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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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Abstract

The elderly are particularly susceptible to trauma, and their outcomes are frequently dismal. Such patients often have complicated clinical courses and ultimately die from infection and sepsis. Recent research has revealed that although elderly subjects have increased baseline inflammation as compared to their younger counterparts, the elderly do not respond to severe infection/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. Subsequent analysis with a murine trauma model also demonstrated that elderly mice had increased mortality after post-trauma Pseudomonas pneumonia. Blood, bone marrow, and bronchoalveolar lavage sample analyses from juvenile and 20–24 month 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, the elderly appeared unable to effectively resolve their inflammatory response to severe injury.

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INTRODUCTION

People of advanced age (greater than 55 years old) have significantly increased morbidity and mortality after trauma (1–3). Since the elderly population is expanding, research into this disease process is increasingly relevant, especially with the escalating economic and health care burdens on our 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 non-infectious inflammation (4–6). However, more 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, neutrophils (PMNs) are replaced after inflammation through a process known as ‘emergency myelopoiesis.’ This occurs after severe injury when bone marrow 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 quite 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 as well as having functional deficits in specific leukocytes (7, 15–19). Early data in less severe trauma-hemorrhage rodent models would suggest that there is indeed some defect in emergency myelopoiesis in elderly mice, as well as with hematopoiesis in general (15, 19). Two key cell types involved in the earliest phases of myelopoiesis are long term (LT-) and short term (ST-) hematopoietic stem cells (HSCs). ST-HSCs have a much more limited capacity for self-renewal than LT-HSCs, but appear to be more vital for rapid myelopoiesis after bone marrow (BM) loss 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 five years of the program included patients over the age of 55 years, 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 better recapitulate the human condition (13).

Our overarching goal was to identify the specific defects in innate immunity and inflammation in the elderly patient after severe trauma that leads to their worsened outcomes to secondary infection. We hypothesized that myeloid dysfunction contributed to the 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 Glue Grant study, we then analyzed myeloid cell function in

young and aged mice following polytrauma and clinically relevant infection (*Pseudomonas* pneumonia). We can conclude that although *'inflammaging'* defined as an age-related increase in systemic chronic inflammation, and promotes many disease processes prevalent in the elderly, including cardiovascular disease, chronic obstructive pulmonary disease and even cancer (27–30), contributes to poor outcomes to injury and infection, it does not translate specifically to an increased inflammatory responses subsequent to trauma or a secondary *Pseudomonas* pneumonia, as well as other clinically relevant insults (7, 31). Rather, the overwhelming data suggests that a failure to initiate an early innate immune response, as well as a subsequent inability to effectively resolve the inflammatory response, leaves the elderly at risk to 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 adult blunt trauma patients in hemorrhagic shock enrolled from seven level 1 trauma centers between 2001–2011 (32). Inclusion criteria required a blunt traumatic mechanism with an Abbreviated Injury Scale (AIS) 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 minutes of emergency department arrival, and blood product transfusion within 12 hours of injury. Exclusion criteria consisted of those with significant mortality risk from severe head injury (AIS head > 4), those evaluated at the trauma center more than six hours 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 (SOPs) for initial resuscitation and supportive care (23, 33). Over the study period, there was an overall SOPs compliance rate of greater than 69%.

As of October 2013, the TRDB contained prospectively collected demographic, clinical and outcomes data on 1,928 blunt trauma patients meeting criteria for this analysis. These patients were separated into two cohorts, either advanced age (≥ 55 years old) or young (< 55 years old) for epidemiologic analysis. This cutoff was utilized based upon previous literature which has shown that an age of 55 years or older is associated with worse outcomes than predicted, even after controlling for other injury factors (34, 35). Using these definitions, there were 1,395 and 533 patients in the "young" and "aged" cohorts, respectively.

Clinical demographics and outcomes analysis

Baseline patient demographics, injury severity, fluid and blood product resuscitation parameters, serial laboratory values and multiple clinical outcomes, ventilator associated

pneumonia (VAP) were obtained from the TRDB. VAP (Table SI) was used rather than ventilator associated events (VAE) as VAE had not been defined by the CDC at the time of study initiation, and was therefore not tracked in the database. Univariate analyses were performed between young and aged cohorts with VAP using Fisher's 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, as well as any significant predictive factors identified by univariate analysis. All patients were included for 28-day mortality modeling. All significance tests were two-sided, with a 0.05 alpha level. Statistical analyses were performed with SAS (v.9.3, Cary, NC).

Mice

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

Murine polytrauma model

Mice underwent 90 minutes of hemorrhagic shock and resuscitation followed by long bone fracture and cecectomy, as previously described (12). Mice were euthanized two hours, one day and three days later. Blood, bone marrow, spleen, and bronco alveolar lavage cells were collected for phenotypic and functional analyses. Genome-wide expression was also performed. Intranasal *Pseudomonas* was instilled to induce pneumonia one day post-trauma. For survival studies, mice were observed up to seven days.

Pneumonia induction

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

Bronchoalveolar lavage (BAL)

The trachea was cannulated and lavaged four times with 800 μl cold PBS containing 2 mM EDTA. Bacterial load was determined by culturing 100 μl of 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 hematocytometer (Hausser Scientific, Horsham, PA).

Histology

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

Flow Cytometry

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

Phagocytosis assay

Cells (2×10^5) were resuspended in 200 μl PBS incubated with 20 μl fluorospheres in 37° C water bath for ten minutes, washed with PBS containing 0.1% BSA and stained with anti-Ly6G and anti-CD11b and analysed by 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, Bellirica, MA). All assays were performed according to the manufacturer's protocols. Cytokine concentrations were determined using BeadView™ software (Millipore)(7).

