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A Detailed Characterization of the Dysfunctional Immunity and Abnormal Myelopoiesis Induced by Severe Shock and Trauma in the Aged

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A Detailed Characterization of the Dysfunctional Immunity and Abnormal Myelopoiesis Induced by Severe Shock and Trauma in the Aged

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The elderly are particularly susceptible to trauma, and their outcomes are frequently dismal. Such patients often have complicated clinical courses and ultimately die of infection and sepsis. Recent research has revealed that although elderly subjects have increased baseline inflammation as compared with their younger counterparts, the elderly do not respond to severe infection or injury with an exaggerated inflammatory response. Initial retrospective analysis of clinical data from the Glue Grant trauma database demonstrated that despite a similar frequency, elderly trauma patients have worse outcomes to pneumonia than younger subjects do. Subsequent analysis with a murine trauma model also demonstrated that elderly mice had increased mortality after posttrauma *Pseudomonas* pneumonia. Blood, bone marrow, and bronchoalveolar lavage sample analyses from juvenile and 20–24-mo-old mice showed that increased mortality to trauma combined with secondary infection in the aged are not due to an exaggerated inflammatory response. Rather, they are due to a failure of bone marrow progenitors, blood neutrophils, and bronchoalveolar lavage cells to initiate and complete an emergency myelopoietic response, engendering myeloid cells that fail to clear secondary infection. In addition, elderly people appeared unable to resolve their inflammatory response to severe injury effectively. *The Journal of Immunology*, 2015, 195: 2396–2407.

People of advanced age (>55 y old) have significantly increased morbidity and mortality after trauma (1–3). Because the elderly population is growing, research into this phenomenon of worsened outcome in the elderly is increasingly relevant, especially with the escalating economic and health care burdens on society (3). Despite decades of promising preclinical and clinical investigations in trauma, our understanding of this entity and why its effects are exacerbated in the elderly remains incomplete, with few therapies demonstrating success in any patient population. Authors have previously argued that age-related immune dysfunction is due to an acute exacerbated response to both infectious and noninfectious inflammation (4–6); however, recent analysis appears to refute these claims (2, 7–9).

Recently, several aspects of innate immunity have been determined to be of vital importance to survival from trauma, and this response may be suboptimal in the aged. Specifically, polymorphonuclear leukocytes (PMNs) are replaced after inflammation through a process known as emergency myelopoietic. This occurs after severe injury when bone marrow (BM) granulocyte stores are rapidly released, and increased stem cell proliferation and differentiation along myeloid pathways results (10, 11). However, our understanding of these responses in the elderly population is still limited, especially in animal models that accurately recapitulate the human condition (12–14). Elderly mice have been shown to have increased mortality to polymicrobial sepsis and to have functional deficits in specific leukocytes (7, 15–19). Early data in

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The datasets reported in the article have been submitted to the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>) under accession number GSE70418.

The work represents a secondary use of the Glue Grant database, which is a public database, and the conclusions and discussion are the authors and do not necessarily represent the views of either Massachusetts General Hospital or the National Institute of General Medical Sciences.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BAL, bronchoalveolar lavage; BM, bone marrow; DFR, distance from reference; GG, Glue Grant; HSC, hematopoietic stem cell; IPA, Ingenuity Pathway Analysis; LSK, lineage⁻ sca-1⁺ c-kit⁺ cell; LT-HSC, long-term hematopoietic stem cell; PMN, polymorphonuclear leukocyte; ST-HSC, short-term HSC; TRDB, GG Trauma Related Database; VAP, ventilator-associated pneumonia.

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less severe trauma-hemorrhage rodent models would suggest that there is indeed some defect in emergency myelopoiesis in elderly mice, and with hematopoiesis in general (15, 19). Two key cell types involved in the earliest phases of myelopoiesis are long-term hematopoietic stem cells (LT-HSCs) and short term (ST)-HSCs. ST-HSCs have a much more limited capacity for self-renewal than LT-HSCs do, but they appear to be more vital for rapid myelopoiesis after loss of BM cells during times of inflammation (20–22). Specific analysis of these cell types after trauma in the elderly is also lacking.

Relatively recently, the Inflammation and the Host Response to Injury Collaborative Research Program, also known as the Trauma Glue Grant (GG), a prospective, multi-institutional observational study with the primary aims of describing the epidemiology, proteomic and leukocyte genomic response in severely injured burn and trauma patients, was completed (23–25). The latter 5 years of the program included patients over the age of 55 y, allowing detailed evaluations of the characteristics and outcomes of the elderly after severe trauma (2, 26). In addition, our laboratory has described a novel murine model of hemorrhage and severe trauma (12), which has allowed us to understand the condition better in humans (13).

Our overarching goal was to identify the specific defects in innate immunity and inflammation in elderly patients after severe trauma that leads to their worsened outcomes to secondary infection. We hypothesized that myeloid dysfunction contributed to increased morbidity and mortality after severe injury with hemorrhagic shock and subsequent pneumonia. After examining outcomes in elderly trauma patients to ventilator-associated pneumonia from the GG study, we then analyzed myeloid cell function in young and aged mice following polytrauma and a clinically relevant infection (*Pseudomonas* pneumonia). We can conclude that although inflammaging (defined as an age-related increase in systemic chronic inflammation) promotes many disease processes prevalent in the elderly, including cardiovascular disease, chronic obstructive pulmonary disease, and cancer (27–30), and contributes to poor outcomes to injury and infection, it does not translate specifically to an increased inflammatory response subsequent to trauma, secondary *Pseudomonas* pneumonia, or other clinically relevant insults (7, 31). Rather, the overwhelming data suggest that a failure to initiate an early innate immune response, as well as a subsequent inability to resolve the inflammatory response, leaves the elderly at risk for subsequent infection and mortality. This failure is imprinted into the transcriptome of HSCs, circulating blood and extravasating bronchoalveolar leukocytes.

Materials and Methods

Approval was obtained from the University of Florida Institutional Review Board to analyze de-identified human data obtained from the GG Trauma Related Database (TRDB) prior to initiation of this study (23).

Human data source and cohort selection

The TRDB contains audited and de-identified data obtained from severely injured adults with blunt trauma and in hemorrhagic shock enrolled from seven level 1 trauma centers between 2001 and 2011 (32). Inclusion criteria required a blunt traumatic mechanism with an Abbreviated Injury Scale score of 2 or greater outside the head region, base deficit of 6 mmol/l or greater, systolic blood pressure of less than 90 mm Hg prehospital or within 60 min of emergency department arrival, and blood product transfusion within 12 h of injury. Exclusion criteria consisted of those with significant mortality risk from severe head injury (Abbreviated Injury Scale score, head > 4), those evaluated at the trauma center more than 6 h from the time of injury, cervical spinal cord injury, and thermal burns of greater than 20% total body surface area. Consistency of patient care between centers was optimized with the development and implementation of standard operating procedures for initial resuscitation and supportive care

(23, 33). Over the study period, there was an overall standard operating procedures compliance rate of greater than 69%.

As of October 2013, the TRDB contained prospectively collected demographic, clinical, and outcome data for 1928 patients with blunt trauma meeting the criteria for this analysis. These patients were separated into two cohorts: either advanced age (≥ 55 y old) or young (< 55 y old) for epidemiologic analysis. This cutoff was used based on previous literature showing that an age of 55 y or older is associated with worse outcomes than predicted, even after controlling for other injury factors (34, 35). Using these definitions, there were 1395 and 533 patients in the young and advanced age cohorts, respectively.

Clinical demographics and outcomes analysis

Data regarding baseline patient demographics, injury severity, fluid and blood product resuscitation parameters, serial laboratory values and multiple clinical outcomes, and ventilator-associated pneumonia (VAP) were obtained from the TRDB. VAP (Supplemental Table I) data were used rather than ventilator-associated events, because ventilator-associated events had not been defined by the Centers for Disease Control and Prevention at the time of study initiation, and were therefore not tracked in the database. Univariate analyses were performed between young and aged cohorts with VAP using Fisher exact test and Wilcoxon two-sample test as appropriate. To determine the role of age as an independent predictor of mortality in patients with VAP, multivariate stepwise logistic regression models were created using prior known and suspected confounding risk factors, and any significant predictive factors identified by univariate analysis. All patients were included for 28-d mortality modeling. All significance tests were two-sided, with a 0.05 α level. Statistical analyses were performed with SAS (version 9.3; Cary, NC).

Mice

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Florida. Male C57BL/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 6–7 wk or the National Institute of Aging at 20–24 mo of age, and allowed to acclimatize for 1 wk before being used for experimental procedures. Mice were maintained on standard rodent food and water ad libitum.

