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# Protective Immunity and Defects in the Neonatal and Elderly Immune Response to Sepsis

Lori F. Gentile,\* Dina C. Nacionales,\* M. Cecilia Lopez,<sup>†</sup> Erin Vanzant,\* Angela Cuenca,\* Alex G. Cuenca,\* Ricardo Ungaro,\* Ben E. Szpila,\* Shawn Larson,\* Anna Joseph,<sup>‡</sup> Frederick A. Moore,\* Christiaan Leeuwenburgh,<sup>‡</sup> Henry V. Baker,<sup>\*,†</sup> Lyle L. Moldawer,\* and Philip A. Efron\*

Populations encompassing extremes of age, including neonates and elderly, have greater mortality from sepsis. We propose that the increased mortality observed in the neonatal and elderly populations after sepsis is due to fundamental differences in host-protective immunity and is manifested at the level of the leukocyte transcriptome. Neonatal (5–7 d), young adult (6–12 wk), or elderly (20–24 mo) mice underwent a cecal slurry model of intra-abdominal sepsis. Both neonatal and elderly mice exhibited significantly greater mortality to sepsis ( $p < 0.05$ ). Neonates in particular exhibited significant attenuation of their inflammatory response ( $p < 0.05$ ), as well as reductions in cell recruitment and reactive oxygen species production (both  $p < 0.05$ ), all of which could be confirmed at the level of the leukocyte transcriptome. In contrast, elderly mice were also more susceptible to abdominal peritonitis, but this was associated with no significant differences in the magnitude of the inflammatory response, reduced bacterial killing ( $p < 0.05$ ), reduced early myeloid cell activation ( $p < 0.05$ ), and a persistent inflammatory response that failed to resolve. Interestingly, elderly mice expressed a persistent inflammatory and immunosuppressive response at the level of the leukocyte transcriptome, with failure to return to baseline by 3 d. This study reveals that neonatal and elderly mice have profoundly different responses to sepsis that are manifested at the level of their circulating leukocyte transcriptome, although the net result of increased mortality is similar. Considering these differences are fundamental aspects of the genomic response to sepsis, interventional therapies will require individualization based on the age of the population. *The Journal of Immunology*, 2014, 192: 3156–3165.

Despite the evolution in our understanding of the human response to sepsis, mortality from severe sepsis and organ failure has only modestly improved over the last several decades (1, 2). Additionally, populations encompassing the extremes of age, including neonates and the elderly, are more vulnerable (3). Neonatal sepsis is the leading cause of mortality in infants, with >1 million deaths per year worldwide (4). Likewise, 60% of septic patients are older than 65 y of age, and age is an independent risk factor for poor outcomes in sepsis (5, 6). The economic burdens of caring for these two groups of patients are not insignificant, as estimates place the annual cost of caring for septic neonatal and elderly patients in the United States at \$700 million and \$17 billion, respectively (5, 7).

The incidence of pediatric sepsis is highest in children <1 y of age, and the neonatal population, specifically premature and very low birth weight infants, is the most susceptible (4, 8). Despite improvements in the quality of neonatal intensive care, the mortality from neonatal sepsis over the last 20 y remains high, with infection being the leading cause of mortality in the first days of life (9, 10). Previously, we have shown that the neonatal murine population is more susceptible to sepsis than their young adult counterparts (11) and that neonatal mice have attenuated inflammatory responses following sepsis and functional defects in their peritoneal myeloid cell populations (12). Despite a modest understanding of the neonatal immune system, there are no currently approved immune-modulating therapies in use to date (8), and research continues to focus on immune responses in neonates, particularly of the innate immune system, as the search for therapeutic strategies continues.

The elderly population also has significantly greater morbidity and mortality following sepsis, and with >80% of adult deaths from sepsis occurring in those over the age of 65; sepsis is becoming known as a disease of the aged (3, 13). Likewise, the elderly are known to be in a state of immunosenescence, in which protective immunity is less able to mount an effective response to invading organisms than the young (14).