Hematopoietic stem and progenitor cells (HSPCs) culture

Bone marrow cells from young and aged mice were aseptically collected one day after trauma. Single cell suspensions were created by passing the cells through 70 μm pore sized cell strainers (BD Falcon, Durham, NC). Erythrocytes were lysed using ammonium chloride lysis buffer and washed with PBS. Cells were stained with anti-biotin Lineage mixture (BD Biosciences, San Jose, CA), anti-ckit and anti-Sca-1 (eBioscience, San Diego, CA). $Lineage^{neg}$ $Sca\text{-}1^{+}ckit^{+}$ cells (LSKs) were sorted using FACSAria™ (BD Biosciences, San Jose, CA). Five hundred LSKs were cultured in methylcellulose media (R&D Systems, Minneapolis, MN) supplemented with either GM-CSF, G-CSF, M-CSF, or IL-7 (R&D

Systems, Minneapolis, MN). Colonies were counted after 10–14 days incubation at 37° C (11).

Genome-wide expression analysis

Blood was collected by intracardiac puncture at two hours, one or three days after trauma and from naïve mice using 1 ml syringes containing 100 μl of 169 mM EDTA. Red blood cells were lysed using Buffer EL (Qiagen, Valencia, CA). Bronchoalveolar lavage cells were collected one day after trauma and from naïve mice. Sorted BM LSK cells from young and aged mice were collected one day after trauma. The cell pellet was homogenized in RLT buffer (Qiagen, Valencia, CA) supplemented with 2-mercaptoethanol and passed through Qiashredder (Qiagen, Valencia, CA). Total RNA was isolated using RNeasy kit (Qiagen, Valencia, CA) and the quality and quantity was assessed using Agilent Bioanalyzer 2000. Nucleic acids were labeled using the 3′ IVT Express Kit and 15 μg of labeled cRNA was hybridized to Mouse Genome 430 2.0 Arrays (Affymetrix, Santa Clara, CA) (7). For bone marrow LSKs, 15 ng total RNA were labeled and amplified using NugenOvation PICO and Encore kits (NuGEN, San Carlos, CA) and 7.5 μg cDNA was hybridized onto MOE 430 2.0 Arrays (Affymetrix, Santa Clara, CA). Arrays were hybridized for 16 hours at 45°C. Following hybridization, 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/aged trauma mice at $p<0.001$ (F test).

Statistics

Differences among groups in flow cytometric analyses were evaluated using Student's 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/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 to the young

The overall GG trauma cohort consisted of 1,928 severely injured patients in hemorrhagic shock. We determined how many of these patients, both young (age < 55 years) and aged (age ≥ 55 years), were diagnosed with ventilator-associated pneumonia (VAP) (Table I). 29% (159 of 533) of aged patients were diagnosed with VAP, as compared to 24% of young patients (345 of 1395). Although the incidence of VAP was not significantly different between the young and the aged, there were some differences in the cohorts, and the

outcomes from the trauma and VAP were profoundly different. Expectedly, elderly patients had an increased number of comorbid conditions at admission (Table I), while young patients were slightly more severely injured. Shock severity, as measured by initial serum lactate 0–6 hours and 12–24 hours from injury, was similar between the two groups. However, elderly patients demonstrated significantly greater evidence of subsequent overall physiologic derangement, as measured by APACHE II at 24 hours from injury (Table I). Additionally, while slightly less severely injured, older patients with VAP had a significantly higher incidence of a complicated clinical trajectory (defined as either an ICU hospitalization of greater than 14 days with evidence of ongoing organ dysfunction, or death after the first 48 hours) (23, 24). Elderly patients with VAP were also more likely to be discharged to skilled facilities rather than home, and had double the 28 day mortality ($p<0.01$). Multivariate logistic regression analysis revealed that age ≥ 55 years old was an independent predictor of mortality in severely injured blunt patients with VAP, after controlling for injury severity, transfusion requirements, shock severity and physiologic derangement, and comorbidities (Table I).

Aging is associated with increased mortality in mice after polytrauma (PT) and subsequent Pseudomonas pneumonia (Pp)

We tested for a similar response of young and aged mice to a more severe model of trauma and hemorrhagic shock (12), and demonstrated that trauma was non-lethal in both age groups. In addition, 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 one day after trauma, there was a significant increase in their mortality compared to young mice (Fig. 1). In this manner, the response by elderly mice recapitulates the findings from elderly human severe trauma patients who develop VAP. We next examined the young and aged mice one day after trauma to examine 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 to young mice (Fig. 2). In fact, elderly mice following trauma mostly trended towards lower concentrations than young mice, albeit not significantly. This was also true for BAL cytokine concentrations obtained one day after trauma or one day 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 one day 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–B). Next, BAL was performed and bacterial colony forming units (CFU) determined.

Surprisingly, aged mice had more bacterial CFUs compared to young mice following polytrauma (Fig. 3C–D). This suggested that even after trauma alone, normal pulmonary protective immunity in aged mice was less effective. Very few CFUs, if any, were found in BAL samples from naïve young and aged mice (*data not shown*).

Neutrophils (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 one day 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 was significantly fewer ($p<0.05$) phagocytic PMNs present in the lavage fluid of aged mice compared to young mice one day after trauma and trauma plus *Pseudomonas* pneumonia (Fig. 4B). This reduction in lung PMN recruitment occurred despite no differences in blood, spleen or bone marrow myeloid cell populations. In fact, there were more splenic PMNs in aged mice as compared to young mice (*data not shown*).

