Original Research *Highlight article*

Mild hypobaric hypoxia influences splenic proliferation during the later phase of stress erythropoiesis

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Impact statement

Environmental stressors can influence processes triggered by tissue trauma and hemorrhagic shock, including stress erythropoiesis and host defense. While aeromedical evacuation enables continuous treatment en route for trauma victims, extended transport may induce these stressors because the aircraft cabin pressure is mildly hypobaric and can yield a lower oxygen content. The objective of this study was to investigate the effect of extended mild hypobaric hypoxia exposure on splenic erythropoiesis in a muscle trauma model and polytrauma model consisting of muscle trauma and hemorrhagic shock. In a polytrauma model, extended mild hypobaric hypoxia exposure reduced the number of proliferating splenic cells during the later phase of stress erythropoiesis; however, this effect was not observed in the muscle trauma model. During intercontinental aeromedical evacuation, spleen immune cells may become dysregulated after polytrauma. Countermeasures or delayed transport may need to be initiated to ensure immune functioning.

Abstract

Tissue trauma and hemorrhagic shock are common battlefield injuries that can induce hypoxia, inflammation, and/or anemia. Inflammation and hypoxia can initiate adaptive mechanisms, such as stress erythropoiesis in the spleen, to produce red blood cells and restore the oxygen supply. In a military context, mild hypobaric hypoxia—part of the environmental milieu during aeromedical evacuation or en route care—may influence adaptive mechanisms, such as stress erythropoiesis, and host defense. In the present study, healthy (control), muscle trauma, and polytrauma (muscle trauma and hemorrhagic shock) mice were exposed to normobaric normoxia or hypobaric hypoxia for ~17.5 h to test the hypothesis that hypobaric hypoxia exposure influences splenic erythropoiesis and splenic inflammation after polytrauma. This hypothesis was partially supported. The polytrauma + hypobaric hypoxia group exhibited more splenic neutrophils, fewer total spleen cells, and fewer splenic proliferating cells than the polytrauma+normobaric normoxia group; however, no splenic erythroid cell differences were detected between the two polytrauma groups. We also compared splenic erythropoiesis and myeloid cell numbers among control, muscle trauma, and polytrauma groups. More reticulocytes at 1.7 days (40 h) post-trauma (dpt) and neutrophils at 4 dpt were produced in the muscle trauma mice than corresponding control mice. In contrast to muscle trauma, polytrauma led to a reduced red blood cell count and elevated serum erythropoietin levels at 1.7 dpt. There were more erythroid subsets and apoptotic reticulocytes in the polytrauma mice than muscle trauma mice at 4 and 8 dpt. At 14 dpt, the red blood cell count of the polytrauma + normobaric normoxia mice was 12% lower than that of the control + normobaric normoxia mice; however, no difference was observed between polytrauma + hypobaric hypoxia and control + hypobaric hypoxia mice. Our findings suggest muscle trauma alone induces stress erythropoiesis; in a polytrauma model, hypobaric hypoxia exposure may result in the dysregulation of splenic cells, requiring a treatment plan to ensure adequate immune functioning.

Keywords: Aeromedical evacuation, hemorrhagic shock, hypoxia, Ki-67, phagocytosis, stress erythropoiesis

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Introduction

Tissue trauma and hemorrhagic shock are common battlefield injuries that can induce inflammation, anemia, and/or hypoxia. Inflammation from severe tissue damage or infection (e.g. bacterial or parasitic) can impede the erythropoietin (EPO) response in bone marrow, suppressing erythroid progenitor cells (EPCs) and increasing EPCs in the peripheral blood after severe injury[1,2]; however, splenic erythropoiesis is initiated to produce new erythrocytes to compensate for the anemia. Similarly, tissue hypoxia resulting from disease and injury,[3,4] high altitude (hypobaric hypoxia (HB)) exposure,[5] or severe hemorrhage (i.e. anemia)[6] can activate EPO and/or the bone morphogenic protein 4 (BMP4)-dependent pathways,[7–10] stimulating splenic erythropoiesis to restore the oxygen supply to hypoxic tissues. Considering mild hypobaric hypoxia exists in the environment of aeromedical evacuation, which is a critical strategy for treating these injuries, hypoxia may influence adaptive mechanisms like stress erythropoiesis and host defense.

Splenic erythropoiesis is a dynamic process.[11] Upon phagocytosing erythrocytes, splenic macrophages produce the chemokine C–C motif chemokine ligand 2 (CCL2), which recruits blood monocytes to the spleen.[11] After migrating to the spleen, blood monocytes accumulate with stress erythroid progenitors (SEPs) to form stress-induced erythroblastic islands.[11] These islands are the sites of the simultaneous proliferation and maturation of both blood monocytes and SEPs.[11] Pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), may be involved in SEP expansion and differentiation.[12] With the maturation of SEPs, reticulocytes and erythrocytes enter the bloodstream to restore the red blood cell (RBC) count.[10,11] Besides the increase in erythroid subsets, myeloid cells also accumulate in the spleen.[13–15] At the same time, apoptosis[13,16–19] and erythrophagocytosis involving both macrophages[12,20,21] and neutrophils[22] may maintain reticulocyte and erythrocyte homeostasis.

Splenic erythropoiesis can be monitored through the expression of cell markers. For example, immature reticulocytes are identified as a $CD71^{+}Ter119^{+}$ population,[22] whereas erythrocytes or more mature reticulocytes are identified as a $CD71^{-}Ter119^{+}$ population.[23] These subpopulations have been detected in both trauma[22] and infection[15] models and correlated with erythrocyte maturation.[23]

Skeletal muscle is prone to damage, and a robust inflammatory response of neutrophils, monocytes, and macrophages develops within hours to days after injury.[24–27] In association with the infiltration of these leukocytes, increased expression of pro-inflammatory cytokines, including TNF-α and IL-1β, occurs.[27] During this pro-inflammatory state after muscle damage, the activation of splenic erythropoiesis may occur. However, muscle trauma-related splenic erythropoiesis has not been investigated.