In this report, we explored the differences in host-protective immunity in response to sepsis in the neonatal and aged murine populations compared with juvenile or young, adult mice. We demonstrate that indeed there are fundamental differences in protective immunity that manifest themselves at the level of the leukocyte transcriptome and contribute to the increased neonatal and elderly mortality after sepsis.

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Abbreviations used in this article: CS, cecal slurry; IPA, Ingenuity Pathway Analysis; ROS, reactive oxygen species.

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## Materials and Methods

### Murine models

Male mice were purchased from either The Jackson Laboratory (Bar Harbor, ME) or the National Institute of Aging, or were bred at the University of Florida, Animal Care Services. Mixed gender neonatal C57BL/6J mice aged 5–7 d, or male mice age 6–12 wk (young adult), or 20–24 mo (elderly) were used in experiments approved by the University of Florida Institutional Animal Care and Use Committee. Mice were housed in pathogen-free facilities and acclimated at least 1 wk prior to use. Neonatal, young adult, and elderly mice underwent the cecal slurry (CS) model of intra-abdominal sepsis revised for mice (11) and were followed for survival. The CS model of polymicrobial sepsis is used in neonatal mice as the “gold standard.” In contrast, cecal ligation and puncture is technically challenging in the neonatal mouse due to its small size and risk for cannibalization by the mother after survival surgery. For a matter of uniformity, we chose to use the CS model of intra-abdominal sepsis across all three of the age groups studied. Briefly, when performing CS, cecal contents are harvested from adult C57BL/6J mice and suspended in 5% dextrose in water to make a CS at a concentration of 80 mg/ml, as previously published. A quantity amounting to 1.1 mg/g body weight was then injected i.p. Mouse whole blood was collected via intracardiac puncture 2 h, 1 d, and 3 d after injection. Blood was used for either complete blood count with differential determination, RNA isolation, or determination of plasma cytokine response, or plated on sheep's blood agar plates for bacterial count determination. The plasma cytokine and chemokine response was measured by multiplex Luminex assay (Austin, TX). Additional animals were followed for 7 d to judge long-term survival.

### Harvest of peritoneal cells

Peritoneal cell isolation from peritoneal lavage specimens was performed, as previously described (12). Peritoneal lavage samples from individual neonatal mice contain small numbers of cells, so peritoneal wash samples from three to five neonatal mice were pooled for analysis and considered to be one sample. Three to four peritoneal wash samples were collected per experiment. Erythrocytes were lysed with ammonium chloride lysis buffer and washed with PBS. Peritoneal bacterial counts were determined by culturing 100  $\mu$ l serially diluted peritoneal lavage sample of sheep's blood agar plates (Thermo Fisher Scientific) at 37°C. Plates were counted after 24 h of culture.

### Flow cytometry for characterization of cell phenotype

Single-cell suspensions were stained with anti-Ly6G allophycocyanin (BD Biosciences, Franklin Lakes, NJ), anti-CD11b PECy7 (BD Biosciences), and anti-F4/80 allophycocyanin (eBioscience, San Diego, CA). Sytox Blue (Invitrogen, Carlsbad, CA) was used for cell viability analysis. Samples were acquired and analyzed on a LSR II flow cytometer (BD Biosciences) and analyzed via FACSDiva (BD Biosciences) software. At least  $1 \times 10^4$  live cells (SYTOX Blue<sup>-</sup>; Invitrogen) were collected for analysis. The absolute numbers of innate immune effector cells were determined by multiplying the percentage of neutrophils (CD11b<sup>+</sup>, Ly6G<sup>+</sup>) and macrophages (CD11b<sup>+</sup>, Ly6G<sup>-</sup>, F4/80<sup>+</sup>) within the total sample population by the total sample cell number. In neonates, these were divided by the total number of mice in the pooled sample. Absolute numbers represent total macrophages or neutrophils per mouse.

### Functional analysis of peritoneal macrophages or neutrophils

To assay for reactive oxygen species (ROS) production, cell suspensions containing  $2 \times 10^6$  cells were stained for cell surface markers, washed with PBS, and then labeled with dihydrorhodamine 123 (Invitrogen) to determine ROS production. Cells were then stimulated with 1  $\mu$ M PMA (Sigma-Aldrich, St. Louis, MO) at 37°C, and aliquots were evaluated by flow cytometry every 10 min for 30 min using a LSR II flow cytometer (BD Biosciences). A minimum of  $1 \times 10^4$  live, nondebris cells was collected for analysis.