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 2,097 probe sets representing 1,649 genes differentiated young and aged trauma and naïve mice at a false-discovery rate (FDR) 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–B). This was unexpected since earlier studies in both humans and mice have demonstrated a *'genomic storm'* in the blood leukocyte transcriptome with the expression of over 70% of the genome changing in response to trauma (13, 24). It is possible that our inability to recapitulate an equivalent injury in the mice may play some role in this genomic response, as some of the human trauma 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 one day following 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 one day after trauma (Fig. 5D). Biocarta™ and Gene Ontology™ analysis also revealed a failure to down regulate ($p<0.05$, t-test) 'negative leukocyte regulated immunity'

and ‘inhibition of matrix metalloproteinases’ pathways in the aged (Figure S1). 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 integrinα6 (cell adhesion/surface mediated signaling); and less downregulation of IL-7 (lymphoid development cytokine) (Table SII). Upstream regulator analysis predicted (−2 < z-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, while 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 two hours, one day and three days) and naïve mice were collected and the genome-wide expression pattern of their circulating leukocytes was analyzed. Pathway analysis of the circulating leukocyte transcriptome two hours after trauma revealed that aged mice were unable to upregulate the expression of many genes important to innate immunity/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 shown to have decreased expression of genes involved in the differentiation of granulocytes/neutrophils, while only young mice were predicted to have increased expression of genes required for the accumulation of granulocytes/myeloid cells, activation of lymphocytes and mononuclear cells, differentiation of phagocytes, immune response of phagocytes, and response of neutrophils (−2 < Z-score > 2) (*data not shown*). Analysis of the expression pattern one day following 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, as well 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 - this corresponds well to the analyzed human data (26).

Bone marrow (BM) hematopoietic stem cells (HSCs) 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. Bone marrow hematopoietic stem cells (HSCs) ($Lin^{-}sca\text{-}1^{+}ckit^{+}$ cells; LSK) were isolated from young and aged mice to compare their phenotype and functional response. There were significantly fewer short term HSCs (ST-HSCs; $CD150^{-}CD135^{+}$ LSK) in the aged compared to the young mice in both naïve and one day after trauma (Fig. 7A). Furthermore, LSKs from aged mice did not proliferate as well as from the young mice when cultured with different growth factors (Fig. 7B).

Genomic analysis of naïve bone marrow 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*). Additionally, genome-wide expression analysis revealed significantly different expression patterns between the young and aged mice one day after trauma (Fig. 7C). Direct comparison of the transcriptome of HSCs from young and elderly mice one day 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 toll-like receptors (TLR1), as well having much greater downregulation of expression of MHCII genes (Table III). Interestingly, HSCs from young mice were much more successful at down-regulating the expression of genes involved in lymphoid development and adaptive immunity in the acute phase after trauma in comparison to 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 down regulation 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 only were predicted to have activation of expression of genes involved in 'leukocyte recruitment' and 'endotoxin shock response pathways' (Z-score > 2), as well as to have G-CSF as an upstream regulator (Z-score > 2) (*data not shown*).

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, the number of aged humans will outnumber those less than 15 years of age. Currently in the United States, humans over the age of 65 use a disproportionate amount of medical resources as compared to their total numbers. This is in part a reflection of the increased morbidity and mortality of infections and non-infectious inflammation (39). Thus, an understanding of the immune dysfunction in and therapeutics tailored to 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 from complications. This includes secondary nosocomial infections, sepsis and the persistent inflammation immunosuppression catabolism syndrome (PICS) (42–46). The "*Inflammation and Host Response to Injury*" GG was 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 (23, 24). Our analysis on the elderly (> 55 years) 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 ICU length of stay (23, 24), were more likely to be discharged to skilled facilities rather than home for continued care, and had twice the 28 day

mortality of similarly injured younger patients. In fact, an age greater than or equal to 55 years old was an independent predictor of mortality in severely injured blunt trauma patients 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 multi-compartmental 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 relevant murine model (12, 14). We have determined that indeed the elderly do not die with an exaggerated inflammatory response and multi-organ failure, but rather, die from a failure of protective immunity and secondary infections.

‘Inflammaging’, 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 even 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, due at least in part to a failure of myeloid cells to be recruited, engulf and kill bacteria during secondary infections. This is evident in both previously functional, aged humans who are subjected to trauma, as well as 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 hematopoietic stem cells, 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 previously argued that age-related immune dysfunction could be due to an exacerbated response in the acute time period to both infectious and non-infectious 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 to 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 pro-inflammatory response in aged mice after trauma. Our data indicates that there is no difference in the plasma or BAL cytokine concentrations between young and aged mice one

day 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 we have determined acutely in aged patients with trauma that had a prolonged ICU course (as compared to the young) (26). Also, our data in this clinically relevant murine model reveals that the leukocyte counts, phenotypes, and the transcriptomic response patterns of young and aged mice after trauma is also consistent with a lack of acute exacerbation of inflammation in the elderly.

So 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, neutrophil (PMN) dysfunction, after severe infectious or non-infectious 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 their lungs as well as 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, bronchoalveolar lavage (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 elderly as compared to 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 may explain the failure to control the infection locally and systemically (Fig. 4B). Interestingly, we did find increased bacterial colony forming units (CFUs) in BAL samples from aged trauma mice even before the *Pseudomonas* was instilled (Fig. 3C), indicating that severe trauma alone may cause an impairment of normal mucosal and innate immunity in the elderly. Analysis of the BAL leukocytes from elderly mice early after trauma indicates they are transcriptomically different (Fig. 5 and Figure S1) 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 bone marrow (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 towards myelopoiesis (52, 53). In addition, it is known, and our laboratory has verified, that aged mammals do not have difficulty expanding bone marrow-derived myeloid cells after severe infection or injury (Figure S1).