In severe trauma situations, hemorrhagic shock can accompany muscle injury or other types of tissue damage. Since hemorrhagic shock and/or tissue damage induce an inflammatory response[6,27,28] and hemorrhagic shock causes systemic hypoxia,[6] polytrauma likely drives splenic erythropoiesis. However, our understanding of the effect of polytrauma on splenic erythropoiesis is limited. In one rat model, polytrauma consisting of lung contusion and hemorrhagic shock did not induce splenic erythropoiesis.[29] However, polytrauma accompanied with chronic stress resulted in splenic erythropoiesis, evidenced by splenomegaly, increased growth of spleen erythroid progenitors, induced *BMP4*, and reduced *EPOr* expression.[29] As these data are from one polytrauma model, other types of polytrauma (e.g. skeletal muscle trauma and hemorrhagic shock) need to be explored.

Polytrauma involving skeletal muscle injury and hemorrhagic shock occurs in both civilian and military populations, but wounded military personnel sustaining polytrauma are often situated in austere environments that are distant from Level I trauma centers. Therefore, the care of wounded soldiers typically entails a 24-h stabilization period and then aeromedical evacuation, or air transport, to an advanced medical center,[30] with the flight time lasting 8–16 h. Although en route care via aeromedical evacuation involves supplemental oxygen for many patients, the presence of mild hypobaric hypoxia within the flight environment (i.e. aircraft cabin pressurized to an altitude of 2,438 m) could influence underlying adaptive mechanisms and, ultimately, treatment plans. For example, Johannigman *et al.*[31] reported that wounded soldiers who endured a 9-h flight without supplemental oxygen experienced reduced blood oxygen saturation en route. Furthermore, data from animal models demonstrate that mild hypobaria can lead to systemic hypoxia.[32,33] In this study, we tested the hypothesis that extended HB exposure influences splenic erythropoiesis and splenic inflammation within the first two weeks after muscle trauma, hemorrhagic shock, and fluid resuscitation. Understanding the effects of mild hypobaria exposure on splenic erythropoiesis after polytrauma involving muscle trauma–hemorrhagic shock and muscle trauma alone will ensure wounded soldiers and other trauma victims requiring air transport receive effective en route care.

Materials and methods

Animals

Male C57BL/6N mice were purchased from Envigo Laboratories (Indianapolis, IN) and Charles River (Hollister, CA) and allowed to acclimate 33.8 ± 0.6 d (mean ± standard error (*SE*)) before the start of the study. Males were studied because the majority of U.S. warfighters are men. The mice were individually housed in a ventilated caging system (Lab Products, Seaford, DE) under a 12:12-h light:dark cycle. Animals were provided a standard laboratory diet with food and water ad libitum. At the time of the procedure, the mouse body mass was 25.5 ± 0.1 g, and the age was 12.9 ± 0.1 weeks. All animal procedures were approved by the University of Nevada, Las Vegas, Institutional Animal Care and Use Committee and the U.S. Army Medical Research and Development Command Animal Care and Use Review Office. Mice

were maintained at the University of Nevada, Las Vegas, and in a specific pathogen-free facility, accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The Guide for the Care and Use of Laboratory Animals and the Office of Laboratory Animal Welfare were followed.

Experimental procedure

Four hundred seventy-two mice were used in this study. Each mouse was assigned to one of six groups of varying trauma status and atmospheric pressure exposure (see below). All mice received two doses of buprenorphine based on a target range of 0.05–0.10 mg/kg subcutaneously (Par Pharmaceutical, Chestnut Ridge, NY), with the second dose administered 8–14 h after the first dose. After the first buprenorphine dose, muscle trauma and polytrauma mice were anesthetized with isoflurane (1–5% to effect; Covetrus, Dublin, OH) and 100% oxygen. These anesthetized mice then underwent a closed crush trauma of the right hindlimb—which targeted the plantarflexor muscle, gastrocnemius, and quadriceps femoris muscle—followed by femoral artery catheterization of the left hindlimb. (Note: Two muscle trauma mice underwent right femoral artery catheterization because the initial left femoral artery catheterization was unsuccessful.) Next, blood was removed from the catheters of polytrauma mice to induce pressure-controlled hemorrhagic shock with a target mean arterial pressure range of 25–45 mmHg for ~30–60 min. These mice received 6% hetastarch (Covetrus) for resuscitation until the mean arterial pressure returned to baseline or 63.8 ± 0.3 mmHg. Approximately 24 h after the first buprenorphine dose, the muscle trauma, polytrauma, and control (no muscle trauma or hemorrhagic shock) mice were exposed to an HB condition (target 565 (560–570) mmHg) or normobaric normoxia (NB) condition ($\geq$690 mmHg) in an acrylic chamber for 17.4 ± 0.02 h to simulate an ultra-long-haul flight. Mice recovered for four time points post-trauma, designated as days post-trauma (dpt): 1.7 (40 h), 4, 8, and 14. Mice that did not survive the trauma/hemorrhage procedure ($n = 1$), did not survive until the scheduled euthanasia ($n = 13$), or were observed to develop comorbid conditions after the protocol ($n = 5$) were excluded from this study.

After euthanasia, the targeted hindlimb muscles were harvested and prepared for leukocyte analysis. The spleen was harvested, weighed to calculate the relative spleen mass (RelSM, mg of spleen mass divided by g of body mass), then maintained in MACS tissue storage solution (Miltenyi Biotec, Auburn, CA) for less than 24 h. Single-cell suspensions were acquired from the gentleMACS tissue dissociator (Miltenyi Biotec) and the spleen dissociation kit (Miltenyi Biotec). Harvested spleen cells were counted using a Z Series Coulter cell counter (Beckman Coulter, Brea, CA) per the manufacturer's instructions. For all samples, the cell counter range was set at 5.5–10.0 μm to ensure spleen cells were enumerated.