### Ex vivo whole-blood cytokine stimulation

Human anticoagulated whole blood was also obtained from three healthy control subjects, age range 35–41 y. Fresh neonatal cord blood samples were obtained from the New York Blood Center National Cord Blood Program (Long Island, NY). Both fresh adult and neonatal cord blood was stimulated ex vivo with 100 ng/ml LPS (ultrapure via ion exchange chromatography *Escherichia coli* O26:B6) (Sigma-Aldrich) in 12-well microtiter plates in triplicate and incubated at 37°C. After 18 h, the plasma was collected for cytokine/chemokine determination by multiplex Lumi-

nex assay. Blood sampling from healthy control subjects and the purchase of fresh cord blood were reviewed and approved by the Institutional Review Board at the University of Florida.

### Gene expression profiling

Leukocytes were isolated from whole blood by lysing erythrocytes with Buffer EL (Qiagen, Germantown, MD) and centrifugation. Cells were lysed with RLT Buffer (Qiagen), and genome-wide expression analysis was performed on 100 ng total RNA (RNeasy; Qiagen) that was labeled and hybridized to GeneChip Mouse Genome 430 2.0 Arrays (Affymetrix, Santa Clara, CA), according to the manufacturer's recommendations. A log<sub>2</sub>-transformed expression matrix was calculated using robust multiarray analysis as implemented in the Partek Genomic Suite 6.6, and gene expression patterns were compared between neonatal, young adult, and elderly mice following CS sepsis using sepsis-responsive genes that were significant at  $p < 0.001$  (F-test). Leave-one-out cross-validation was performed to compute the misclassification rate, and Monte Carlo simulation was used to determine whether the miscalculation rate was significantly better than predicted by chance. Once significant genes were identified, fold changes were calculated between each murine age group (neonate, young adult, and elderly) and a control group (neonate or adult).

Functional pathway analysis was performed using Ingenuity Pathway Analysis (IPA, Redwood City, CA). IPA Systems performs functional pathways analysis as part of its tools available to researchers. IPA identifies those pathways that are overrepresented, indicating that their expression is affected by the intervention. Significance was determined using a Z score. Values of  $2 < Z < -2$  are considered significant and correspond to a 95% confidence interval.