Hematopoiesis involves many stem and progenitor cells (20). Even though LT-HSCs can reconstitute HSCs almost indefinitely at very low numbers, more 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 bone marrow loss (21). At baseline, HSCs from aged mice have reduced regeneration, reconstitution and bone marrow

homing potential, which could be due to multiple causes, including accumulated DNA damage (39). We have previously demonstrated that the bone marrow 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 bone marrow is significantly different in regards 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 (ex. multipotent progenitors (MPPs) and common myeloid progenitors (CMPs)) are vital for appropriate, rapid myelopoiesis after bone marrow loss during times of non-infectious or infectious acute inflammation (20–22). Recent data from the Baltimore laboratory has illustrated that the ST-HSC's 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 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 elderly patient outcomes in the future, with promise existing for specific areas of intervention, including manipulation of progenitor cells that still exhibit plasticity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this article

BAL	bronchoalveolar lavage
BM	bone marrow
GG	Glue Grant

H&E	Hematoxylin and eosin
HSC	hematopoietic stem cells
ST-HSC	short term hematopoietic stem cells
LSKs	lineage^{-} sca-1^{+} c-kit^{+} cells
PMN	neutrophils
Pp	Pseudomonas pneumonia
PSB	protected specimen brush
PT	polytrauma
SOP	standard operating procedure

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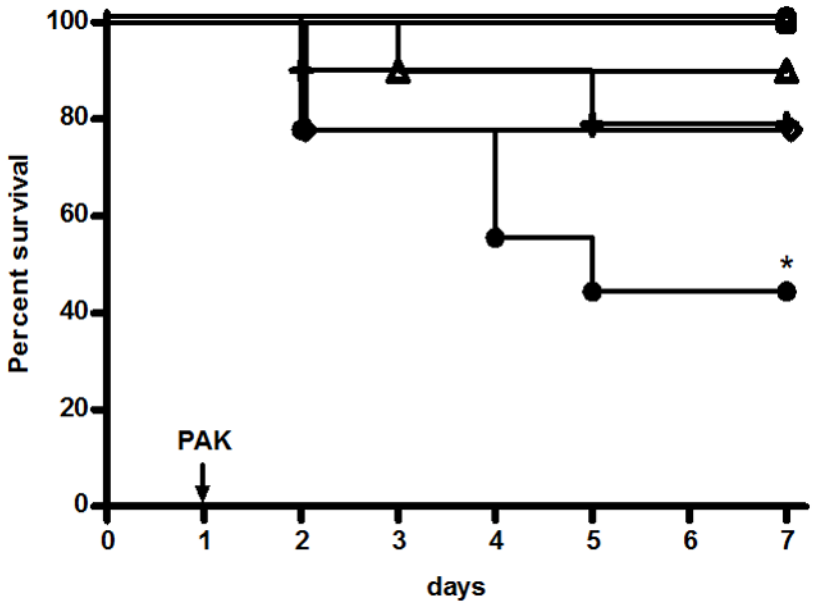

Figure 1. Murine survival rates after trauma or trauma and Pseudomonas pneumonia

Aged mice have a significantly lower survival rate when exposed to Pseudomonas pneumonia (Pp) one day after trauma (PT). Young (6–10 weeks old) and aged (20–24 months old) C57Bl/6 mice underwent trauma and/ or exposed to *Pseudomonas aeruginosa* (PAK, 10^7 CFU) and survival was monitored. (PT young□;PT aged○; Pp young△; Pp aged +; PT+Pp young◇; PT+Pp aged●). This figure is the combination of five separate experiments; n=9–10; *p<0.05, Log-rank (Mantel-Cox) test.

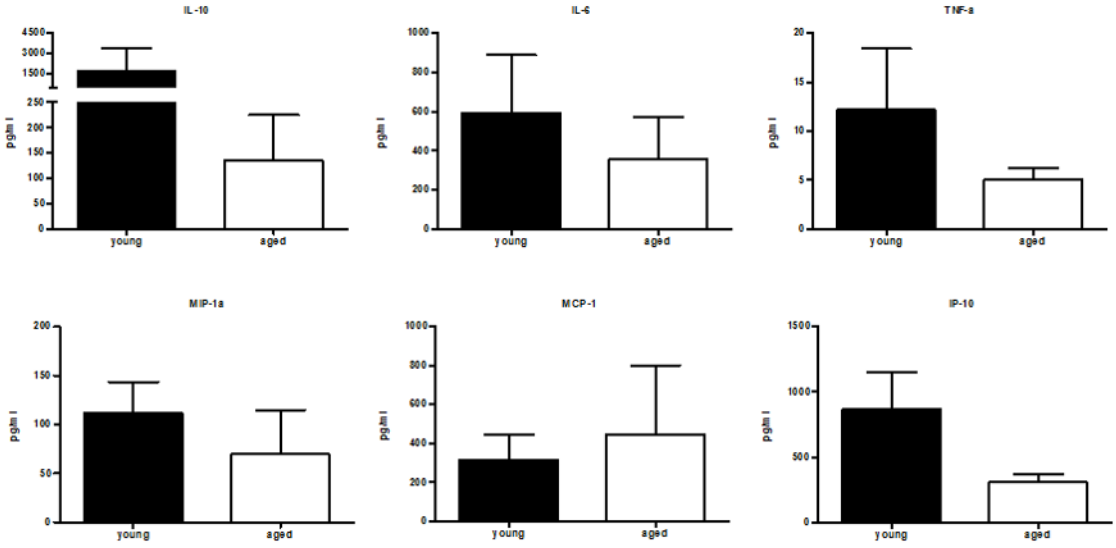

Figure 2. Plasma cytokine concentrations after PT

There was no significant statistical difference in the plasma cytokine concentrations between young and aged mice one day after PT. Plasma from young and aged mice were collected one day after PT and cytokine/chemokine production was evaluated by Luminex (n=3).