Murine polytrauma model

Mice underwent 90 min of hemorrhagic shock and resuscitation followed by long bone fracture and cecectomy, as described previously (12). Mice were euthanized 2 h, 1 d, and 3 d later. Blood, BM, spleen, and bronchoalveolar lavage cells were collected for phenotypic and functional analyses. Genome-wide expression was also performed. Intranasal *Pseudomonas* was instilled to induce pneumonia 1 d after trauma. For survival studies, mice were observed up to 7 d.

Pneumonia induction

Pneumonia was induced using *Pseudomonas aeruginosa* as described previously (PAK strain) (36). Briefly, PAK was grown overnight, transferred to fresh medium, and grown to mid log phase. The bacterial density was measured at OD 600 λ (DU 640 Spectrophotometer; Beckman Coulter, CA) and washed with PBS. The mice were given 1×10^7 bacteria per 50 μ l intranasally.

Bronchoalveolar lavage

The trachea was cannulated and lavaged four times with 800 μ l cold PBS containing 2 mM EDTA. Bacterial load was determined by culturing 100 μ l of bronchoalveolar lavage (BAL) fluid on sheep's blood agar plates (Thermo Fisher Scientific) at 37°C in 5% CO₂. Plates were counted after overnight culture. The rest of the BAL fluid was centrifuged, and the supernatant stored at -80°C until analysis. The BAL cells were counted using a hemacytometer (Hausser Scientific, Horsham, PA).

Histology

Lungs were inflated with 10% paraformaldehyde, harvested, and processed for H&E staining (University of Florida Molecular Pathology Core). Histologic evaluation was completed on the stained sections to assess the degree of acute lung injury. The degree of inflammation was quantified by an independent pathologist who was blinded to the group assignments. Each sample was given a histologic score for acute lung injury ranging from 0 to 4: 0 = no inflammation, 1 = mild, 2 = moderate, and 3 = severe inflammation based on the degree of perivascular or peribronchial neutrophilic infiltrate, consolidation, necrosis, and fibrin deposition.

Flow cytometry

Spleen, blood, BM, and BAL cells were harvested, and single-cell suspensions were created by passing the cells through 70- μ m pore-sized cell strainers (BD Falcon, Durham, NC). Erythrocytes were then lysed using ammonium chloride lysis buffer and washed two times using PBS without calcium, phenol red, or magnesium. Cells were stained with the following Abs for flow cytometric studies: PE Cy7 anti-CD11b, APC anti-Gr-1, and Pacific Blue anti-Ly6G (BD Pharmingen, Billerica, MA). Additional Abs used were anti-lineage mixture (Lin; BD Biosciences, San Jose, CA), anti-c-kit, anti-Sca-1, anti-CD135, and anti-CD150 (eBioscience, San Diego, CA). Sytox Blue (Invitrogen, Carlsbad, CA) was used for cell viability analysis. Samples were acquired and analyzed with an LSRII flow cytometer (BD Biosciences) and FACSDiva (BD Biosciences) (37, 38).

Phagocytosis assay

Cells (2×10^5) were resuspended in 200 μ l PBS incubated with 20 μ l fluorospheres in a 37°C water bath for 10 min, washed with PBS containing 0.1% BSA, stained with anti-Ly6G and anti-CD11b, and analyzed with flow cytometry (7).

Cytokine production

Plasma and BAL supernatant were collected and stored at -80°C until the time of analysis. Cytokine concentrations were determined using a commercially available multiplexed Luminex kit (MILLIPLEX MAP, Mouse Cytokine/Chemokine Panel; Millipore, Billerica, MA). All assays were performed according to the manufacturer's protocols. Cytokine concentrations were determined using BeadView software (Millipore) (7).

Hematopoietic stem and progenitor cell culture

Bone marrow cells from young and aged mice were aseptically collected 1 d after trauma. Single-cell suspensions were created by passing the cells through 70- μ m pore-sized cell strainers (BD Falcon). Erythrocytes were lysed using ammonium chloride lysis buffer and washed with PBS. Cells were stained with anti-biotin Lineage mixture (BD Biosciences), anti-c-kit and anti-Sca-1 (eBioscience, San Diego, CA). Lineage^{neg} Sca-1⁺ckit⁺ cells (LSKs) were sorted using FACSAria (BD Biosciences). Five hundred LSKs were cultured in methylcellulose media (R&D Systems, Minneapolis, MN) supplemented with GM-CSF, G-CSF, M-CSF, or IL-7 (R&D Systems, Minneapolis, MN). Colonies were counted after 10–14 d incubation at 37°C (11).

Genome-wide expression analysis

Blood was collected by intracardiac puncture at 2 h, 1 or 3 d after trauma and from naive mice using 1-ml syringes containing 100 μ l of 169 mM EDTA. RBCs were lysed using Buffer EL (Qiagen, Valencia, CA). Bronchoalveolar lavage cells were collected 1 d after trauma and from naive mice. Sorted BM LSK cells from young and aged mice were collected 1 d after trauma. The cell pellet was homogenized in RLT buffer (Qiagen, Valencia, CA) supplemented with 2-ME and passed through Qias shredder (Qiagen, Valencia, CA). Total RNA was isolated using RNeasy kit (Qiagen) and the quality and quantity was assessed using Agilent Bioanalyzer 2000. Nucleic acids were labeled using the 3' IVT Express Kit, and 15 μ g labeled cRNA was hybridized to Mouse Genome 430 2.0 Arrays (Affymetrix, Santa Clara, CA) (7). For BM LSKs, 15 ng total RNA was labeled and amplified using NugenOvation PICO and Encore kits (NuGEN, San Carlos, CA) and 7.5 μ g cDNA was hybridized onto MOE 430 2.0 Arrays (Affymetrix). Arrays were hybridized for 16 h at 45°C. After hybridization,

Table I. Univariate and multivariate analysis of young and aged, severely injured, blunt trauma patients with hemorrhagic shock who developed ventilator-associated pneumonia

Demographics	Patient Demographics and Outcomes		p Value
	Young (Age < 55 y; n = 345)	Aged (Age \geq 55 y; n = 159)	
	N (%)	N (%)	
Sex, male	264 (76.5%)	118 (74.2)	0.88
\geq 1 major medical comorbidity	233 (67.5)	140 (88.1)	<0.001
Major surgical procedures	328 (95.1)	145 (91.2)	0.78
	Mean (95% CLM)	Mean (95% CLM)	
NISS	37 (35.6–38.4)	34.4 (32.4–36.4)	0.035
BMI (kg/m ²)	29.4 (27.7–29.1)	29.5 (28.4–30.7)	0.075
Max. APACHE II score (0–24 h)	30.0 (29.4–30.6)	33.1 (32.1–34.0)	<0.001
Lowest prehospital SBP	89.9 (86.3–93.5)	88.2 (82.7–93.7)	0.61
Lowest ED SBP (mm Hg)	84.3 (81.8–86.8)	76.0 (72.3–79.7)	<0.001
Max lactate, 0–6 h (mmol/L)	5.88 (5.5–6.3)	5.16 (4.7–5.6)	0.16
Max lactate, 12–24 h (mmol/L)	3.8 (3.4–4.1)	3.8 (3.4–4.2)	0.22
Total blood, 0–12 h (ml)	2870 (2550–3190)	2530 (2121–2939)	0.83
Total crystalloid, 0–12 h (ml)	11,877 (11,167–12,586)	11,185 (10,335–12,034)	0.25
	N (%)	N (%)	
Outcomes			
ICU tracheostomy	165 (47.8)	89 (56)	0.10
ICU readmission	45 (13)	14 (8.9)	0.18
Complicated recovery	225 (65.2)	120 (75.5)	0.02
Discharge: home or rehabilitation	205 (59.4)	49 (30.1)	<0.001
Discharge: long-term care facility	96 (27.8)	75 (47.1)	0.003
28-d mortality	30 (8.7)	17 (10.6)	0.006
	Mean (95% CLM)	Mean (95% CLM)	
Maximum Marshall MOF score	6.9 (6.6–7.1)	6.8 (6.4–7.1)	0.73
Maximum Denver 2 MOF score	3.3 (3.1–3.5)	3.5 (3.2–3.8)	0.18
Ventilator days	19.1 (17.8–20.3)	18.9 (17.2–20.6)	0.87
ICU LOS (d)	23.7 (22.1–25.3)	22.3 (20.5–24.1)	0.29
	Multivariate Analysis		
Risk Factor	Odds Ratio (95% Confidence Interval)		p Value
	28-d Mortality ^a		
Age \geq 55 y old	2.41 (1.36–4.28)		<0.004
Total blood > 9.5 (U), 0–12 h	2.16 (1.18–3.97)		<0.011

The p values considered significant at <0.05 are designated in boldface.