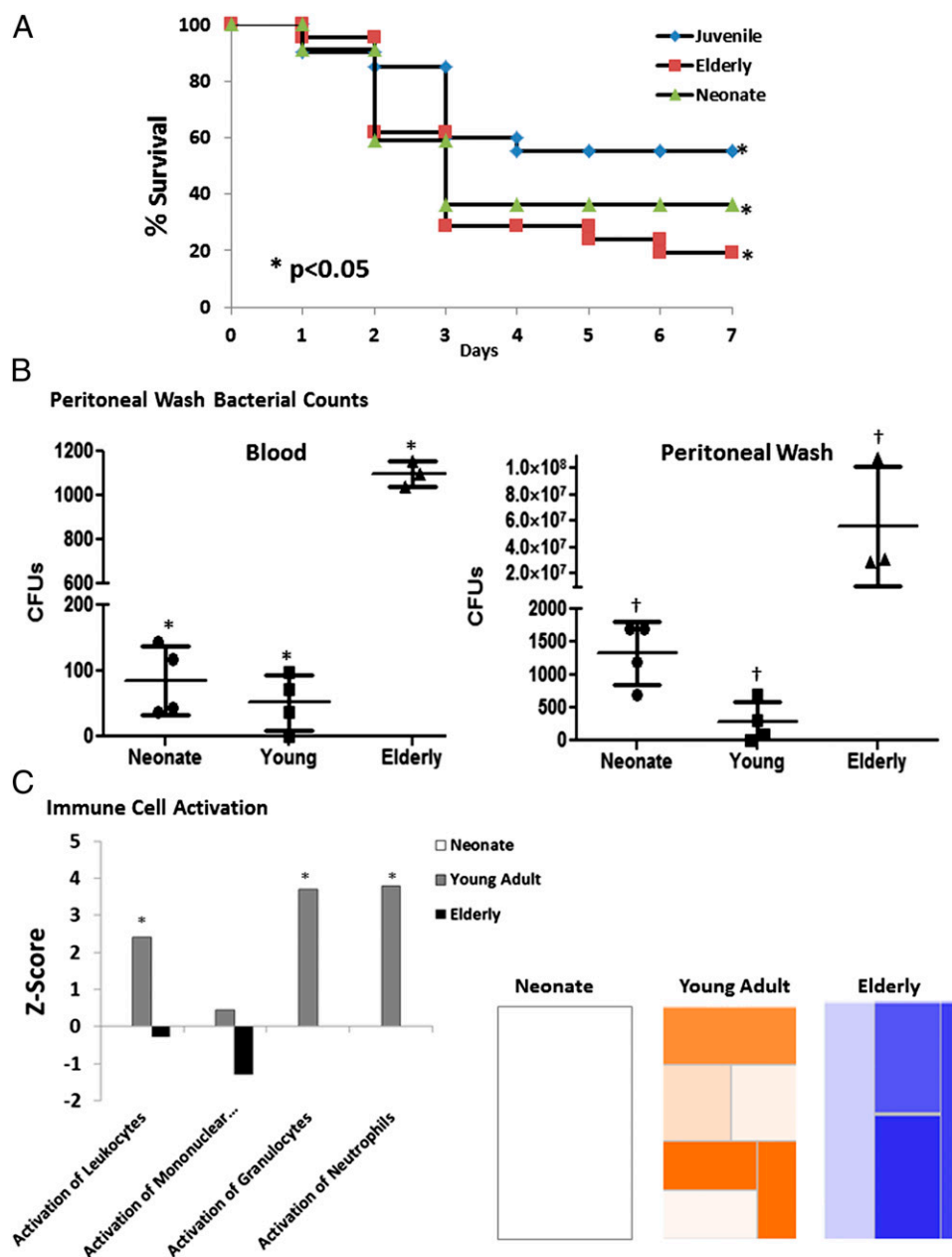
### Additional statistical analysis

Continuous nongenomic variables were tested for normality and equality of variances. Differences in survival were determined by Fisher's exact test. Differences among groups were evaluated by either one-way ANOVA with Dunn's or Tukey post hoc analysis, two-way ANOVA with Bonferroni's post hoc analysis, or Student *t* test. Significance was determined at the 95% confidence level.

## Results

### Elderly and neonatal mice have impaired ability to clear bacteria from the peritoneum and an inability to activate immune cells after CS, leading to increased mortality compared with young adult mice

Murine studies have demonstrated that neonatal and elderly mice are more susceptible to the same insult of polymicrobial abdominal sepsis compared with their young adult counterparts (11, 15), but a direct comparison between neonates and the elderly is lacking. We simultaneously evaluated survival in neonatal, young adult, and elderly mice using an i.p. CS dose of 1.1 mg/g, known to produce a LD<sub>30–40</sub> lethality in young adult mice. As expected, both neonatal and elderly mice had increased mortality compared with young adult mice with the same insult (Fig. 1A) ( $p < 0.05$ ). The elderly mice had the greatest susceptibility, with 1.8 times greater mortality than young adult mice, followed by neonatal mice, which had a 1.4 times greater mortality. To confirm that this was due to a failure of protective immunity, we examined bacterial counts in peritoneal washes 1 d after CS sepsis, and we found that elderly mice had significant impairments in the ability to clear bacteria from the site of infection compared with juvenile mice, followed by neonatal mice ( $p < 0.0001$ , one-way ANOVA) (Fig. 1B). These findings were substantiated at the level of the leukocyte transcriptome, as elderly mice actually had downregulation of genes involved in the activation of leukocytes, whereas young adult mice had significant upregulation of these same genes (Fig. 1C). Interestingly, neonatal mice completely failed to exhibit any significant change in a number of genes involved in leukocyte activation at a significance level of  $p < 0.001$ , thus confirming their attenuated immune response (Fig. 1C).

