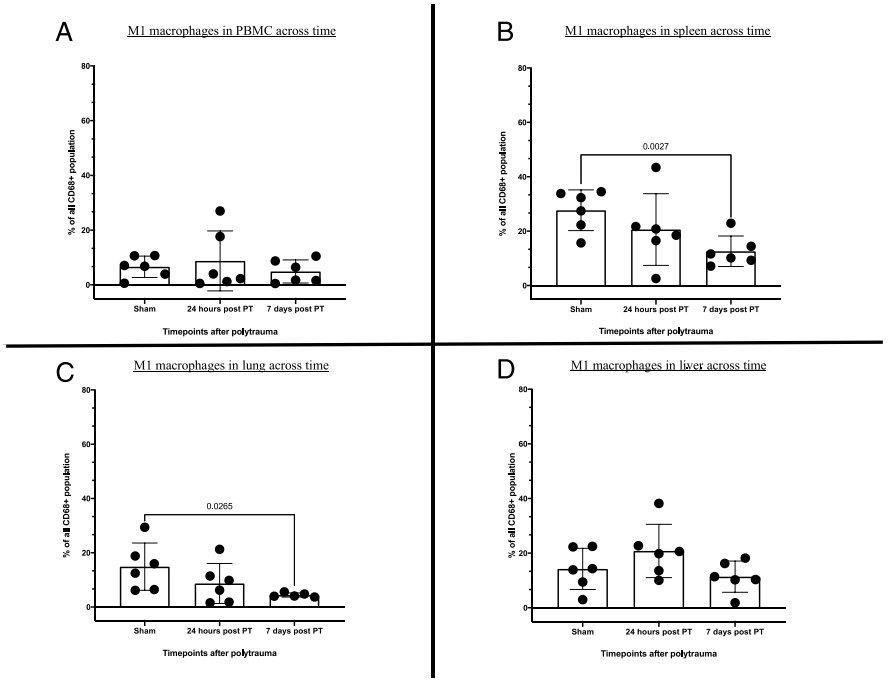

Fig. 2. **M1 macrophages in tissues over time.** Data presented correspond to mean ± SD. A, Graph showing the presence of M1 macrophages in PBMC at different timepoints after polytrauma. B, Graph showing the presence of M1 macrophages in spleen at different timepoints after polytrauma. Significant results through *t* test analysis and one-way ANOVA. C, Graph showing the presence of M1 macrophages in the lung at different timepoints after polytrauma. Significant results through *t* test analysis. D, Graph showing the presence of M1 macrophages in the liver at different timepoints. PBMC, peripheral blood mononuclear cells; PT, polytrauma; Sham, out-of-box controls.