Peripheral blood was collected into BD Microtainer® blood collection tubes with dipotassium ethylenediaminetetraacetic acid (BD Biosciences, San Jose, CA) by heart puncture, then analyzed for RBC count and hemoglobin (Hgb) levels using a Coulter AcT diff2 hematology analyzer (Beckman Coulter).

Flow cytometry

Single-cell suspensions (10^6 cells/mL) were stained with Fc block (0.5 μg, Clone 2.4G2; BD Biosciences) for 10 min, then stained with fluorescent surface markers for 30 min at 4°C. For intracellular staining,[8] the cells were fixed in 1% paraformaldehyde (Alfa Aesar, Tewksbury, MA) immediately after the surface marker staining, then permeabilized using 0.1% saponin buffer (Alfa Aesar). Intracellular staining was performed in the dark for 60 min at 4°C. After washing, cells underwent flow cytometry acquisition and analysis using a BD Biosciences AriaFusion. The BD FACSDiva software 8.0 (BD Biosciences) was used for data analysis, and less than 0.5% of the events in the unstained samples were used to identify the false-positive events. The percentage of cells in the singlets gate was multiplied by the number of single cells to generate an absolute cell count. Antibodies and flow cytometry markers are listed in Supplemental Tables 1 and 2. $CD45^-$ erythroid cells were used since these cells are more terminally differentiated erythroid cells and, unlike $CD45^+$ cells, have not been assigned immunosuppressive activities.[34]

Serum EPO levels

Serum EPO levels were measured using the MECY2MAG-73K multiplex antibody-bead assay kit (MilliporeSigma, Burlington, MA) and Luminex 200 instrument (Austin, TX) with Belysa 1.1 software (MilliporeSigma). Samples were run in triplicates; however, the sample mean value was calculated using duplicates. Standards were run in duplicates. Standard curves were generated for each kit, and the standard curve parallelism was determined. The standard curve with a parallelism value closest to 1 was used to analyze all sample levels. The six-point standard curve ranged from 49 to 50,000 pg/mL.

Statistical analysis

SAS® 9.4 software was used for statistical analysis.[35] Extreme outliers from the dataset of phagocytosed reticulocytes were detected using the interquartile range rule with an adjusted coefficient (instead of 1.5), then deleted. The adjusted coefficient allowed us to differentiate between moderate and extreme outliers and keep all observations in small groups.

Two approaches were used. In one approach, we conducted an overall comparison between the three groups—i.e. control (uninjured), muscle trauma, and polytrauma—with each pressure condition tested separately. This analysis was conducted using the Kruskal-Wallis chi-square (CS) at an α level of 5%. If this overall test yielded a P value <0.05, we performed two additional tests using Bonferroni correction to account for multiple comparisons. Specifically, we tested muscle trauma against polytrauma and polytrauma against control. The adjusted significance level was $0.05 \div 2 = 2.5\%$ for each test. A second approach

compared (a) polytrauma + NB against polytrauma + HB and (b) muscle trauma against control. For each comparison, we used the Kruskal-Wallis CS test at an α level of 5%. All statistical tests were two-tailed, and values are expressed as mean ± *SE*. We reported test results as exact *P* values or $P < 0.001$.

Results

Leukocytes in damaged plantarflexor muscle

Mice in the muscle trauma groups demonstrated an increased number of $CD11b^+$ cells in the damaged plantarflexor muscle at 1.7 dpt (40 h) and 4 dpt when compared to the control groups (Figure 1). At the same time points, there were more $CD11b^+$ cells in the polytrauma mice than in the control mice (Figure 1). However, there was no difference in the number of muscle $CD11b^+$ cells between the muscle trauma and polytrauma groups.

Muscle trauma and HB effects

At 1.7 dpt, splenic $CD71^+Ter119^+$ cells (reticulocytes) increased similarly in both muscle trauma groups compared to corresponding control groups (Figure 2(a)). At 4 dpt, mice in the muscle trauma + HB group produced more reticulocytes than mice in the control + HB group (Figure 2(b)). At the same time, more of these reticulocytes were phagocytosed by neutrophils (Figure 2 (c)) or monocytes (Figure 2(d)) in the muscle trauma + HB compared to the control + HB group. However, the RBC count of the muscle trauma mice did not decrease compared to control mice at these time points. The RBC counts of the control + NB and control + HB groups were 9.37 ± 0.16 and 9.76 ± 0.09 at 1.7 dpt, respectively, and 9.19 ± 0.09 and 9.26 ± 0.11 at 4 dpt, respectively.

At 1.7 dpt, the neutrophil number was higher in the muscle trauma + NB group compared to the control + NB group (Figure 3(a)). At 4 dpt, the neutrophil number increased in both muscle trauma groups compared to the corresponding control groups (Figure 3(b)). This increased neutrophil number was accompanied by a greater RelSM (Figure 3(c)).

Polytrauma and HB effects

Compared to muscle trauma, polytrauma led to a ~30% reduction in the RBC count (Figure 4(a)), ~35% decrease in Hgb levels (Supplemental Table 4), 22% increase in RelSM (Figure 4(b)), and a 5–10-fold elevation in serum EPO levels (Figure 4(c)) at 1.7 dpt. From 1.7 to 4 dpt, there was a two-fold increase in RelSM in the polytrauma mice (Figure 4(b)). At 4 dpt, mice in the polytrauma groups demonstrated increased numbers of $CD71^+Ter119^-$ (Figure 5(a)), $CD71^+Ter119^+$ (Figure 5(b)), and apoptotic $CD71^+Ter119^+$ (Figure 5(c)) cells compared to the muscle trauma groups. At 8 dpt, the RBC count and RelSM began to reflect recovery (Figures 4(a) and (b)), and mice in the polytrauma + HB group exhibited more phagocytosed reticulocytes than the muscle trauma + HB group (Figure 5(d)). This finding is similar to that observed between muscle trauma + HB and control + HB mice at 4 dpt (Figure 2(d)).