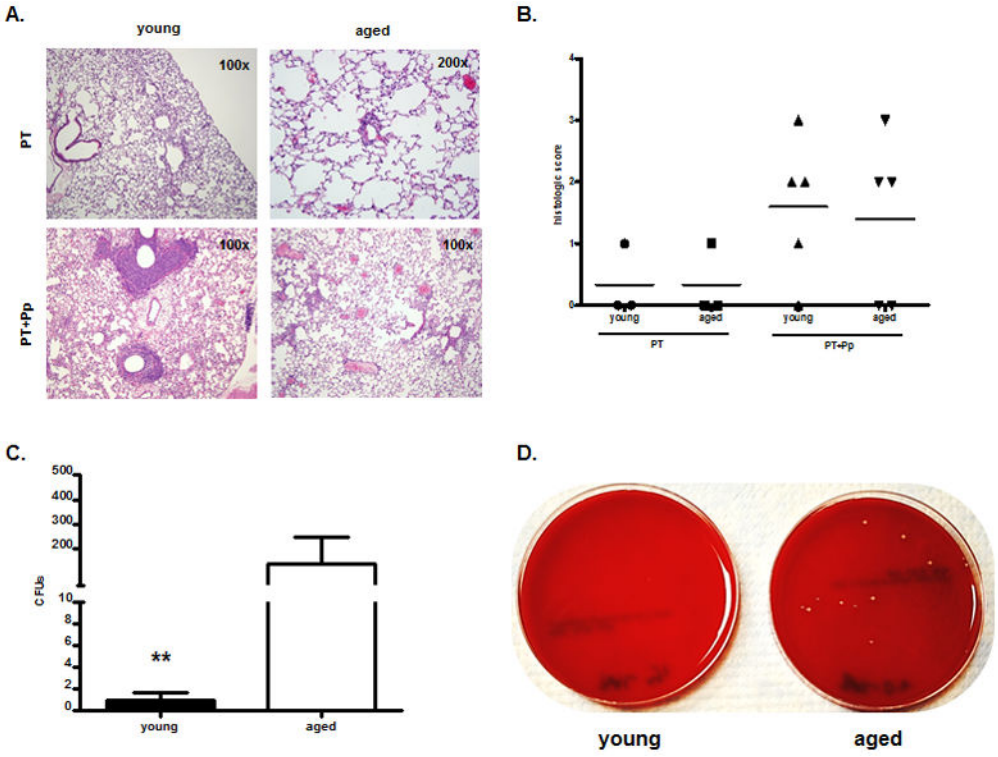

Figure 3. Lung histology and bacterial clearance in young and aged mice after trauma

(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 0–3 with 0=no inflammation, 1=mild, 2=moderate, and 3=severe. Representative sections are shown (n=3–5/group). (C) Bronchoalveolar lavage (BAL) fluid was collected and bacterial colony forming units (CFUs) were determined by plating on sheep blood agar. The experiment was performed at least twice (n=6); ** $p<0.01$, Mann Whitney t-test. (D) Examples of bacterial CFUs on sheep blood agar plates from BAL samples one day after trauma.

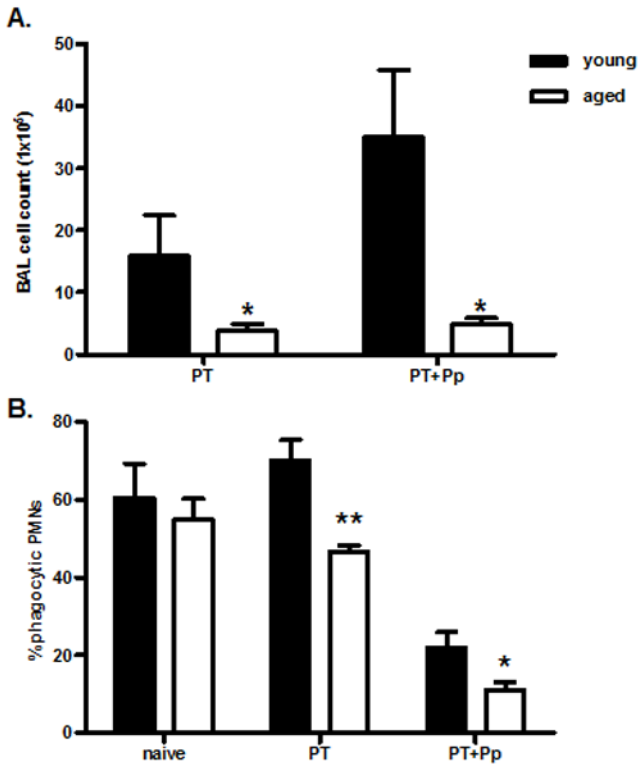

Figure 4. Total BAL leukocytes and functional capacity and in young and aged mice after trauma

(A) Young and aged mice underwent trauma (PT) or trauma and Pseudomonas pneumonia (PT+Pp) and sacrificed one day later. BAL fluid was collected and cells were counted using a hematocytometer. 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) 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/group); *$p<0.05$, **$p<0.01$, unpaired t-test.