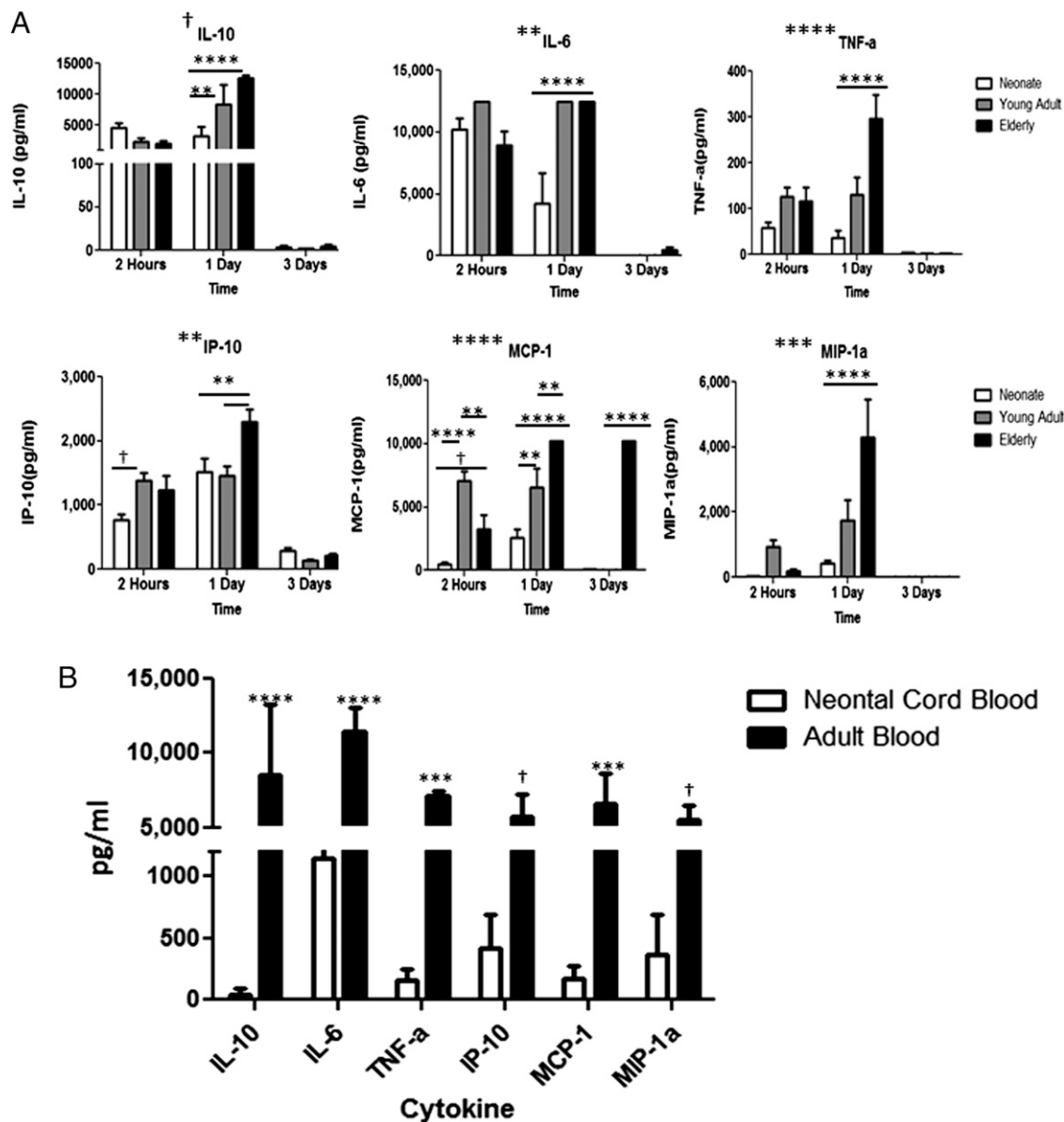


**FIGURE 1.** Neonatal and elderly mice have increased mortality compared with young adult mice with impaired control of bacteria at the site of infection and decreased activation of leukocytes. **(A)** Neonatal and elderly mice had increased mortality after CS sepsis compared with young adult mice with the same insult ( $*p < 0.05$ ) over 7 d. Neonate ( $n = 22$ ), young adult ( $n = 20$ ), elderly ( $n = 21$ ). **(B)** One day after sepsis, both neonatal and elderly mice had significantly increased bacterial counts in the blood ( $*p < 0.0001$ , one-way ANOVA) and in the peritoneum ( $^{\dagger}p = 0.019$ , one-way ANOVA), with elderly mice having the most severe impairments in clearing bacteria (blood,  $*p < 0.001$ ; peritoneal wash,  $^{\dagger}p < 0.05$ ; Tukey's multiple comparisons post hoc test) ( $n = 3-4$ ; results shown are from two or more independent experiments). **(C)** Functional pathway analysis showed that elderly mice had downregulation of genes involved in the activation of leukocytes, whereas young adult mice had significant upregulation of these same genes. Neonatal mice neither upregulated nor downregulated a large enough number of genes involved in leukocyte activation at a significance level of  $p < 0.001$ . Heat maps show the gene expression of the functional category Immune Cell Trafficking-Activation 1 d after sepsis in neonatal, young adult, and elderly mice. Orange represents pathways with an overexpression of genes leading to the activation of the pathway, whereas blue represents pathways with an overexpression of genes whose activation will lead to downregulation of the pathway. White represents genes that are neither upregulated or downregulated to significance level of  $p < 0.001$ . Graph shows the various subcategories and their corresponding significance level (Z score) of upregulation or downregulation. From left to right, categories include Activation of Leukocytes, Activation of mononuclear Leukocytes, Activation of Granulocytes, and Activation of Neutrophils ( $*Z$  score  $> 2$ ). See Supplemental Table II for a complete list of genes in presented pathways.

*Significant differences in the phenotypic and functional responses by the neonatal and elderly mouse to an identical sepsis challenge*