At 14 dpt, the RelSM of mice in the polytrauma groups (Figure 4(b)) was similar to control group values (2.71 ± 0.07 and 2.79 ± 0.09 for control + NB and control + HB, respectively). The Hgb levels in the polytrauma groups were similar to the corresponding control groups (Supplemental Table 4). The RBC counts of the polytrauma groups were 7.3% (NB) and 4% (HB) lower than the corresponding control groups, but no difference was observed between the two polytrauma groups (Figure 4(a)). The RBC count of the polytrauma + NB group was lower than that of the muscle trauma + NB group, whereas the RBC counts of the polytrauma + HB and muscle trauma + HB groups were similar (Figure 4(a)).

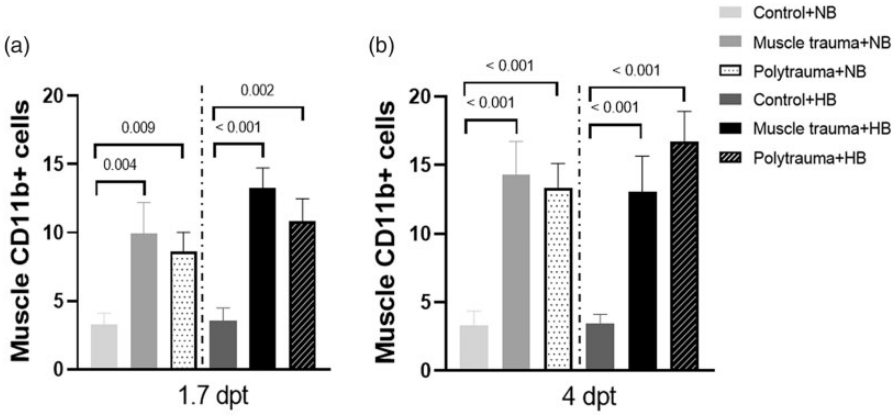

Figure 1. $CD11b^+$ cells in the plantarflexor muscle after trauma or polytrauma and NB or HB exposure. Both muscle trauma and polytrauma groups show an increase in $CD11b^+$ cells (10^5 cells/g) in the plantarflexor muscle at 1.7 dpt (40 h, a) and 4 dpt (b) compared to control groups. However, there is no difference in the number of muscle $CD11b^+$ cells between muscle trauma and polytrauma groups. Kruskal-Wallis CS test with Bonferroni correction for multiple tests ($\alpha = 0.025$) was used to compare polytrauma vs. control groups and polytrauma vs. muscle trauma groups. Kruskal-Wallis CS test ($\alpha = 0.05$) was used to compare control vs. muscle trauma groups. *P* values are listed above the bracket for each comparison.
dpt: days post-trauma; NB: normobaric normoxia; HB: hypobaric hypoxia; vs.: versus; $n = 8$–10.

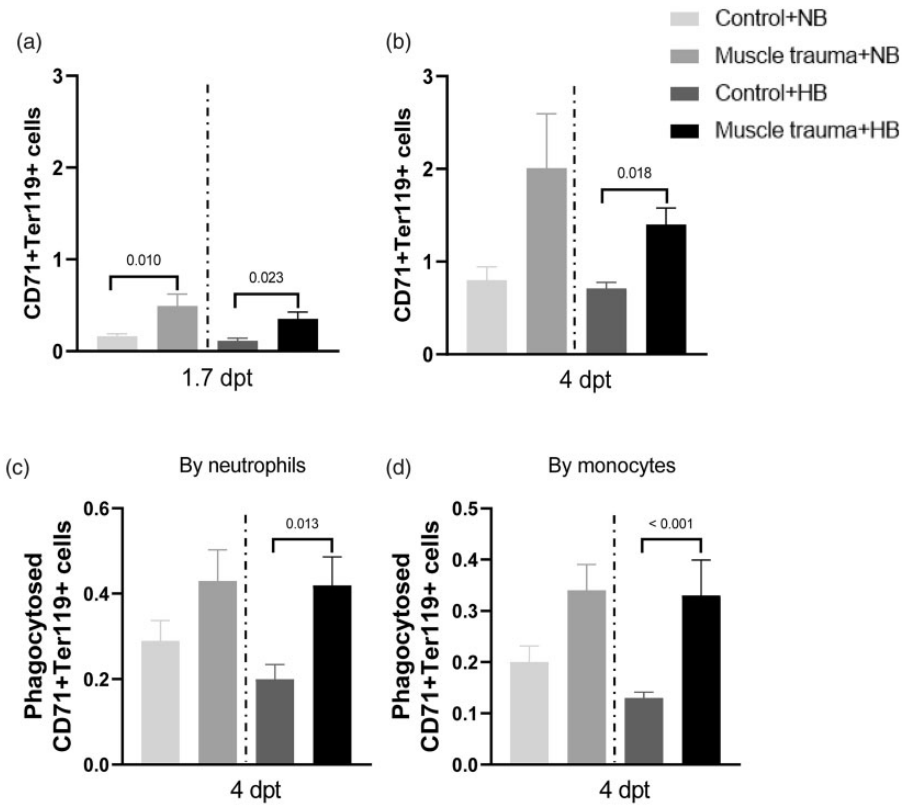

Figure 2. Splenic erythroid cell numbers after muscle trauma and NB or HB exposure. In both trauma groups, the splenic $CD71^{+}Ter119^{+}$ cell number increases at 1.7 dpt (a, $n = 6$–9), whereas there are more cells in the muscle trauma + HB group than the control+HB group (b, $n = 5$–7) at 4 dpt. More $CD71^{+}Ter119^{+}$ cells are phagocytosed by neutrophils (c, $n = 7$–11) and monocytes (d, $n = 7$–11) at 4 dpt in the muscle trauma+HB group compared to the control+HB group. Cell number is in units of 10^{6}. Kruskal-Wallis CS test was used to compare control vs. muscle trauma groups. Same bracket notation and abbreviations as in Figure 1.