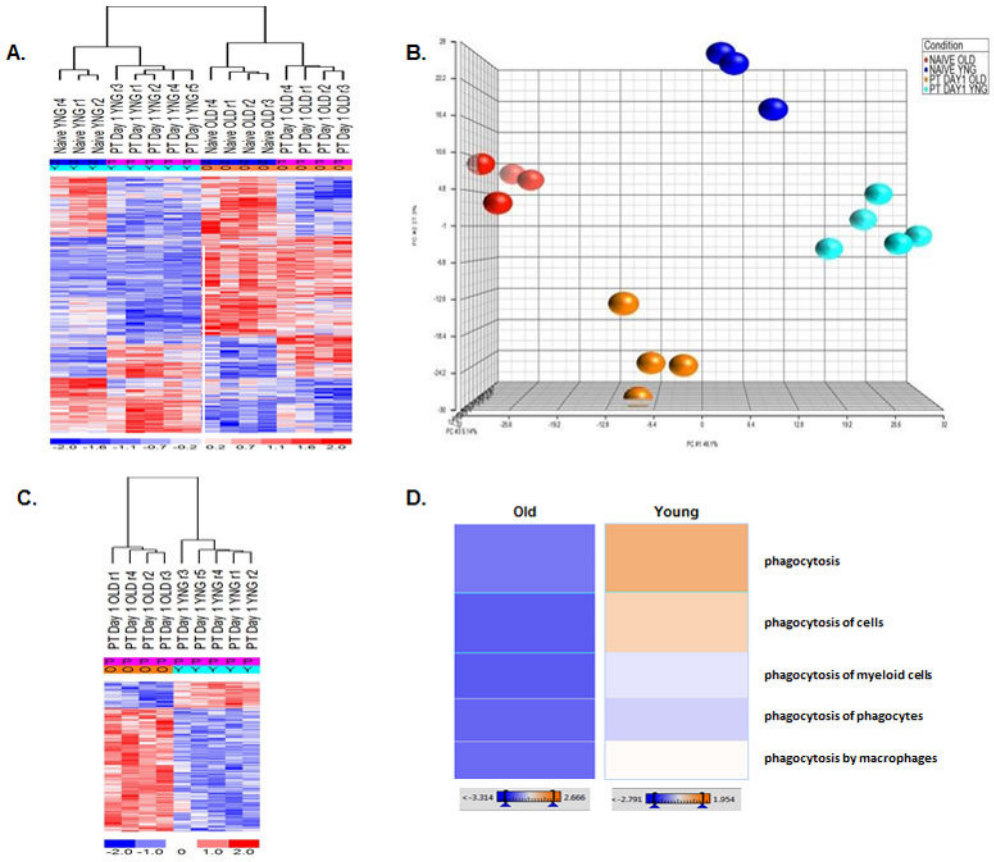

Figure 5. Microarray analysis of bronchoalveolar lavage (BAL) cells

The genomic response of BAL leukocytes of young and aged mice that were sacrificed one day after trauma. (A) Heat maps of the hierarchical clustering of gene expression patterns and variation between naïve and aged and young trauma (PT) mouse BAL leukocytes. (B) Conditional principal component analysis of naïve and aged and young trauma mouse BAL leukocytes gene expression patterns. (C) Heat maps of the hierarchical clustering of gene expression patterns and variation between aged and young PT mouse BAL leukocytes. (D) Heat maps show the fold change (from naïve) gene expression of the functional category "phagocytosis pathways" (IPA®) in young and aged mice one day after trauma (fold change expression versus naïve, $p<0.001$; orange = upregulation, blue = downregulation, white = neither significantly up nor down regulated).

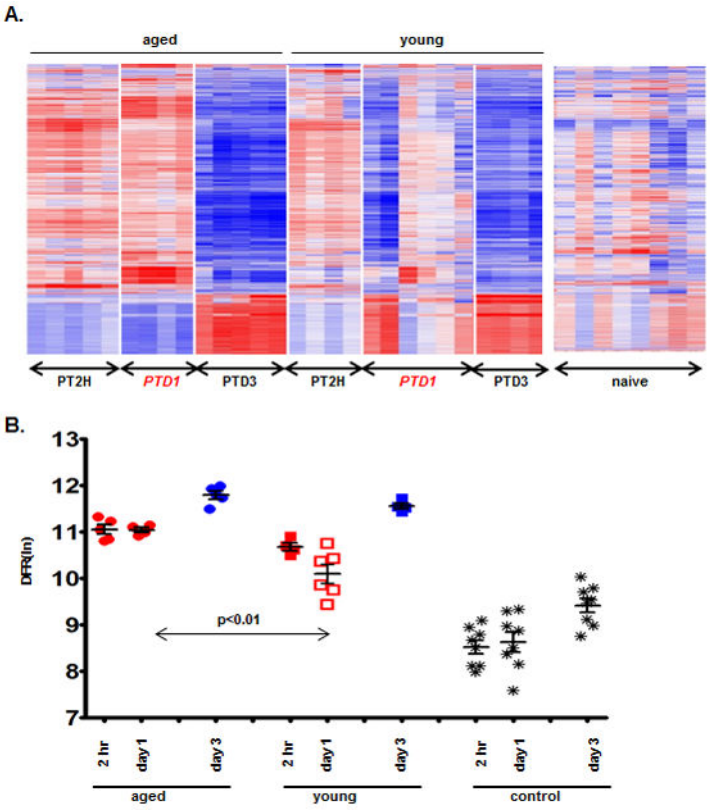

Figure 6. Microarray analysis of circulating leukocytes after trauma in young and aged mice

(A) Heat map of genomic response of aged and young mice after trauma (PT) and compared to naïve control. Most of the genomic changes are upregulated (red), as compared to control in both aged and young mice 2 hours after trauma. After one day, the expression pattern of young mice is more similar to control as compared to 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 down regulation (blue) of specific gene expression patterns as compared to control. (B) Distance from reference (DFR) calculations confirm that young, but not aged, mice are genomically more similar to control/naïve mice one day after trauma.