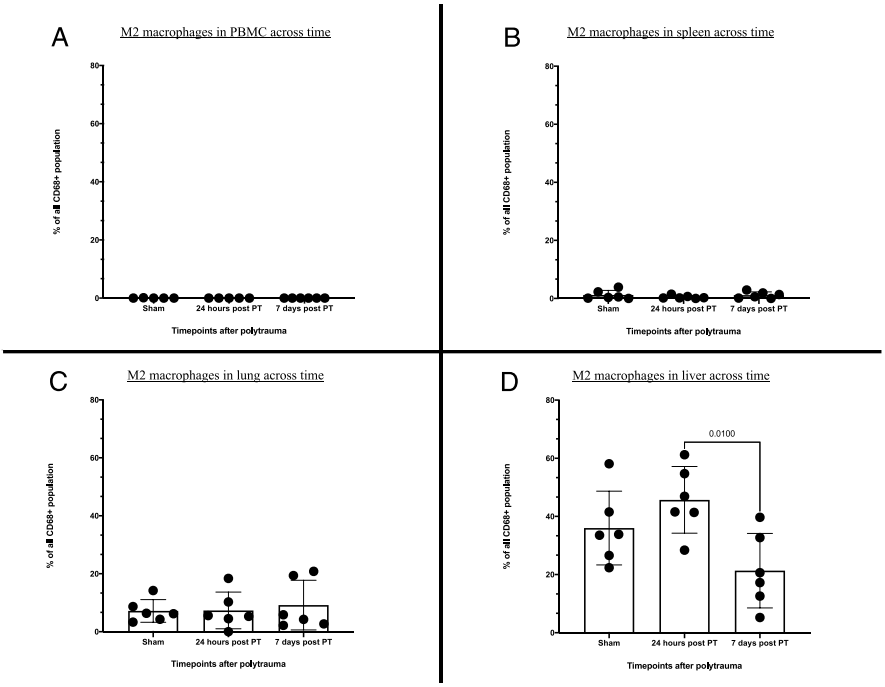

FIG. 3. **M2 macrophages in tissues over time.** Data presented correspond to mean ± SD. A, Graph showing the presence of M2 macrophages in PBMC at different timepoints after polytrauma. B, Graph showing the presence of M2 macrophages in spleen at different timepoints after polytrauma. C, Graph showing the presence of M2 macrophages in the lung at different timepoints after polytrauma. D, Graph showing the presence of M2 macrophages in the liver at different timepoints. Results significant through one-way ANOVA. PBMC, peripheral blood mononuclear cells; PT, polytrauma; Sham, out-of-box controls.

drops to 21.4% (±12.8%) at 7 days. These changes were not statistically significant.

c. Ratio of M2/M1 macrophages in the lung and liver over time (Fig. 4):

The baseline ratio of the average lung M2/M1 is 0.48, demonstrating M1 macrophage predominance at baseline. This changes 24 h after trauma with the ratio increasing to 0.84, as lung macrophages shift to an M2 phenotype. The ratio increases to 2.07 at 7 days after PT, showing an even larger M2 predominance.

In the liver, the baseline ratio of the average lung M2/M1 is 2.53, demonstrating M2 macrophage predominance at baseline. This remains relatively constant 24 h PT, with a ratio of 2.19 and trending downward to 1.88 at 7 days after PT, indicating a trend toward an increase in M1 response.

Oxidative burst

We evaluated oxidative burst in the liver and lung macrophages at baseline, 24 h, and 7 days after PT. Results are shown in Figure 5.

At baseline, 2.0% (±7.9%) of $CD68^+$ lung cells performed oxidative burst. Twenty-four hours after PT, 6.9% (±3.6%) of $CD68^+$ lung macrophages were undergoing oxidative burst. At 7 days after PT, 22.1% (±11.5%) of lung macrophages were undergoing oxidative burst, respectively (compared with baseline, 24 h: $P = 0.0028$; day 7: $P = 0.0258$).

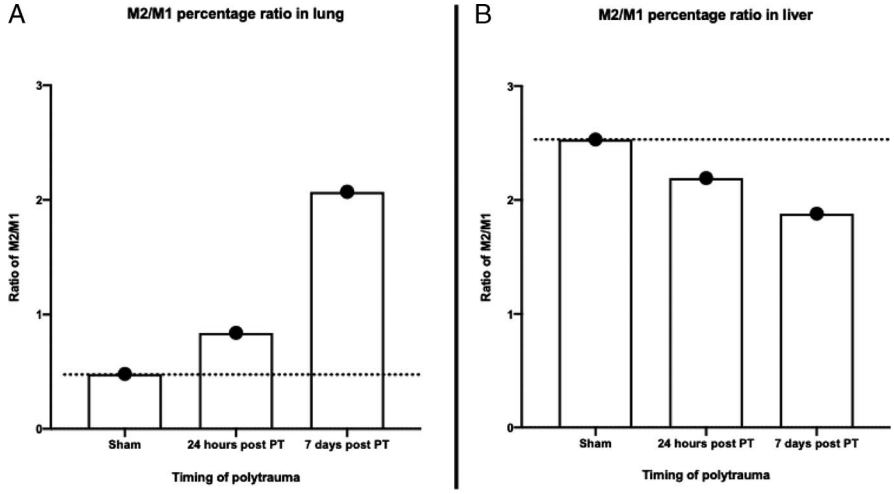

FIG. 4. **Ratio of M2 to M1 percentages in the lung and liver.** A, Graph showing ratios of average M2 to average M1 in the lung over time after polytrauma. B, Graph showing ratios of average M2 to average M1 in the liver over time after polytrauma. PT, polytrauma; Sham, out-of-box controls.