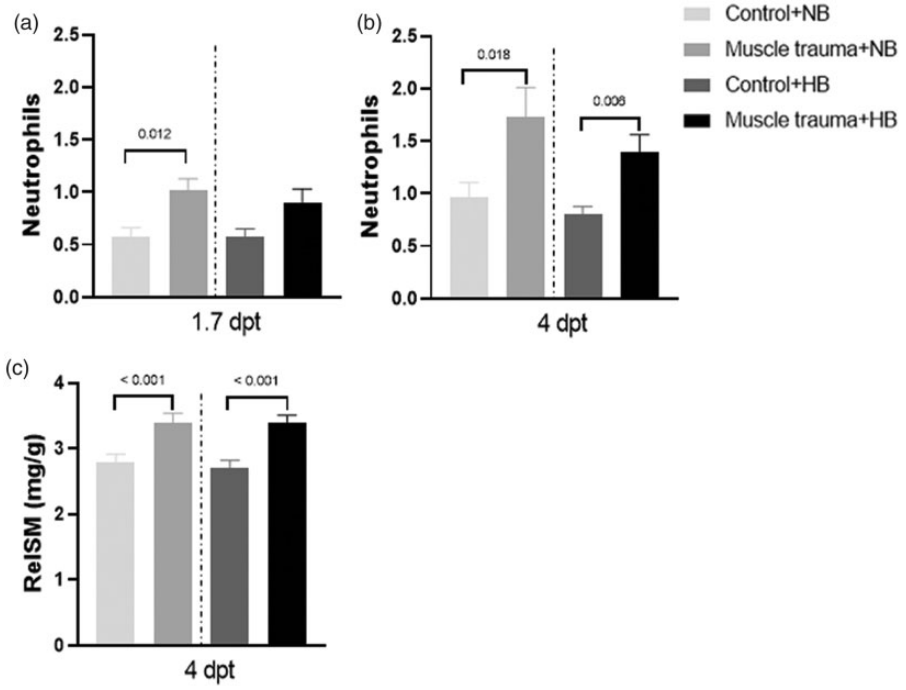

Figure 3. Splenic neutrophil number and RelSM after muscle trauma and NB or HB exposure. At 1.7 dpt, there are more neutrophils in the muscle trauma+NB group compared to the control+NB group (a, $n = 11$–14). Neutrophils (b, $n = 10$–12) and RelSM (c, $n = 19$–21) increase at 4 dpt in both muscle trauma groups compared to the corresponding control groups. Cell number is in units of 10^{6}. Kruskal-Wallis CS test was used to compare control vs. muscle trauma groups.
RelSM: relative spleen mass. Same bracket notation and abbreviations as in previous figures.

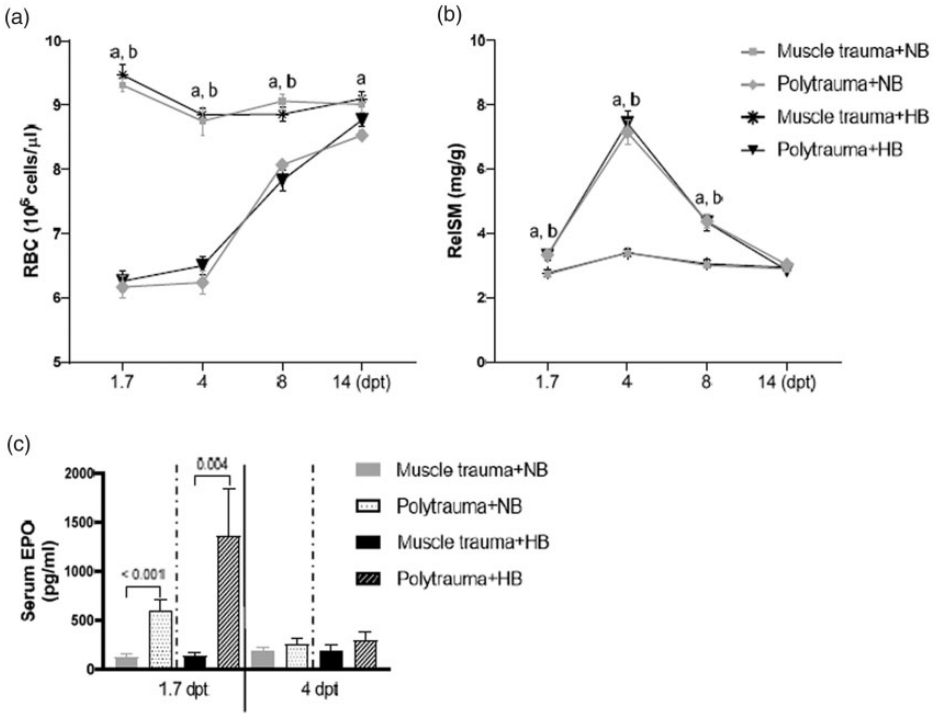

Figure 4. RBC count, RelSM, and serum EPO levels after muscle trauma or polytrauma and NB or HB exposure. The circulating RBC count in the polytrauma groups is low at 1.7 and 4 dpt (a, $n = 15–20$). The RelSM (B, $n = 19–21$) peaks at 4 dpt in both polytrauma groups. At 1.7 dpt, serum EPO levels are higher in the polytrauma groups (c, $n = 7–14$) than in the muscle trauma groups. (a) $P < 0.01$, muscle trauma+NB vs. polytrauma+NB groups; (b) $P < 0.01$, muscle trauma+HB vs. polytrauma+HB groups. Kruskal-Wallis CS test with Bonferroni correction for multiple tests ($\alpha = 0.025$) was used to compare polytrauma vs. muscle trauma groups. RBC: red blood cell; EPO: erythropoietin. Same bracket notation and abbreviations as in previous figures.