^aModel fit statistics: area under the curve, $c = 0.775$; Akaike information criterion = 1433; likelihood ratio test, $p < 0.0001$.

AMA, against medical advice; APACHE, Acute Physiology and Chronic Health Evaluation; BMI, body mass index; COPD, chronic obstructive pulmonary disease; ED, emergency department; ICU, intensive care unit; ISS, injury severity scale; LOS, length of stay; MOF, multiple-organ failure; NISS, new injury severity scale; SBP, systolic blood pressure.

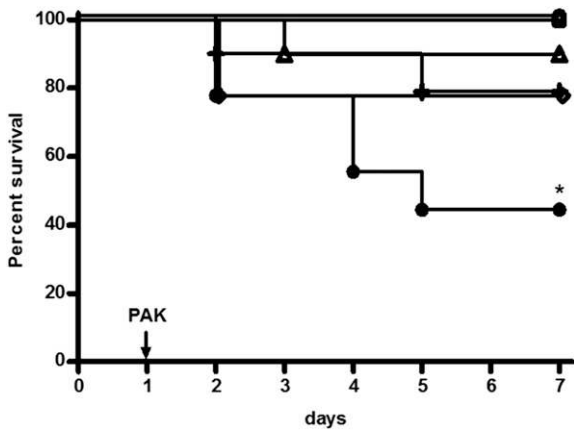


FIGURE 1. Murine survival rates after trauma or trauma and *Pseudomonas pneumonia*. Aged mice have a significantly lower survival rate when exposed to *Pseudomonas pneumonia* (Pp) 1 d after polytrauma (PT). Young (6–10 wk old) and aged (20–24 mo old) C57BL/6 mice underwent trauma or were exposed to *P. aeruginosa* (PAK, 10^7 CFU), or both, and survival was monitored. This figure is the combination of five separate experiments ($n = 9–10$). * $p < 0.05$, log-rank (Mantel–Cox) test. □, polytrauma (PT) young; ○, PT aged; △, Pp young; +, Pp aged; ◇, PT+Pp young; ●, PT+Pp aged.

arrays were stained and washed using an FS450 Affymetrix fluidics station and Affymetrix FlexFS 450-0004 protocol. Arrays were then scanned in an Affymetrix GeneChip scanner 7G Plus. Genome-wide expression was performed on total blood leukocytes. Expression patterns were compared between healthy and young or aged trauma mice at $p < 0.001$ (F test).

Statistics

Differences among groups in flow cytometric analyses were evaluated using Student *t* test. Additional statistics were performed using one-way ANOVA and two-way ANOVA. Post hoc comparisons were performed using Student Neuman-Keuls test. Significance was determined at the 95% confidence interval using a two-sided test. Blood leukocyte genome-widen expression patterns were compared between healthy and young or aged trauma mice using a false discovery adjusted F test ($p < 0.001$) with BRB

Tools. We also calculated the distance from reference (DFR) based on the studies of Warren et al. (25). The DFR calculation derives a single metric for the overall differences in gene expression calculated as the natural log of the sum of the differences in gene expression for each probe set divided by the pooled variance for that individual probe set.

Results

After severe trauma, pneumonia is associated with worse outcomes in elderly humans as compared with the young

The overall GG trauma cohort consisted of 1928 severely injured patients in hemorrhagic shock. We determined how many of these patients, both young (age < 55 y) and aged (age ≥ 55 y) had a diagnosis of VAP (Table I). Twenty-nine percent (159 of 533) of aged patients were diagnosed with VAP, as compared with 24% of young patients (345 of 1395). The incidence of VAP was not significantly different between the young and the aged. However, there were differences in the aged and young cohorts who had trauma and developed VAP. As expected, elderly patients had more comorbid conditions at admission (Table I), whereas young patients were slightly more severely injured. Shock severity, as measured by initial serum lactate 0–6 h and 12–24 h after injury, was similar between the two groups. However, elderly patients demonstrated significantly greater evidence of subsequent overall physiologic derangement, as measured with the Acute Physiology and Chronic Health Evaluation II at 24 h after injury (Table I). In addition, although slightly less severely injured, older patients with VAP had a significantly higher incidence of a complicated clinical trajectory (defined as either an ICU hospitalization > 14 d with evidence of ongoing organ dysfunction, or death after the first 48 h) (23, 24). Elderly patients with VAP were also more likely to be discharged to skilled facilities rather than home, and they had double the 28-d mortality ($p < 0.01$). Multivariate logistic regression analysis revealed that age ≥ 55 y old was an independent predictor of mortality in severely injured patients with blunt trauma and VAP, after controlling for injury severity, transfusion requirements, shock severity and physiologic derangement, and comorbidities (Table I).

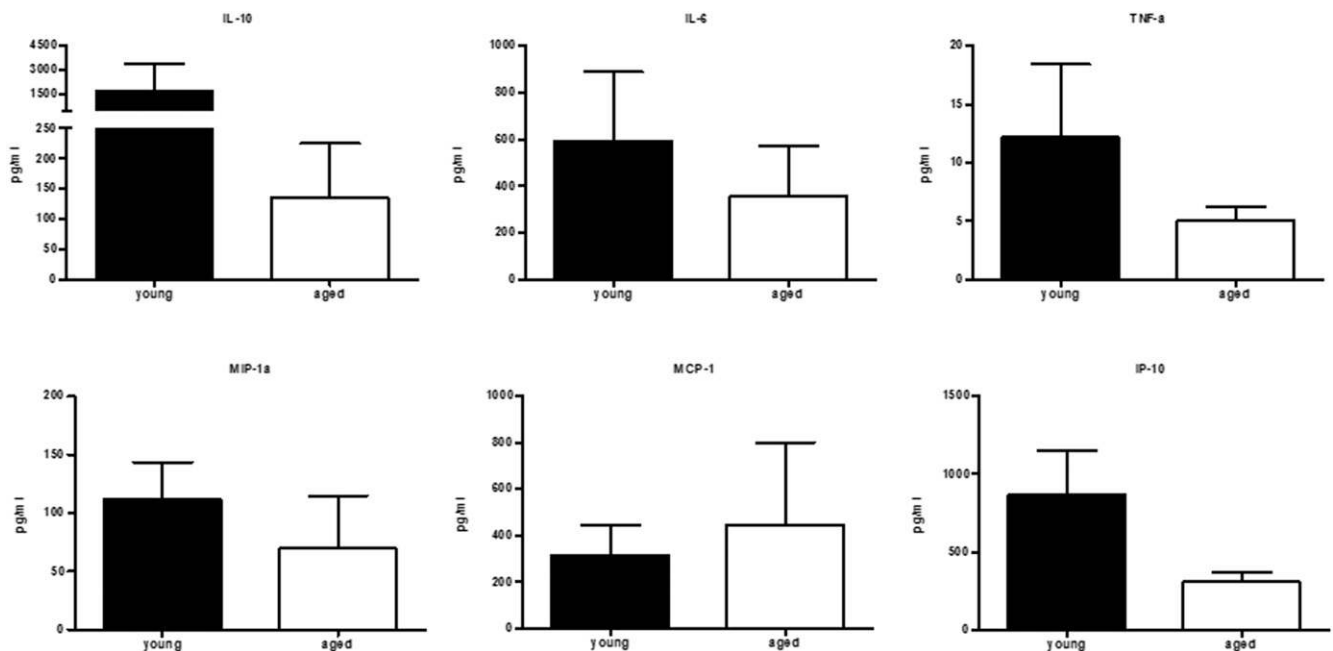


FIGURE 2. Plasma cytokine concentrations after polytrauma (PT). There was no significant statistical difference in the plasma cytokine concentrations between young and aged mice 1 d after PT. Plasma from young and aged mice were collected 1 d after PT, and cytokine–chemokine production was evaluated by Luminex ($n = 3$).

Aging is associated with increased mortality in mice after polytrauma and subsequent Pseudomonas pneumonia

We tested for a similar response among young and aged mice in a more severe model of trauma and hemorrhagic shock (12), and demonstrated that trauma was nonlethal in both age groups. There was no significant difference in mortality in young and elderly mice who received *Pseudomonas pneumonia* alone. However, when elderly mice were exposed to *Pseudomonas pneumonia* 1 d after trauma, there was a significant increase in their mortality compared with young mice (Fig. 1). In this manner, the response by elderly mice recapitulates the GG findings determined in elderly human severe trauma patients who develop VAP. We next examined the young and aged mice 1 d after trauma to identify the potential mechanisms that could explain the increase in susceptibility of aged mice exposed to *Pseudomonas pneumonia* after trauma.