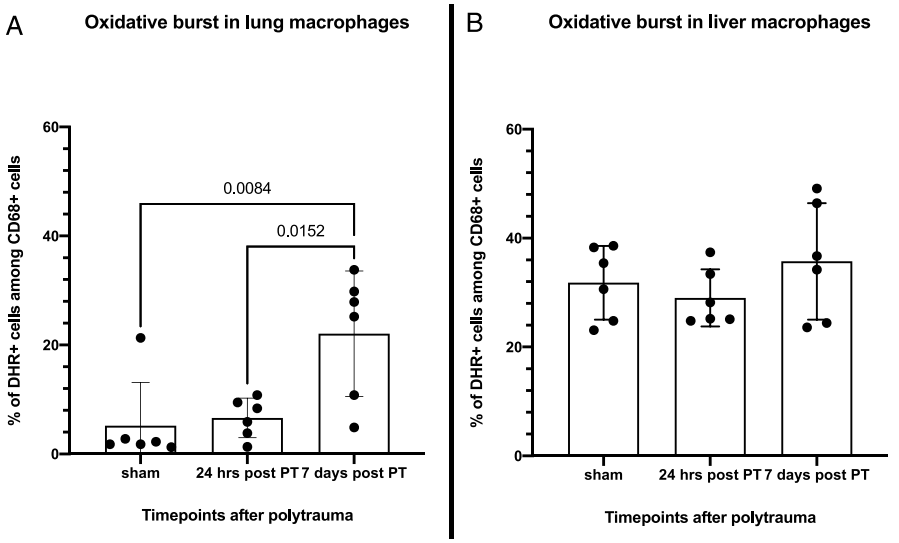

FIG. 5. **Macrophage oxidative burst in the lung and liver.** A, Graph showing oxidative burst in the lung macrophages over time after polytrauma. Results are significant through one-way ANOVA. B, Graph showing oxidative burst in the liver macrophages over time after polytrauma. PT, polytrauma; Sham, out-of-box controls.

In contrast, liver resident macrophages did not undergo a functional change after PT; 31.8% (±6.8%) of $CD68^+$ macrophages underwent oxidative burst at baseline, 29.8% (±5.3%) of macrophages underwent oxidative burst 24 h after PT, and 35.7% (±10.7%) at 7 days after PT. The changes seen in the liver were not statistically significant.

DISCUSSION

Our present study characterized the distribution of macrophage phenotype and changes in polarization in specific organs in a murine model of PT and hemorrhage. Myeloid cell contributors to systemic inflammatory response syndrome after trauma include mast cells (15) and polymorphonuclear leukocytes (16), but few studies have examined the association of macrophage polarization with posttraumatic immunopathology. We found a baseline presence of M1 macrophages in PBMCs, with the percentage only marginally changing over time after PT. Very few M2 macrophages were found in the PBMC compartment. A similar trend is found in the spleen, where we discovered a high M1 presence that decreases over time after PT, along with low M2 presence. The presence of M1 macrophages was consistent with our hypothesis, with the addition of a potential baseline priming effect secondary to spleen pathogen filtering. The white pulp of the spleen is where cells from the immune system will face foreign material and start responding to them (17). The decrease in M1 macrophages in the spleen over time after trauma could be due to a decrease in stimulation after the initial trauma or mobilization of these circulating cells to end organs. The low M2 macrophage presence in circulating PBMCs as well as the spleen was not unexpected, as these are not end organs requiring tissue repair or angiogenesis after injury.

The liver has the largest fixed macrophage population in the body, with tissue-specific macrophages (Kupffer cells) accounting for 40% to 65% of the total liver nonparenchymal cells (18). In this study, we evaluated the resident rather than the intravascular macrophages in the liver, because of the nature of the digestion of the tissue. We saw a baseline level of both M1 and M2 phenotype macrophages. This is expected as these cells are in contact with circulating blood from the portal vein and the hepatic artery and can therefore have a certain degree of activation, all while preserving a "tolerogenic" phenotype (18). The liver also has a large regenerative capacity (19), making the presence of the M2 phenotype at baseline reasonable.

We saw a baseline M1 macrophage phenotype present in the liver that is not significantly changed after PT and a baseline M2 phenotype that is stable in the first 24 h after trauma and decreases at 7 days after trauma. This decrease caused the shift of the ratio of M2/M1 macrophages at 7 days after PT toward the M1 phenotype. These observations point us to the fact that these changes might be specific to the crush injury as the response is different when compared with other models of liver injury such as the I/R injury model. In the I/R model, a predominance of M1 macrophages is seen in the early stages after the insult (18), with a shift to an M2 predominant phenotype in later stages (20). In contrast, the crush injury model showed an earlier infiltration of wound-healing macrophages and persistence of M1 macrophages for debris clearance. It is possible that there would be a change in the ratio toward the M2 phenotype, consistent with "late-stage" M2 predominance as seen in I/R, if the mice were followed for a longer period.