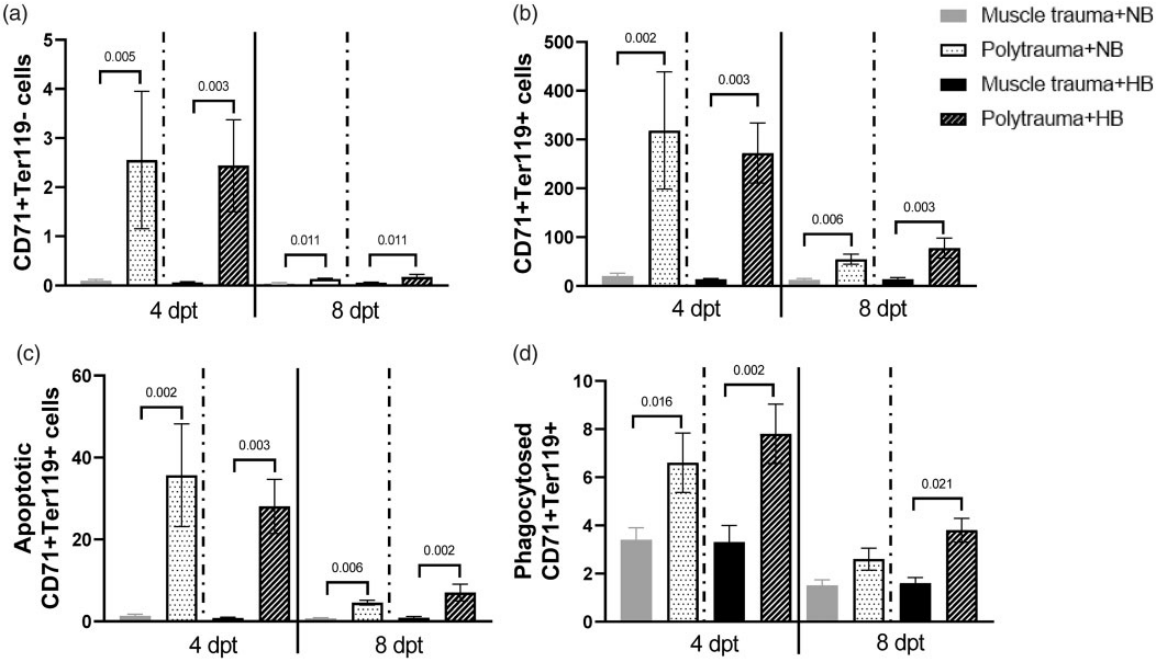

Figure 5. Number of splenic non-apoptotic and apoptotic erythroid subsets and reticulocytes phagocytosed by monocytes after polytrauma and NB or HB exposure. Compared to muscle trauma groups, $CD71^+Ter119^-$ cells (a, $n = 5–8$), $CD71^+Ter119^+$ cells (b, $n = 5–8$), and apoptotic $CD71^+Ter119^+$ cells (c, $n = 5–8$) in the polytrauma groups increase in number at 4 and 8 dpt. At 4 dpt, more $CD71^+Ter119^+$ cells are phagocytosed by monocytes in the polytrauma groups than in the muscle trauma groups, and at 8 dpt, there are more phagocytosed $CD71^+Ter119^+$ cells in the polytrauma+HB group compared to the muscle trauma+HB group (d, $n = 7–11$). Control+NB values (4 dpt) for $CD71^+Ter119^-$ cells and apoptotic $CD71^+Ter119^+$ are 0.05 ± 0.03 and 0.47 ± 0.10, respectively. Control+NB values (4 dpt) for $CD71^+Ter119^+$ cells and phagocytosed $CD71^+Ter119^+$ cells are provided in Figure 2(b) and (d), respectively. Cell number is in units of 10^5. Kruskal-Wallis CS test with Bonferroni correction for multiple tests ($\alpha = 0.025$) was used to compare polytrauma vs. muscle trauma groups.
Same bracket notation and abbreviations as in previous figures.

Effect of hypobaric hypoxia on splenic cells in the polytrauma model

Although no erythroid cell differences were detected between the polytrauma + NB and polytrauma + HB groups, we observed differences in splenic proliferating cells at 8 and 14 dpt (Figure 6(a) and (b)), total spleen cells at 14 dpt (Figure 6(c)), and neutrophils at 8 dpt (Figure 6(d)) between these two polytrauma groups. Specifically, the HB mice exhibited more neutrophils and fewer proliferating cells and total spleen cells. We further identified the phenotype of these proliferating cells with double immunostaining of Ki-67 and markers of B cells (CD19), T cells (CD3 and CD4), monocytes/neutrophils (Ly6C), or macrophages (F4/80). These proliferating cells were B and T cells (Supplemental Figure 1). Co-localization of Ly6C and Ki-67 markers or F4/80 and Ki-67 markers was not observed (data not shown).

Discussion

We hypothesized that HB exposure would influence splenic erythropoiesis and splenic inflammation after polytrauma. This hypothesis was partially supported. Although HB exposure did not affect splenic erythropoiesis, this exposure increased the splenic neutrophil number. Other significant findings are that (a) muscle trauma alone induced splenic erythropoiesis; (b) polytrauma, which consisted of muscle trauma and hemorrhagic shock, induced greater splenic erythropoiesis than muscle trauma alone, even though the initial leukocyte infiltration was similar between the two trauma groups; and (c) as the RBC count recovered after polytrauma, HB exposure had an inhibitory effect on splenic proliferating cells but an enhancing effect on neutrophils. These findings are novel in demonstrating the splenic response to muscle trauma with or without hemorrhagic shock and exposure to mild hypobaric hypoxia.