Aged mice do not manifest an exaggerated inflammatory response after trauma

We looked for evidence of an exaggerated local or systemic inflammatory response after trauma, but observed no significant evidence of either. The difference in the concentration of plasma cytokines was not different in aged as compared with young mice (Fig. 2). In fact, after trauma, elderly mice mostly trended toward lower concentrations than young mice did, albeit not significantly. This was also true for BAL cytokine concentrations obtained 1 d after trauma or 1 d after trauma and *Pseudomonas pneumonia* in young and aged mice (data not shown).

Aged mice do not have increased lung injury but fail to clear bacteria after trauma or trauma followed by Pseudomonas pneumonia

Lung tissue was isolated and fixed 1 d after trauma or trauma and pneumonia from both young and aged mice. These samples were evaluated in a blinded fashion for lung injury by an independent pathologist. We found no differences in the level of lung injury (Fig. 3A, 3B). Next, BAL was performed and bacterial CFUs were determined. Surprisingly, aged mice had more bacterial CFUs compared with young mice after polytrauma (Fig. 3C, 3D). This result suggested that even after trauma alone, normal pulmonary protective immunity in aged mice was less effective. Few CFUs, if any, were found in BAL samples from naive young and aged mice (data not shown).

PMNs from aged mice have impaired acute phagocytic and chemotaxis ability

In an effort to explain the inability to kill bacteria in the lungs of elderly mice, BAL fluid was harvested from aged and young mice 1 d after trauma or trauma and *Pseudomonas pneumonia*. Significantly fewer cells could be recovered from the lavage fluid of elderly mice after trauma or trauma and *Pseudomonas pneumonia* (Fig. 4A). There were significantly fewer ($p < 0.05$) phagocytic PMNs present in the lavage fluid of aged mice compared with young mice 1 d after trauma and trauma plus *Pseudomonas pneumonia* (Fig. 4B). This reduction in lung PMN recruitment occurred despite no differences in blood, spleen, or BM myeloid cell populations. In fact, there were more splenic PMNs in aged mice as compared with young mice (data not shown).

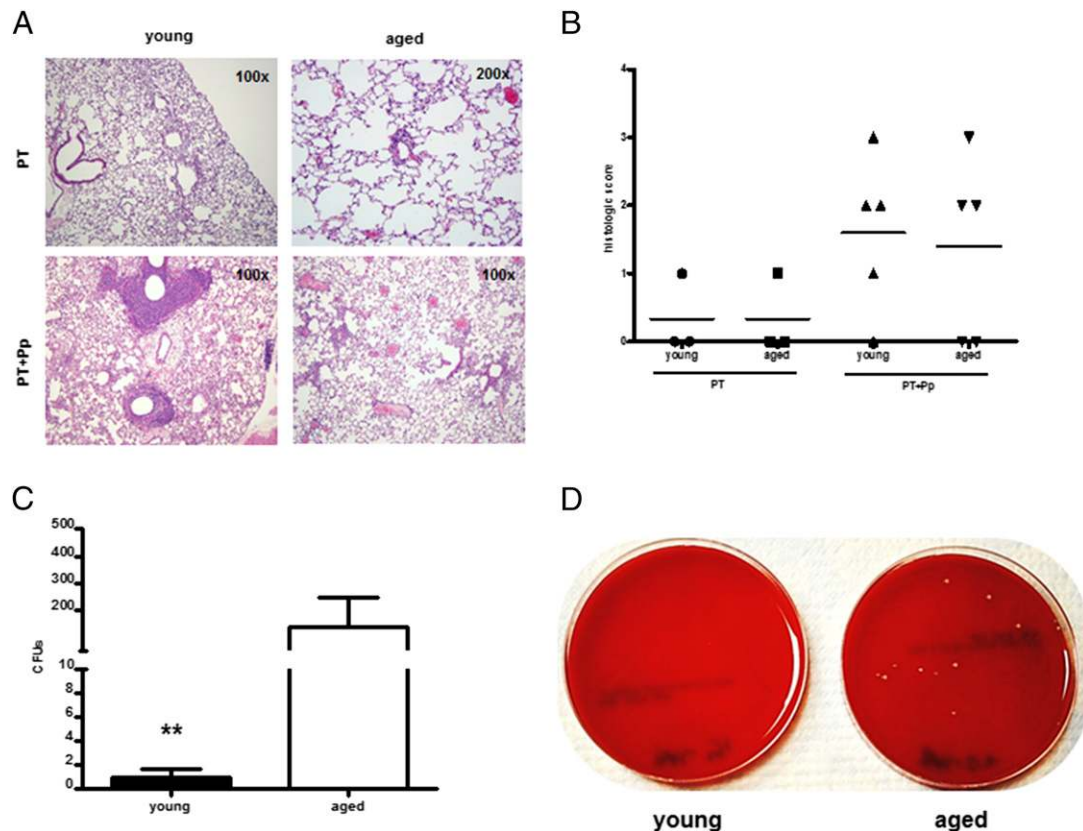


FIGURE 3. Lung histology and bacterial clearance in young and aged mice after trauma. **(A)** Histologic evaluation was performed on H&E sections from lung tissue to assess the degree of acute lung injury. **(B)** Histologic score ranged from 0 to 3; 0 = no inflammation, 1 = mild, 2 = moderate, and 3 = severe. Representative sections are shown ($n = 3-5$ per group). **(C)** Bronchoalveolar lavage (BAL) fluid was collected, and bacterial CFUs were determined by plating on sheep blood agar. The experiment was performed at least twice ($n = 6$). **(D)** Examples of bacterial CFUs on sheep blood agar plates from BAL samples 1 d after trauma. $**p < 0.01$, Mann-Whitney t test.

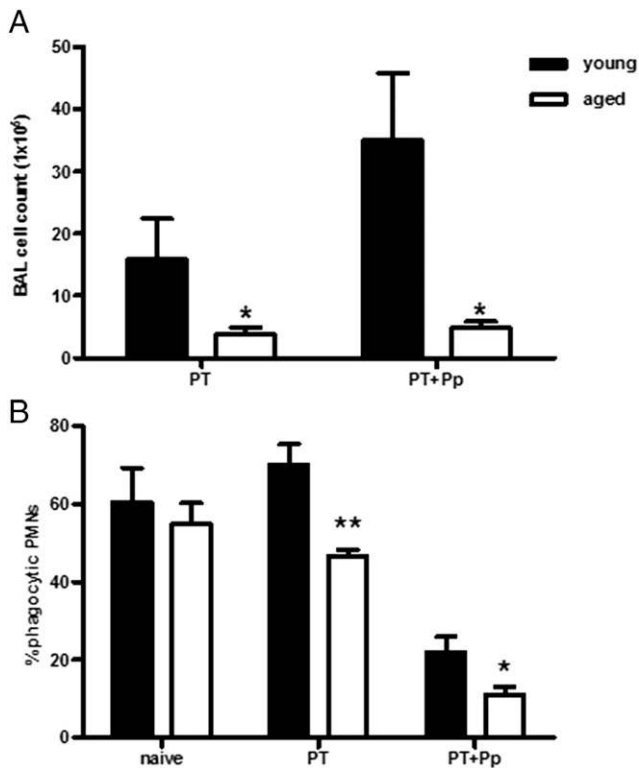


FIGURE 4. Total BAL leukocytes and functional capacity in young and aged mice after trauma. **(A)** Young and aged mice underwent poly-trauma (PT) or trauma and *Pseudomonas pneumonia* (PT+Pp) and sacrificed 1 d later. BAL fluid was collected and cells were counted using a hemacytometer. An average of two experiments is shown ($n = 6$). * $p < 0.05$, Mann-Whitney t test. **(B)** BAL cells from young (solid bars) and aged (empty bars) mice were incubated with FITC latex beads and stained for PMNs (Ly6G⁺CD11b⁺). FITC⁺ cells were considered phagocytic. This figure contains at least three separate experiments ($n = 6$ –10 per group). * $p < 0.05$, ** $p < 0.01$, unpaired t test.