Macrophage polarization in the lung after trauma is important to study, particularly to understand their role in the increased vulnerability of hospitalized trauma patients to nosocomial infections including pneumonia (21). We found a baseline presence of both M1 and M2 macrophages in the lung. This is to be expected as the lung both clears respiratory pathogens and participates in tissue repair at baseline (22). Interestingly, we saw a significant decrease in percentages of M1 macrophages after trauma in the lung. This was unexpected, given the association of M1 macrophage polarization with systemic inflammation in the setting of insults such as pancreatitis (15) and thermal injury (16) and that macrophages isolated from posttraumatic patients exhibit enhanced inflammatory response to LPS (17). This prompted us to look at their function at these different timepoints. Even though the number of M1 macrophages significantly decreased, we saw a significant increase in the oxidative burst capability of these macrophages. This could be demonstrative of a separate subset of macrophages, uncaptured by our phenotyping, with increases in inflammatory capacity after

injury. In our understanding of macrophage physiology, it is becoming increasingly evident that macrophage polarization is malleable. There is a multitude of different macrophage subtypes that could be responsible for this discrepancy between phenotype and function. These fall on a spectrum of polarization, with clearly defined M1 and M2 being the two extreme poles, and further study using multiplexing assays such as CyTOF or RNA sequencing may better delineate these subtypes. The unexpected discrepancy between function and phenotype also prompts us to think about the possibility of using functional changes in macrophages rather than phenotypic changes to characterize macrophages. Targeting macrophages based on their function could also open up new therapeutic potentials.

LIMITATIONS

This study has several limitations. Although we were able to identify the clearly polarized M1 and M2 macrophages in the different tissues, we were unable to capture the evolving phenotypes that have not reached a mature polarization state. These include different subtypes of macrophages that are on the spectrum of polarization. This study also did not look at macrophages through a single-cell perspective, which might have offered a more detailed phenotypic background. In addition to that, being that this study is the first to evaluate the dynamic changes in macrophage population over time after PT, a sample size of six was chosen as a pilot study. These results will be utilized to power future analyses. In addition, the study was conducted with male C57B6 mice and therefore may not be generalizable. We also acknowledge that we chose to concentrate on $CD68^{+}$ cells and did not focus solely on tissue resident ($F4/80^{+}$) macrophages, to include evaluation of more immature circulating macrophages. We also chose to represent the M1 and M2 phenotypes by their most classical markers, as others (CCR2 and CD206) were much more variable in nature, possibly due to the fluctuating phenotype of these cells.

CONCLUSION

Macrophages play an important immunomodulatory role, and their ability to respond to external stimulus allowed them to become a target of therapeutic potential. We evaluated macrophages after trauma in different organ tissues and focused on the two main phenotypes: the M1 proinflammatory phenotype and the M2 anti-inflammatory phenotype. We found a discrepancy in macrophage phenotype and function in the lung, which may signify a change in subset polarization after injury. An important next step will be to confirm these findings in the setting of a secondary bacterial infection in the trauma recovery phase, to better understand the response to infection after trauma.

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Appendix 1

Per 1 ready solution: Per 1 stock solution, diH_2O, 2.23 μM EGTA (ethylene glycol-*bis*(2-aminoethylether)-N,N,N′,N′-tetraacetic acid)
Per 1 stock solution: diH_2O, 1.42 M sodium chloride (NaCl), 67 mM potassium chloride, 100 mM HEPES
Per 2 ready solution: Per 2 stock solution, 60 mg of collagenase H (cat. no. 11087789001; Roche, Basel, Switzerland).Per 2 stock solution: 19 mM calcium chloride, diH_2O, 151.52 mM albumin, 67 mM sodium chloride, 6.7 mM potassium chloride, 100 mM HEPES

Appendix 2

Flow cytometry markers

Marker	Color	Product detail
Live/dead fixable stain—405-nm excitation	Aqua	Thermo Fisher Scientific L34957
F4/80	BUV395	BD Biosciences 565,614
CD68	PE-Cy7	Biolegend 137,016
iNOS	Fluorescein isothiocyanate	Thermo Fisher Scientific 53-5,920-82
iNOS isotype control: rat Immunoglobulin G2a κ isotype control	Alexa Fluor 488	Thermo Fisher Scientific 53-4,321-80
Arginase 1	Allophycocyanin	R&D IC5868A
Arginase 1 isotype control	Sheep immunoglobulin G	R&D 1C016A