Although both neonatal and elderly mice have increased mortality compared with young adult mice, the mechanisms of this increased mortality, as exhibited both functionally, and at the level of the

leukocyte transcriptome, are unique. For example, when examining the magnitude of the inflammatory response to a similar sepsis challenge using the plasma cytokine response, we found that 1 d after sepsis neonatal mice had significantly attenuated production of the inflammatory cytokines IL-10, IL-6, and TNF- $\alpha$  ( $p < 0.01$ ) (Fig. 2A) and had significantly decreased production of chemo-



**FIGURE 2.** Inflammatory and anti-inflammatory chemokine and cytokine responses. **(A)** Neonatal, young adult, and elderly mice have significant differences in the production of inflammatory and anti-inflammatory cytokine and chemokine responses across all time points after CS sepsis. Significance determined using two-way ANOVA with Bonferroni multiple comparisons post hoc test. Symbols placed adjacent to graph titles indicate that age was a significant interaction accounting for a source of variation. Symbols placed within the graph show all significant differences between the age groups for specific cytokine.  $^{\dagger}p < 0.05$ ,  $^{**}p < 0.01$ ,  $^{***}p < 0.001$ ,  $^{****}p < 0.0001$ . **(B)** Neonatal cord blood had an attenuated cytokine response compared with adult blood following ex vivo stimulation with LPS across all cytokines studied.  $^{\dagger}p < 0.05$ ,  $^{***}p < 0.001$ ,  $^{****}p < 0.0001$ .

kines MCP-1 and MIP-1 $\alpha$  at all time points after sepsis ( $p < 0.0001$ ) (Fig. 2A). Similar results were seen when human cord blood was stimulated ex vivo with LPS and compared with LPS-stimulated whole blood from young adults. Proinflammatory cytokine production was dramatically attenuated in the cord blood (Fig. 2B).

Similarly, we found that recruitment of neonatal peritoneal mouse CD11b $^{+}$ Ly6G $^{+}$  neutrophils and CD11b $^{+}$ Ly6G $^{-}$  F480 $^{+}$  macrophages to the peritoneum was significantly diminished ( $p = 0.001$ , Tukey's multiple comparison) (Fig. 3), with the recruited cells having decreased ability to produce ROS compared with adult mice 1 d after sepsis ( $p < 0.0001$ , two-way ANOVA) (Fig. 4A).