BAL gene expression data reveals age-associated genomic differences

In an effort to explain why BAL leukocytes had reduced phagocytosis, genome-wide expression analysis was performed on leukocytes obtained from lavage fluid from elderly and young healthy mice, and those subjected to trauma. The mRNA abundance of 2097 probe sets representing 1649 genes differentiated young and aged trauma and naive mice at a false-discovery rate adjusted $p < 0.001$. Surprisingly, the major node of separation in genome-wide expression from BAL leukocytes was not the presence or absence of trauma, but rather, the age of the mice (Fig. 5A, 5B). This was unexpected because earlier studies in both humans and mice have demonstrated a genomic storm in the blood leukocyte transcriptome, with the expression of >70% of the genome changing in response to trauma (13, 24). It is possible that our inability to re-create an equivalent injury in the mice might have some role in this genomic response, as some of the human trauma patients had much greater injury severity scores (12, 26). Regardless, the changes seen here were dramatically less in BAL leukocytes and were overshadowed by the baseline differences in gene expression between leukocytes from elderly and young animals. For example, direct comparison of leukocytes obtained from lavage of healthy young and aged mice showed that gene expression patterns differed (322 probe sets representing 250 genes; t test $p < 0.001$; data not shown). Further comparison of the transcriptomic response of lavage leukocytes from young and

aged mice after trauma revealed 429 probe sets or 327 genes whose expression ($p < 0.001$) could differentiate between the two groups 100% of the time using leave-one cross-validation and Monte Carlo simulation (Fig. 5C).

The genes whose expression differed between BAL leukocytes from young and aged naive mice and 1 d after trauma were subjected to Ingenuity Pathway Analysis (IPA) transcriptomic analysis. Pathway analysis confirmed at the level of the transcriptome that gene expression changes involved in phagocytosis were not similarly upregulated in the elderly mice 1 d after trauma (Fig. 5D). Biocarta and Gene Ontology analysis also revealed a failure to downregulate ($p < 0.05$, t test) negative leukocyte regulated immunity and inhibition of matrix metalloproteinases pathways in the aged (Supplemental Fig. 1). Individual fold gene analysis revealed the following in aged mice: greater downregulation of CD74 (MHCII formation and transportation); greater upregulation of CXCL13 (B cell chemoattractant) and IL18bp (inhibitor of proinflammatory/T_{H18} cytokine), attenuated upregulation of haptoglobin (acute phase protein) and integrin α 6 (cell adhesion/surface mediated signaling); and less downregulation of IL-7 (lymphoid development cytokine; Supplemental Table II). Upstream regulator analysis predicted ($-2 < Z\text{-score} > 2$) that only the elderly would exhibit inhibition of IL-12, T_{H1} cytokines, CCL5, CCR9, CSF1, IL-1, and TLR2/3/4/9, whereas only the young predicted activation of CXCL4 (data not shown). In general, the aged transcriptome illustrates an inability to upregulate innate immune functions to the same magnitude as their younger counterparts in the acute post-trauma period.

Genome-wide expression analysis of circulating leukocytes

Blood from mice following trauma (at 2 h, 1 d, and 3 d) and naive mice were collected, and the genome-wide expression pattern of their circulating leukocytes was analyzed. Pathway analysis of the circulating leukocyte transcriptome 2 h after trauma revealed that aged mice were unable to upregulate the expression of many genes important to innate immunity and inflammation to the same capacity as young mice (Table II). For the expression pathways present in the Hematological System Development and Function category (IPA), only elderly mice were predicted to have decreased expression of genes involved in the differentiation of granulocytes/neutrophils, whereas only young mice were predicted to have increased expression of genes required for the accumulation of granulocytes and myeloid cells, activation of lymphocytes and mononuclear cells, differentiation of phagocytes, immune response of phagocytes, and response of neutrophils ($-2 < Z\text{-score} > 2$; data not shown). Analysis of the expression pattern 1 d after trauma demonstrates that the leukocyte transcriptome in young mice had returned to patterns more closely associated with healthy mice than the transcriptome of aged animals (Fig. 6). Thus, young mice are able to initiate a more robust early innate immune response at the level of the transcriptome than elderly mice are. In addition, young mice repress expression of adaptive immunity pathways in the acute phase of inflammation. At the same time, changes in gene expression were more transient in young animals, suggesting that they could re-establish homeostasis more readily than aged mice could; this corresponds well to the analyzed human data (26).

BM HSC phenotype and function

The question that arises is whether these differences in the transcriptome and phenotype of blood and alveolar leukocytes from the aged mice in response to trauma reflect differences in their ontogeny. BM HSCs (Lin⁻sca-1⁺ckit⁺ cells; LSK) were isolated from young and aged mice to compare their phenotype and functional response.

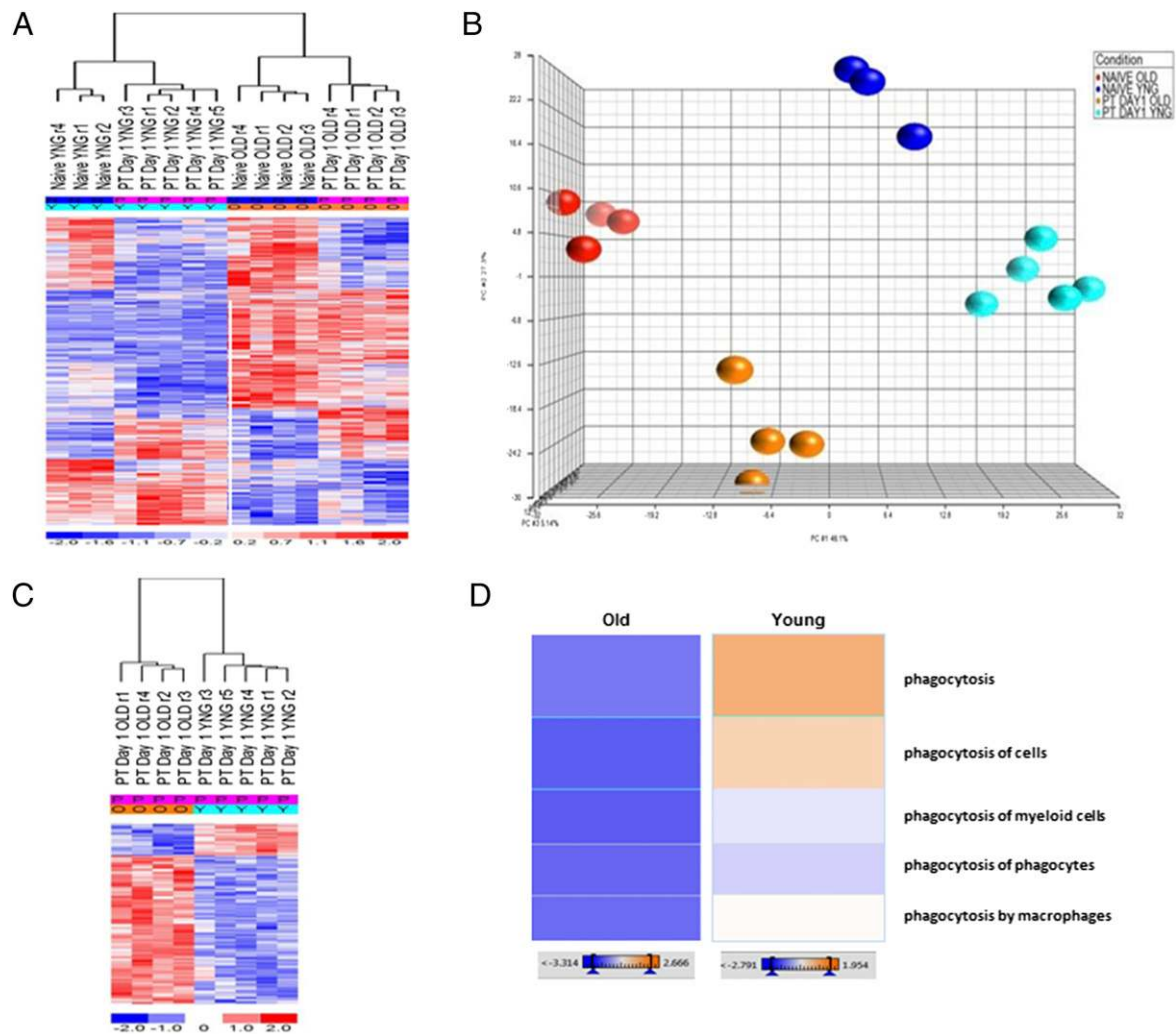


FIGURE 5. Microarray analysis of BAL cells showing the genomic response of BAL leukocytes of young and aged mice that were sacrificed 1 d after trauma. **(A)** Heat maps of the hierarchical clustering of gene expression patterns and variation between naive and aged and young polytrauma (PT) mouse BAL leukocytes. **(B)** Conditional principal component analysis of naive, old and young trauma mouse BAL leukocyte gene expression patterns. **(C)** Heat maps of the hierarchical clustering of gene expression patterns and variation between old and young PT mouse BAL leukocytes. **(D)** Heat maps show the fold change (from naive) gene expression of the functional category phagocytosis pathways (IPA) in young and old mice 1 d after trauma (fold change expression versus naive). $p < 0.001$. Orange indicates upregulation; blue indicates downregulation; white indicates neither significantly upregulated nor downregulated).