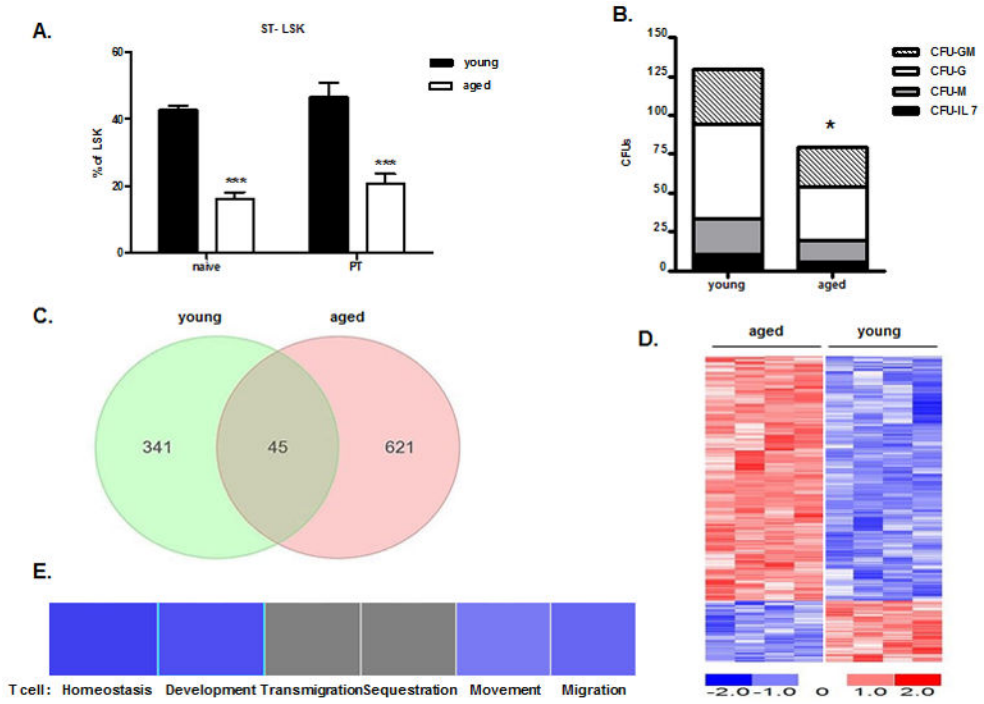

Figure 7. Murine hematopoietic cell numbers, function, and transcriptomic expression from young and aged mice one day after trauma

(A) One day after trauma, BM from young and aged mice were analyzed for LSK ($lin^{-}sca\text{-}1^{+}ckit^{+}$) and ST-HSCs ($CD150^{-}CD135^{+}$LSK); ***p<0.001 by two-way ANOVA. (B) BM LSKs from young and aged mice were sorted and cultured in methylcellulose media with indicated cytokines. Colonies were counted 10–14 days later; *p<0.05 by paired t-test. (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 % correct classification (leave-one-out validation). (E) IPA® of the Hematopoiesis Pathway reveals down regulation of the cell-mediated immune responses pathways in young mouse HSC as compared to murine HSCs after trauma.

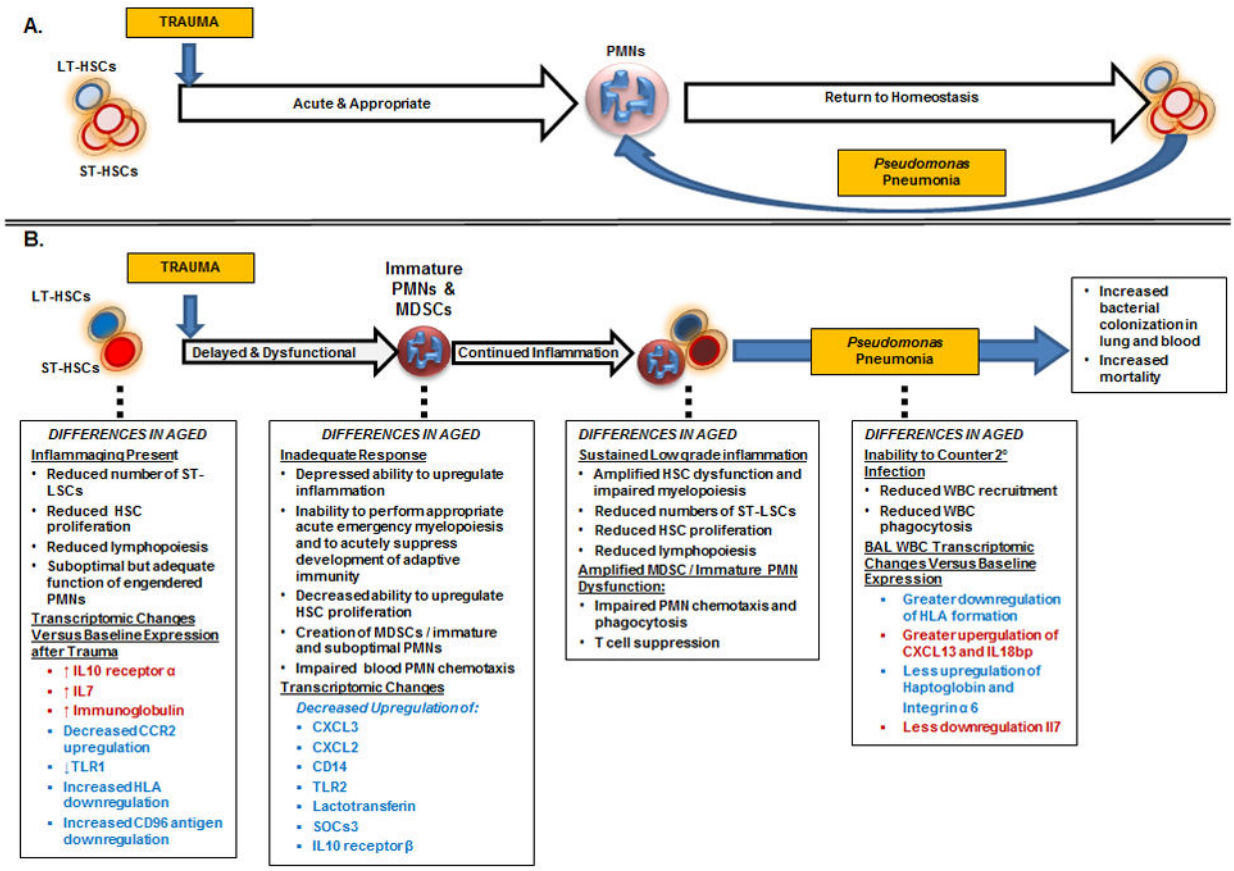

Figure 8. Summary of differences in young and aged emergency myelopoietic responses after severe hemorrhagic shock and injury

(A) Bone marrow cells from young animals have overall increased numbers of ST-HSCs as well as an increased functional capacity of their HSCs as compared to the elderly. 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 functional 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 appropriately handle secondary infections.