As part of this study, we report that crush muscle trauma alone led to extramedullary (spleen) erythropoiesis, evidenced by an increase in $CD71^{+}Ter119^{+}$ reticulocytes at 1.7 dpt (Figure 2(a)). This result is more likely due to the crush muscle trauma and not the femoral artery catheterization since phlebotomy does not usually produce a significant wound[36] in contrast to the crush muscle trauma. In the crush muscle trauma model, the associated inflammation appears to induce SEP production and differentiation because $CD71^{+}Ter119^{+}$ reticulocytes arise from SEPs.[7,19] However, since there was no decrease in the circulating RBC count associated with muscle trauma alone, the possibility exists that these reticulocytes were phagocytosed via neutrophils and monocytes (Figure 2(c) and (d)). Therefore, inflammation appears to be driving splenic erythropoiesis following muscle trauma as a protective mechanism for the possibility of significant blood loss. In the absence of substantial blood loss, muscle inflammation-induced splenic

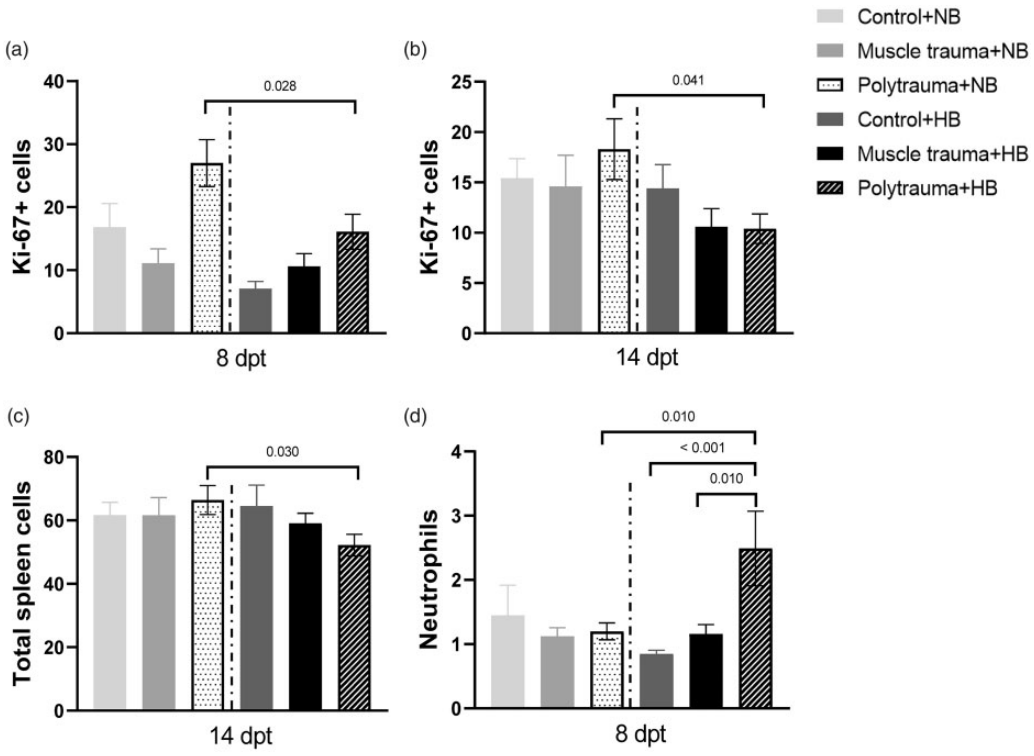

Figure 6. Number of splenic proliferating cells, total spleen cells, and splenic neutrophils after muscle trauma or polytrauma and NB or HB exposure. Splenic Ki-67 $^{+}$ cells decreased at 8 dpt (a, $n = 4$–8) and 14 dpt (b, $n = 6$–10) in the polytrauma + HB group compared to the polytrauma + NB group. The number of total spleen cells is reduced in the polytrauma + HB group compared to the polytrauma + NB group (c, $n = 14$–16) at 14 dpt. Splenic neutrophils increase in the polytrauma + HB group compared to the polytrauma + NB group (d, $n = 9$–10) at 8 dpt. Kruskal-Wallis CS test with Bonferroni correction for multiple tests ($\alpha = 0.025$) was used to compare polytrauma vs. control groups and polytrauma vs. muscle trauma groups. Kruskal-Wallis CS test was used to compare the polytrauma + NB vs. polytrauma + HB groups.
Same bracket notation and abbreviations as in previous figures.

erythropoiesis may be curtailed through multiple mechanisms, including phagocytosis, as demonstrated in this study, and increased apoptosis via FAS-L.[18]

Another possible explanation is that splenic erythropoiesis may accompany muscle trauma to ensure oxygen availability for mitochondria. With muscle trauma, the sarcolemma can be destroyed, leading to the loss of myoglobin from muscle cells. Decreased myoglobin will limit the amount of oxygen available for mitochondrial processes. Splenic erythropoiesis may generate more immature reticulocytes (identified as $CD71^{+}Ter119^{+}$ as in this study) and/or more mature reticulocytes (identified as $CD71^{-}Ter119^{+}$ or EryC cells), and these cells will contain hemoglobin.[23] This hemoglobin may serve as an alternative oxygen source for mitochondrial processes. Additional research is needed to investigate whether immature and/or mature reticulocytes positive for hemoglobin increase in the spleen or peripheral blood after muscle trauma and/or in the injured muscle itself.