There were significantly fewer ST-HSCs (CD150⁻CD135⁺ LSK) in the aged compared with the young mice in both naive and 1 d after trauma (Fig. 7A). Furthermore, LSKs from aged mice did not proliferate as well as those from the young mice when cultured with different growth factors (Fig. 7B).

Genomic analysis of naive BM HSCs from young and aged mice illustrated that these cells were transcriptomically unique: 228 probe sets, representing 179 genes ($p < 0.001$) could differentiate the two groups 100% of the time using leave-one-out cross-validation and Monte Carlo simulation (data not shown). In ad-

Table II. Fold change expression of genes related to innate immunity in circulating leukocytes two hours after trauma

Young	Aged	Symbol	Name
167.6	119.3	Cxcl3	Chemokine (C-X-C motif) ligand 3
136.6	77.3	Cxcl2	Chemokine (C-X-C motif) ligand 2
47.6	33.7	Cd14	CD14 Ag
31.2	17.1	Tlr2	Toll-like receptor 2
33.4	16	Ltf	Lactotransferrin
23.7	13.3	Socs3	Suppressor of cytokine signaling 3
15.9	13.2	Cebpb	CCAAT/enhancer binding protein (C/EBP), β
18.1	9.6	Il1f9	IL-1 family, member 9
10.7	9.4	C5ar1	Complement component 5a receptor 1
15.4	8.8	Tnfaip2	TNF, α -induced protein 2
6.2	5.4	Il10rb	IL-10 receptor, β

Young mice (bold) are more likely to have greater upregulation (fold change compared with control) of genes related to innate immunity. The values in columns one and two indicate the fold change expression compared to control samples.

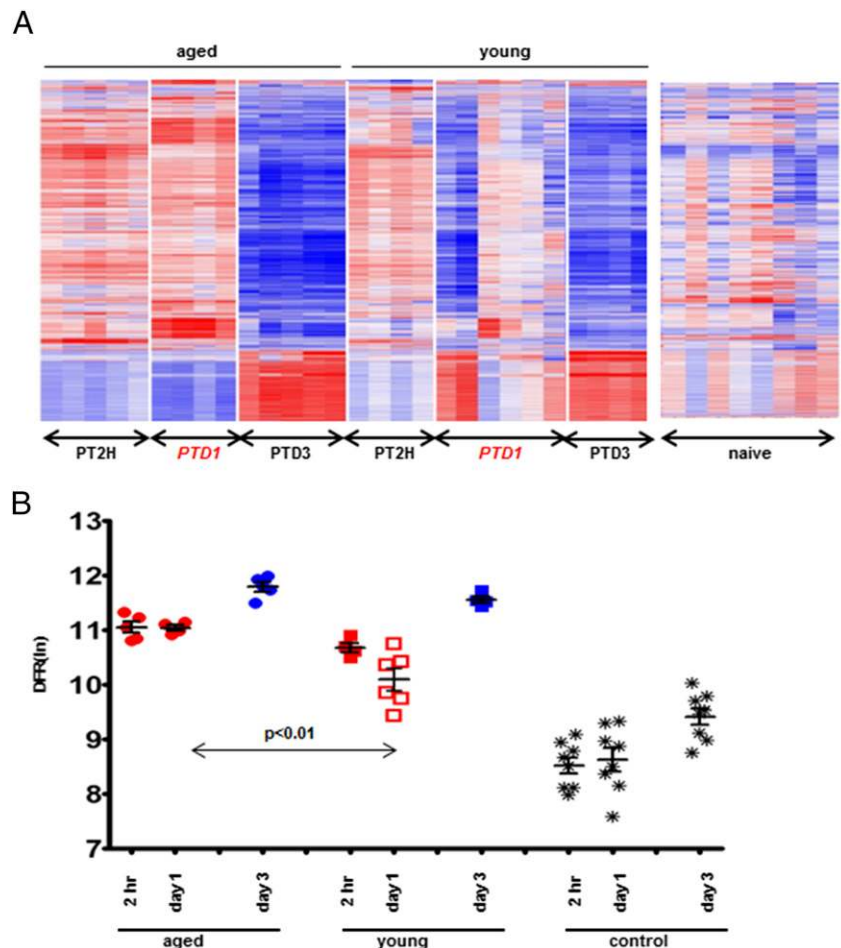


FIGURE 6. Microarray analysis of circulating leukocytes after trauma in young and aged mice. **(A)** Heat map of genomic response of aged and young mice after polytrauma (PT) and compared with naive control. Most of the genomic changes are upregulated (red), as compared with control in both aged and young mice 2 h after trauma. After 1 d, the expression pattern of young mice is more similar to control as compared with aged mice. Three days after the trauma, the genomic differences between the aged and young mice become similar to each other, most of which represents downregulation (blue) of specific gene expression patterns as compared with control. **(B)** DFR calculations confirm that young, but not aged, mice are genomically more similar to control (naive) mice 1 d after trauma.

dition, genome-wide expression analysis revealed significantly different expression patterns between the young and aged mice 1 d after trauma (Fig. 7C). Direct comparison of the transcriptome of HSCs from young and elderly mice 1 d after trauma showed that the two groups could also be differentiated with 100% certainty by leave-one-out cross-validation using 593 probe sets representing 426 genes ($p < 0.001$; Fig. 7D). Individual gene analyses illustrated that HSCs from elderly mice failed to upregulate expression of specific innate immunity genes related to chemotaxis (CCR2) and TLRs (TLR1), as well as having much greater downregulation of expression of MHCII genes (Table III). Interestingly, HSCs from young mice were much more successful at downregulating the expression of genes involved in lymphoid development and adaptive immunity in the acute phase after trauma in comparison with HSCs from aged mice (Table III). IPA also illustrated this reprioritization toward myeloid pathways and innate immunity in HSCs from young animals after trauma, as exhibited by downregulation of the cell-mediated immune responses pathways (including T cell homeostasis, development, transmigration, sequestration, movement, and migration; Fig. 7E). Further analysis revealed that HSCs from juvenile mice were predicted to have only activation of expression of genes involved in leukocyte recruitment and endotoxin shock response pathways (Z-score > 2), and to have G-CSF as an upstream regulator (Z-score > 2 ; data not shown). We have summarized our findings in Fig. 8.

Discussion

It is estimated that before 2050, the human population over the age of 60 will be 2 billion (39). In addition, by the start of the latter half of the 21st century, aged humans will outnumber those younger

than 15 y. Currently in the United States, humans over the age of 65 use a disproportionate amount of medical resources as compared with the make up of their total population. This is in part a reflection of the increased morbidity and mortality from infections and noninfectious inflammation (39). Thus, an understanding of the immune dysfunction in and therapeutics tailored for the aged patient population has the potential to significantly improve cost utilization of precious medical resources.

Severe traumatic injury is responsible for a major percentage of deaths worldwide (40), and elderly patients are known to have greater morbidity and mortality than their younger counterparts (1, 41). Although advances in critical care have substantially improved the initial mortality associated with trauma, many patients who survive the initial injury, especially those who are aged, go on to succumb to complications. This includes secondary nosocomial infections, sepsis, and persistent inflammation immunosuppression catabolism syndrome (42–46). The Inflammation and Host Response to Injury GG was a prospective, multi-institutional observational study with the primary aims of describing the epidemiologic, proteomic, and leukocyte genomic response in severely injured burn and trauma patients (23, 24). Our analysis of the elderly (>55 y) and young cohorts from the GG has demonstrated that although older patients did not develop VAP more frequently than the young, those aged individuals who developed VAP had a much greater incidence of prolonged length of stay in the intensive care unit (23, 24), were more likely to be discharged to skilled facilities rather than home for continued care, and had twice the 28 d mortality of similarly injured, younger patients. In fact, an age greater than or equal to 55 y was an independent predictor of mortality in severely injured blunt trauma patients