In contrast, the response by the elderly mice was fundamentally different. First, their early inflammatory cytokine response was not different from seen in juvenile or young adult animals (Fig. 2). We

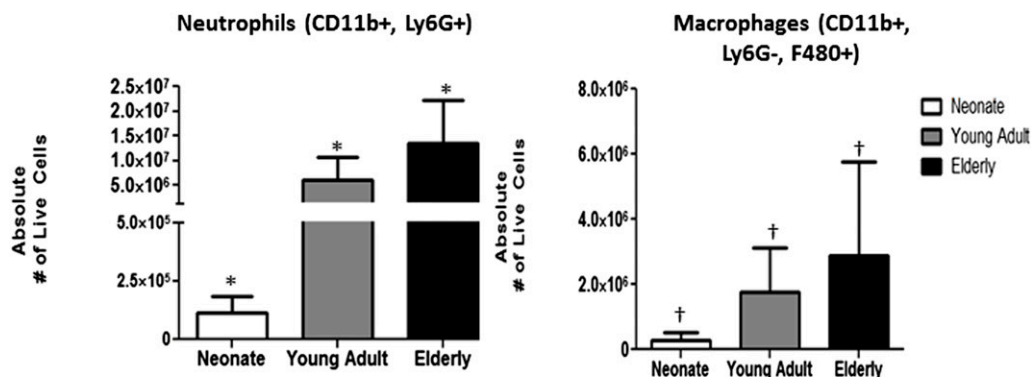
also examined the absolute numbers of CD11b $^{+}$ Ly6G $^{+}$  neutrophils and CD11b $^{+}$ Ly6G $^{-}$  F4/80 $^{+}$  macrophages found in the peritoneum 1 d after sepsis and found that elderly mice had an increased ability to recruit innate immune effector cells to the peritoneum, whereas neonatal mice had a significantly decreased ability to recruit these same cells ( $p < 0.0001$ ,  $p = 0.001$ , one-way ANOVA) (Fig. 3).

#### *Differences in baseline gene expression compared among healthy neonate, young adult, or elderly mice*

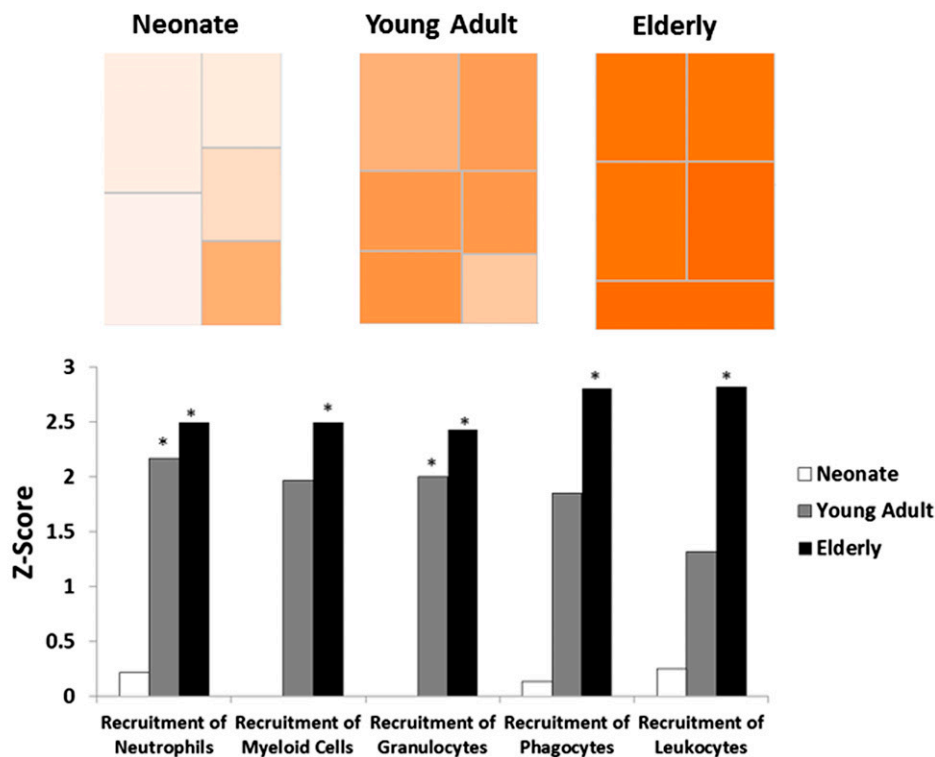
We compared the baseline gene expression of naive neonatal, young adult, and elderly mice to help determine our control populations for genomic comparison after sepsis. Surprisingly, we found that expression patterns from healthy, young adult, and elderly naive mice could not be distinguished from one another by cluster analysis using Pearson correlations (data not shown), de-