(B) 'Inflammaging' 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 less ST-HSCs. Granulocytes created through myelopoiesis in this 'inflammaging' environment are released into the circulation have inferior bacterial homing and killing functions. However, these granulocyte functions are adequate enough to overcome typical infections and are overcompensated by increased overall myelopoiesis.

After severe trauma, aged mice are unable to acutely upregulate inflammation in a similar manner to young mice. In addition, aged HSCs are unable to undergo adequate acute emergency myelopoiesis due to multiple causes, including relatively decreased overall ST-

HSC proliferation and numbers. Also, after injury and shock, aged HSCs engender more immature granulocytes with sub-optimal function - we hypothesize 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 now maintain a chronic low grade inflammation. This further worsens their HSC and granulocyte function. In addition, these immature granulocytes contribute to this ‘viscous cycle’ of continued low grade inflammation and further creation of dysfunctional myeloid cells. While 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.

Table I

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

Patient Demographics and Outcomes			
	Young (Age <55 years) (n = 345)	**Aged (Age ≥55 years) (n = 159)**	
Demographics			**p-value**
	N (%)	**N (%)**	
Gender, male	264 (76.5%)	118 (74.2)	0.88
≥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 hours	30.0 (29.4–30.6)	33.1 (32.1–34.0)	**<0.001**
Lowest Pre-Hospital SBP	89.9 (86.3–93.5)	88.2 (82.7–93.7)	0.61
Lowest ED SBP (mmHg)	84.3 (81.8–86.8)	76.0 (72.3–79.7)	**<0.001**
Max Lactate 0–6 hours (mmol/L)	5.88 (5.5–6.3)	3.8 (3.4–4.1)	0.16
Max Lactate 12–24 hours (mmol/L)	5.16 (4.7–5.6)	3.8 (3.4–4.2)	0.22
Total Blood 0–12 hours (cc)	2870 (2550–3190)	2530 (2121–2939)	0.83
Total Crystalloid 0–12 hours (cc)	11877 (11167–12586)	11185 (10335–12034)	0.25
Outcomes			**p-value**
	N (%)	**N (%)**	
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/Rehabilitation	205 (59.4)	49 (30.1)	**<0.001**
Discharge - Long Term Care Facility	96 (27.8)	75 (47.1)	**0.003**
28-day Mortality	30 (8.7)	17 (17.6)	**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 (days)	19.1 (17.8–20.3)	18.9 (17.2–20.6)	0.87
ICU LOS (days)	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-day Mortality[1]		
Age ≥ 55 years old	2.41 (1.36–4.28)	**<0.004**	
Total Blood > 9.5 (U) 0–12 hours	2.16 (1.18–3.97)	**<0.011**	

Abbreviations: ISS, injury severity scale; NISS, new injury severity scale; BMI, body mass index; ED, emergency department; SBP, systolic blood pressure; COPD, chronic obstructive pulmonary disease; MOF, multiple-organ failure; LOS, length of stay; VAP, ventilator-associated pneumonia; TBI, traumatic brain injury; SNF, skilled nursing facility; AMA, against medical advice. *Note:* P-value considered significant at < 0.05 designated by values in bold.

[1]Model fit statistics: (AUC, c=0.775; AIC=1433; Likelihood Ratio Test, P<0.0001)

Table II

Gene 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 antigen
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), beta
18.1	9.6	Il1f9	interleukin 1 family, member 9
10.7	9.4	C5ar1	complement component 5a receptor 1
15.4	8.8	Tnfaip2	tumor necrosis factor, alpha-induced protein 2
6.2	5.4	Il10rb	interleukin 10 receptor, beta

Young mice (bold) are more likely to have greater upregulation (fold change compared to control) of genes related to innate immunity.

Table III

Fold change (versus control) expression BM HSCs genes one day after trauma

Young	Aged	Symbol	Name
Chemotaxis			
4.9	2.4	CCR2	Chemokine (C-C Motif) Receptor 2
Toll-like Receptors			
1.8	-1.3	TLR1	Toll-like receptor 1
Antigen Presentation			
−1.4	−2.7	H2-Eb	Histocompatibility 2, class II antigen E beta
−3.4	−3.7	H2-Ob	Histocompatibility 2, O region beta locus
−1.3	−5.2	H2-Aa	Histocompatibility 2, class II antigen A
−1.7	−2.9	H2-Aa	Histocompatibility 2, class II antigen A
−1.8	−3.4	H2-Aa	Histocompatibility 2, class II antigen A
Immunosuppression			
−1.4	10.4	Il10ra	Interleukin 10 receptor, alpha
Lymphoid Development			
−20.7	1.5	Ighg	Immunoglobulin heavy chain (gamma polypeptide)
−20.6	2.0	Ighg	Immunoglobulin heavy chain (gamma polypeptide
−2.3	−1.3	Cd96	CD96 antigen
−1.1	1.3	Il7	Interleukin 7

Up or down regulated BM HSCs genes in young (bold) and aged mice one day post PT. Positive values indicate upregulation and negative values mean downregulation. (BM-bone marrow; HSC-hematopoietic stem cells; PT-polytrauma)