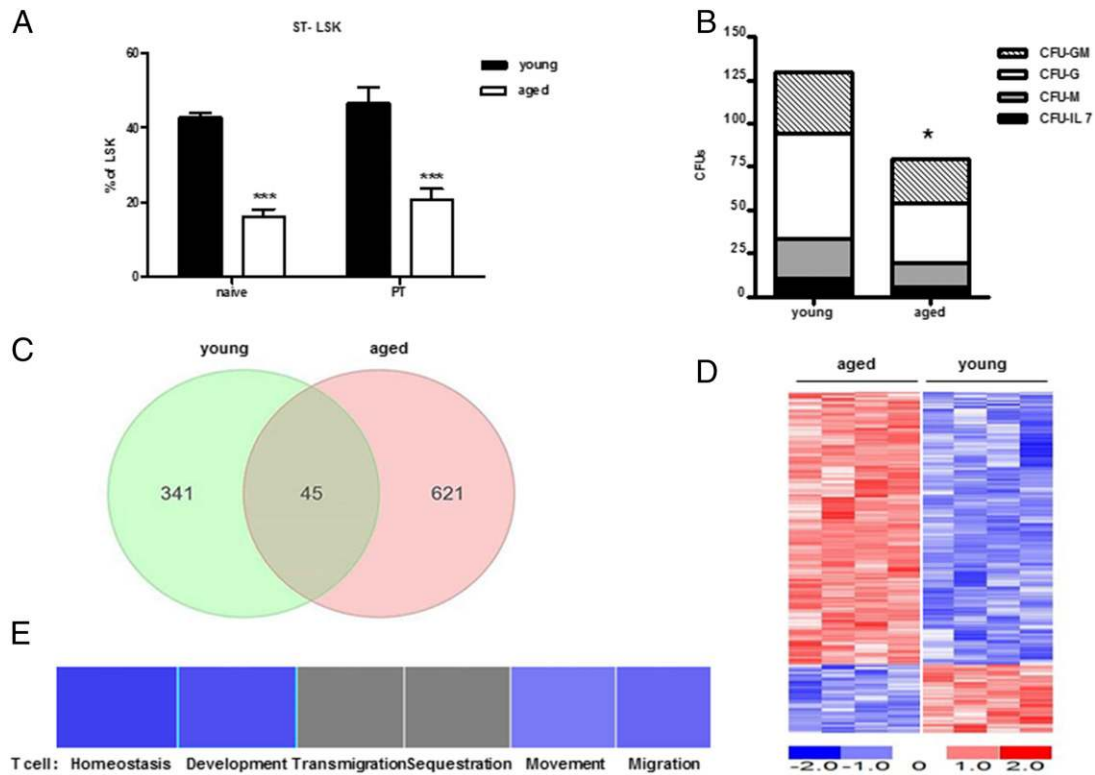


FIGURE 7. Murine hematopoietic cell numbers, function, and transcriptomic expression from young and aged mice 1 d after trauma. **(A)** One day after trauma, BM from young and aged mice were analyzed for LSK and ST-HSCs (CD150⁺CD135⁺LSK). **(B)** BM LSKs from young and aged mice were sorted and cultured in methylcellulose media with indicated cytokines. Colonies were counted 10–14 d later. **(C)** Most of the genes that have significant fold expression changes are dissimilar between aged and young HSCs ($p < 0.0001$). **(D)** Supervised analysis reveals that 593 probe sets (426 genes; $p < 0.001$) can differentiate between aged and young HSCs with 100% mean percent correct classification (leave-one-out validation). **(E)** IPA of the Hematopoiesis Pathway reveals downregulation of the cell-mediated immune responses pathways in young mouse HSCs as compared with murine HSCs after trauma. * $p < 0.05$, paired t test; *** $p < 0.001$, two-way ANOVA.

with pneumonia, after controlling for injury severity, transfusion requirements, shock severity, physiologic derangement, and comorbidities (Table I). Taken together, this shows that aged patients are less able to compensate for, and recover from, the physiologic stress and subsequent complications of severe trauma than younger, more robust individuals. Using a murine trauma model that includes hemorrhagic shock and multicompartmental injury and more closely recapitulates human trauma (12, 13), we have demonstrated that elderly mice have a similar increased

mortality to trauma and pneumonia as their human counterparts (Fig. 1). Given recent publications highlighting the differences between rodents and humans regarding inflammation (47, 48), we believe it is essential to perform this type of comparative research in animal models that attempts to best recapitulate the human condition being investigated (12–14). In this manner, we sought to address the topic of elderly patients who suffer severe trauma and then subsequently have much worse clinical trajectories and long-term outcomes than their juvenile counterparts using a clinically

Table III. Fold change (versus control) expression of BM HSC genes 1 d after trauma

Young	Aged	Symbol	Name
4.9	Chemotaxis 2.4	CCR2	Chemokine (C-C Motif) receptor 2
1.8	TLRs -1.3	TLR1	TLR 1
-1.4	Ag Presentation -2.7	H2-Eb	Histocompatibility 2, class II Ag E β
-3.4	-3.7	H2-Ob	Histocompatibility 2, O region β locus
-1.3	-5.2	H2-Aa	Histocompatibility 2, class II Ag A
-1.7	-2.9	H2-Aa	Histocompatibility 2, class II Ag A
-1.8	-3.4	H2-Aa	Histocompatibility 2, class II Ag A
-1.4	Immunosuppression 10.4	Il10ra	IL-10 receptor, α
-20.7	Lymphoid Development 1.5	Ighg	IgH (γ polypeptide)
-20.6	2.0	Ighg	IgH (γ polypeptide)
-2.3	-1.3	Cd96	CD96 Ag
-1.1	1.3	Il7	IL-7

Upregulated or downregulated BM HSC genes in young (bold) and aged mice 1 d after polytrauma. Positive values indicate upregulation, and negative values mean downregulation. The values in columns one and two indicate the fold change expression compared to control samples.