We found that greater splenic erythropoiesis (except for serum EPO levels) occurred in our polytrauma model, which consisted of both muscle trauma and hemorrhagic shock, than muscle trauma alone at 4 dpt (Figure 5(a) and (b)), even though the initial leukocyte infiltration was similar between the muscle trauma and polytrauma groups at the same time point. Hemorrhagic shock induces tissue hypoxia, which stimulates the kidneys to produce EPO.[36,37] We detected higher serum EPO levels in the polytrauma groups compared to the muscle trauma groups under both NB and HB conditions, indicating that hemorrhagic shock—and not polytrauma consisting of muscle trauma and hemorrhagic shock—induced high serum EPO levels. In addition, we found an increased number of $CD71^{+}Ter119^{-}$ and $CD71^{+}Ter119^{+}$ cells in polytrauma groups compared to the muscle trauma groups (Figure 5 (a) and (b)). Collectively, these data suggest that hemorrhagic shock in a polytrauma model may increase EPO. This increased EPO is associated with splenic erythropoiesis, which prompts the restoration of the RBC count. It should also be mentioned that additional studies, such as functional data related to erythroid colony growth in the spleen, reticulocyte count in the peripheral blood, and comparison of erythropoiesis in the bone marrow and the spleen, will be helpful to better understand the relationship between polytrauma and splenic erythropoiesis.

In contrast, hemorrhagic shock did not induce splenic erythropoiesis in a rat polytrauma model. Alamo *et al.*[29] reported similar splenic weight, splenic erythroid colony growth, splenic hematopoietic progenitor cell number, and *BMP4* and *EPOr* expression between uninjured rats and rats that underwent lung contusion and hemorrhagic shock. One possible reason for the difference between our findings and those of Alamo *et al.*[29] relates to resuscitation. Alamo *et al.*[29] resuscitated rats with shed RBCs after hemorrhagic shock, which may have limited the degree of stress erythropoiesis and anemia because of the immediate restoration of circulating RBCs. In contrast, we resuscitated mice with a plasma expander, which resulted in a severe anemic condition post-shock. Another explanation for the discrepancy may be species related. In mice, stress erythropoiesis mainly occurs in the liver (neonates) and spleen,[38] whereas bone marrow is a major site for stress erythropoiesis in rats.[39]

Regarding the muscle trauma groups, we observed an increased neutrophil number and splenomegaly at 4 dpt. In another model of inflammation-induced erythropoiesis, there was a positive correlation between spleen mass and neutrophil accumulation.[40] Therefore, the splenomegaly in muscle trauma groups may be related to the increased number of neutrophils, which phagocytose reticulocytes.

Another consideration regarding the difference in splenic erythropoiesis between muscle trauma and polytrauma involves blood lactate. Soon after hemorrhagic shock, blood lactate levels temporarily increase.[41] In addition, Luo *et al.*[42] found that an *in vivo* treatment of lactic acid stimulated erythropoiesis in the bone marrow of mice. These findings suggest that greater splenic erythropoiesis may occur in polytrauma groups than muscle trauma groups because polytrauma may yield greater blood lactate levels than muscle trauma alone. Further research examining factors associated with the stimulation of splenic erythropoiesis could compare blood lactate levels between muscle trauma and polytrauma groups during and after several hours of hemorrhagic shock.

In the spleen, we also found reduced cellular proliferation at 8 and 14 dpt, decreased total cell number at 14 dpt, and an increased neutrophil number at 8 dpt in the polytrauma + HB group than the polytrauma + NB group. An increased neutrophil number may have secondary effects on lymphocytes as well. Neutrophils can downregulate B and T cells under physiological and pathological conditions,[43–46] including suppressing proliferation after interferon-γ stimulation.[47] This neutrophil inhibitory effect may explain the reduced total cell count and cellular proliferation since the proliferating cells appeared to be B and T cells. In addition, hypoxia may account for the decreased cellular proliferation as this environmental stressor reduces T-cell proliferation, survival, and cytokine production.[48–50] Follow-up flow cytometry studies are needed to determine whether fewer splenic B and T cells are present in the polytrauma + HB mice compared to polytrauma + NB mice at 14 dpt and whether mild hypobaric hypoxia in the polytrauma model affects $CD8^{+}$ T cell activation and cytokine production.[49]

This study had limitations. One limitation is that the experiments conducted on the control groups were performed at an altitude of 610 m rather than at sea level. While there are no data to support meaningful differences in the splenic erythropoiesis response between sea level and an altitude of 610 m, we cannot be certain that the altitude at which the current study was performed yielded sea-level results. The lack of supplemental oxygen is another limitation. We acknowledge that wounded soldiers are likely to receive supplemental oxygen during en route care, and supplemental oxygen administered to polytrauma + NB and polytrauma + HB groups would have served as more rigorous control groups. However, since polytrauma + HB models similar to ours had not been tested previously, we believed the first step was to determine whether mild hypobaric hypoxia without

supplemental oxygen would have effects. A third limitation is we used hetastarch as the resuscitative fluid instead of RBCs. An RBC transfusion immediately after hemorrhage may minimize the overall splenic erythropoiesis response or hypobaric hypoxia's effects on the splenic erythropoiesis response. The fourth limitation is we used only one approach for hypothesis testing. In alignment with the American Statistical Association, we note that the *P* values reported in this study are contingent on the stated hypotheses, and *P* values less than 5% indicate that the data gathered in this animal study are incompatible with the specified null hypotheses (of no effect).[51]

Unlike most military ground-based medical care situations, en route care must account for hypobaria, which may affect the successful management of acute anemia during the air transport of wounded soldiers. While our finding is that an ultra-long-haul flight does not impair stress erythropoiesis, such flight conditions may contribute to the risk of infectious complications post-trauma. Further, we found that splenic immune cell populations, which are essential for mounting a host defense, may change under different environmental conditions. Therefore, greater attention to host defense may be necessary during an ultra-long-haul flight, and patients at risk for infectious complications may warrant prolonged field care rather than en route care within 24 h after trauma.[52]

AUTHORS' CONTRIBUTIONS

LZ, JNS, CCC, and BSS participated in the design, data analysis and interpretation, and article drafting; LZ and SP participated in data collection; SP provided additional critical revision.

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SUPPLEMENTAL MATERIAL

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