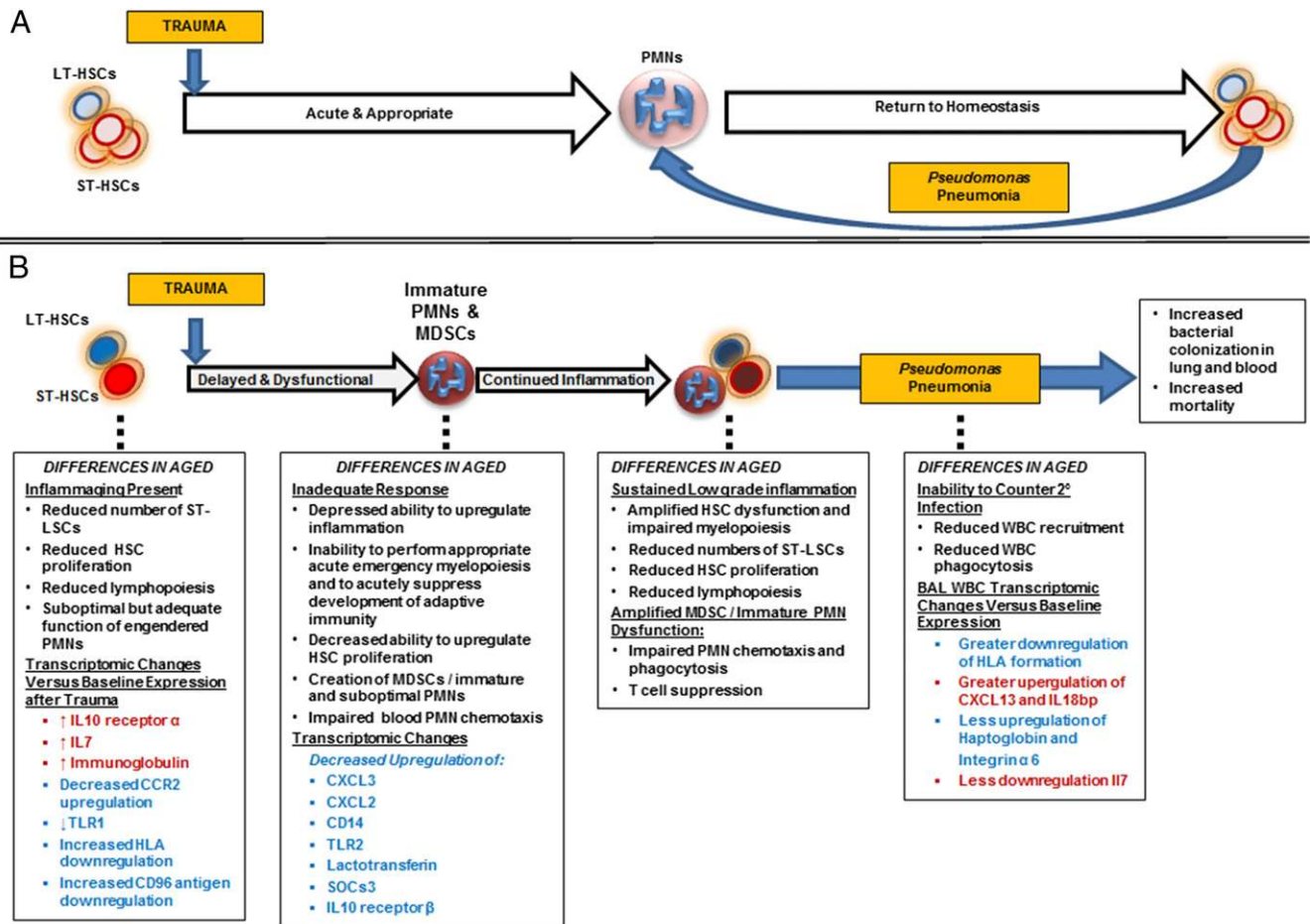


FIGURE 8. Summary of differences in young and aged emergency myelopoietic responses after severe hemorrhagic shock and injury. **(A)** Bone marrow cells from young animals have overall increased numbers of ST-HSCs and an increased functional capacity of their HSCs as compared with older animals. After injury, young mice can rapidly upregulate the expression of genes involved in innate immunity. Their HSCs, especially ST-HSCs, are capable of acutely diverting all their genomic resources to rapid myelopoiesis and creating well-functioning myeloid cells (specifically PMNs). Subsequently, the immune system in the young is more able to return closer to homeostasis than their elderly counterparts, allowing them to handle secondary infections. **(B)** Inflammation in the aged engenders genomic and epigenomic HSC changes, as well as local and systemic alterations, inducing the following in HSCs: lower functional frequency, delayed proliferative response, reduced efficiency for BM homing, myeloid-skewed cell production, and relatively fewer ST-HSCs. Granulocytes created with myelopoiesis in this inflammatory environment are released into the circulation have inferior bacterial homing and killing functions. However, these granulocyte functions are sufficient to overcome typical infections and are overcompensated by increased overall myelopoiesis. After severe trauma, aged mice are unable to upregulate inflammation acutely in a manner similar to young mice. In addition, aged HSCs are unable to undergo adequate acute emergency myelopoiesis because of multiple causes, including relatively decreased overall ST-HSC proliferation and numbers. After injury and shock, aged HSCs engender more immature granulocytes with suboptimal function; we hypothesize that these include myeloid-derived suppressor cells. Regardless, these cells are able to overcome some previous dysfunction, such as creation of reactive oxygen species. Unlike young mice that can rapidly return to baseline transcriptomic expression levels in their HSCs and leukocytes after severe injury, aged murine HSCs maintain a chronic, low-grade inflammation. This inflammation further worsens their HSC and granulocyte function. In addition, these immature granulocytes contribute to this vicious cycle of continued low-grade inflammation and further creation of dysfunctional myeloid cells. Although young mice are able to again undergo appropriate emergency myelopoiesis and combat secondary infections with functional granulocytes, aged mice eventually succumb to sources of sepsis, such as bacterial pneumonia infections.

relevant murine model (12, 14). We have determined that indeed the elderly do not die with an exaggerated inflammatory response and multiorgan failure; rather, they die of a failure of protective immunity and secondary infections.

Inflammation, an age-related increase in systemic chronic inflammation, contributes to many disease processes prevalent in the elderly, including cardiovascular disease, chronic obstructive pulmonary disease, and cancer (27–30); however, this does not translate to an exaggerated inflammatory response to infection or injury. Thus, the increased mortality in the elderly after trauma and subsequent pneumonia is secondary to reduced inflammation and protective immunity, because of a failure of myeloid cells to be recruited and engulf and kill bacteria during secondary infections. This is evident in both previously functional, aged humans

who are subjected to trauma and in elderly mice. In the former, these elderly patients have a more deleterious outcome, including disposition to long-term care facilities and increased death. Elderly mice subjected to trauma have greater lethality to *Pseudomonas* pneumonia. This dysfunctional response in mice appears programmed into the transcriptome as early as HSCs, and this dysfunctional response continues through to terminal neutrophils.

It is clear that severely injured or infected patients who develop multiple organ failure often demonstrate a failure in protective immunity (7, 49), and it is presumed that advanced age exacerbates these impairments in immune function (50); however, the mechanisms behind this remain unclear. Historically, authors have argued that age-related immune dysfunction could be due to an exacerbated response in the acute period to both infectious and

noninfectious inflammation (4, 6). However, there has been a shift in the more current literature regarding aging immune dysfunction (7–9, 39). After intra-abdominal sepsis, the cytokine response of aged mice, as compared with young mice, was found to be similar when comparing models with similar mortality among the cohorts (5). Our work in sepsis has verified this (7), and we have found a similar lack of an exacerbated proinflammatory response in aged mice after trauma. Our data indicate that there is no difference in the plasma or BAL cytokine concentrations between young and aged mice 1 d after trauma (Fig. 2). In fact, there is a trend for the aged mice to have lower cytokine concentrations; this recapitulates the lower plasma cytokine concentrations that we have determined acutely in aged patients with trauma that had a prolonged ICU course (as compared with the young) (26). In addition, our data in this clinically relevant murine model reveal that the leukocyte counts, phenotypes, and transcriptomic response patterns of young and aged mice after trauma is also consistent with a lack of acute exacerbation of inflammation in the elderly.

The question that arises is “Why do elderly animals die more frequently from trauma and subsequent infection?” Similar to recent reports from the Kovacs laboratory, we have found that aged mice have suboptimal myeloid cell function and, more specifically, PMN dysfunction after severe infectious or noninfectious inflammation (7, 31). The Kovacs laboratory revealed that despite increased chemokine levels in the lung after *Pseudomonas pneumonia* in elderly mice, there were fewer PMNs in the lungs and decreased myeloperoxidase activity (31). Our work using the trauma model has revealed a similar phenomenon, although through somewhat different mechanisms. We found no differences in the level of lung injury in young or aged mice after trauma or after trauma and *Pseudomonas pneumonia* (Fig. 3). However, BAL samples after trauma revealed defects in the function of PMNs from aged animals. These defects did not include a reduction in ROS production, but significantly fewer leukocytes in the lung after trauma and trauma and pneumonia (Fig. 4A), indicating defective recruitment in the aged as compared with the young. This latter phenomenon is similar to what was described by the Kovacs laboratory (31). We also identified dysfunctional phagocytosis following trauma and pneumonia in elderly mice, which might explain the failure to control the infection locally and systemically (Fig. 4B). Interestingly, we found increased bacterial CFUs in BAL samples from aged trauma mice, even before the *Pseudomonas* was instilled (Fig. 3C), indicating that severe trauma alone might cause an impairment of normal mucosal and innate immunity in elderly mice. Analysis of the BAL leukocytes from elderly mice early after trauma indicates they are transcriptomically different (Fig. 5, Supplemental Fig. 1) from those of young mice, and this pattern was also identified in circulating blood leukocytes (Fig. 6).

Circulating PMNs have relatively short half-lives, and they require continual replacement with functional myeloid cells from the BM (51). An appropriate myelopoietic response to inflammation is essential to host survival in the young adult (36, 38). This also appears to be deranged in the elderly after trauma, similar to what we have previously observed in the phenotype and function of HSCs from elderly mice after polymicrobial sepsis (7). Prior to infection, aged populations already have a predilection for myelopoiesis (52, 53). In addition, it is known, and our laboratory has verified, that aged mammals do not have difficulty expanding BM-derived myeloid cells after severe infection or injury (Supplemental Fig. 1).

Hematopoiesis involves many stem and progenitor cells (20). Although LT HSCs can reconstitute HSCs almost indefinitely at very low numbers, recent data from the transplantation literature

suggest that ST-HSCs, although more limited in their self-renewing potential, are more vital for appropriate, rapid myelopoiesis after BM loss (21). At baseline, HSCs from aged mice have reduced regeneration, reconstitution, and BM homing potential, which could be due to multiple causes, including accumulated DNA damage (39). We have previously demonstrated that the BM response of young mice to polymicrobial sepsis includes a marked expansion in both the relative percentage and absolute number of LSK cells, including both LT- and ST-HSCs (11). Although elderly mice demonstrate a similar trend, the composition and function of their BM is significantly different in regard to the numbers of LT- and ST-HSCs before and after trauma (Fig. 7A). ST-HSCs from aged animals have also been shown to have multiple functional defects by other authors (53). ST-HSCs and their immediate downstream progenitor cells (e.g., multipotent progenitors, common myeloid progenitors) are vital for appropriate, rapid myelopoiesis after BM loss during times of noninfectious or infectious acute inflammation (20–22). Recent data from the Baltimore laboratory has illustrated that the ST-HSC response to danger signals is vital to an appropriate hematopoietic response (20). Finally, genomic analysis of HSCs from both young and aged mice after trauma revealed significantly different gene expression patterns (Fig. 7C–E). Thus, from progenitor to downstream effector cells, it would appear that the aged response to severe infection or injury deviates from that of their younger counterparts at the level of the transcriptome.

This inappropriate protective immune response clearly leaves them at risk to subsequent infection (Fig. 8). A proper understanding of this phenomenon is critical to improving outcomes for elderly patients in the future, with promise existing for specific areas of intervention, including manipulation of progenitor cells that still exhibit plasticity.

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Disclosures

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