### A Peritoneal Cell Numbers



### B Cell Recruitment

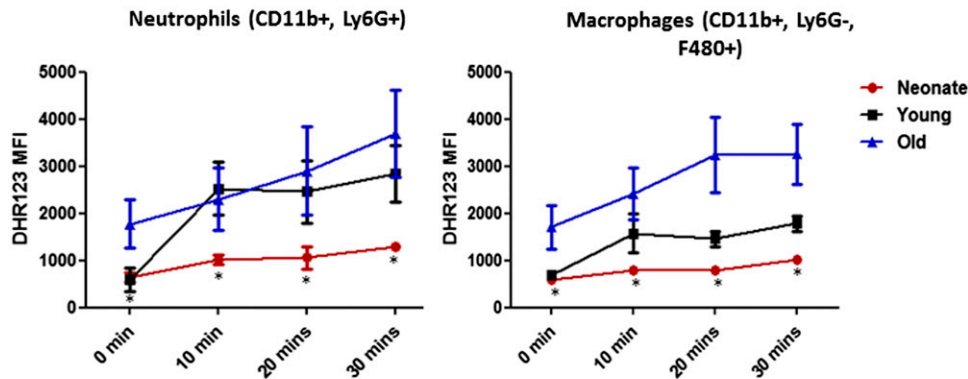
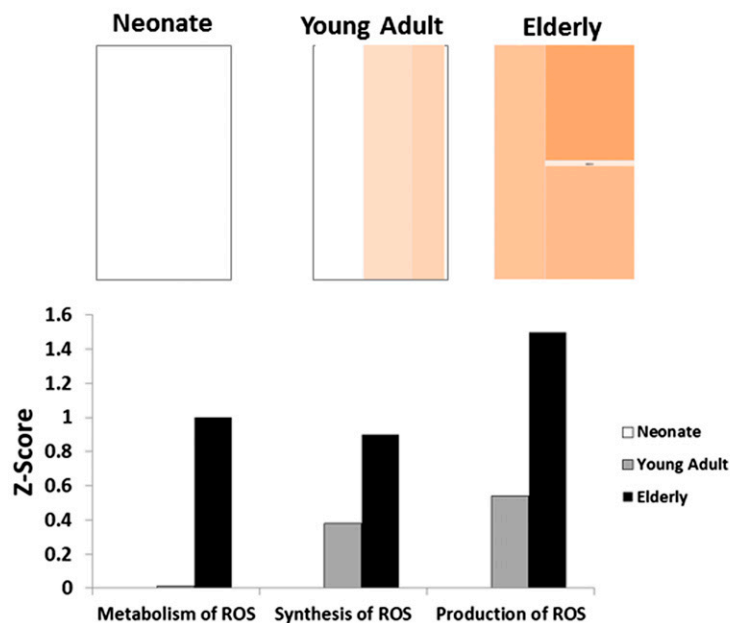


**FIGURE 3.** Elderly mice have increased ability to recruit innate immune effector cells to the peritoneum, whereas neonatal mice have a significantly decreased ability to recruit those same cells. **(A)** Elderly mice have increased absolute numbers of neutrophils (CD11b<sup>+</sup>, Ly6G<sup>+</sup>) (\* $p < 0.0001$ ; one-way ANOVA) and macrophages (\* $p < 0.05$ ; one-way ANOVA) (CD11b<sup>+</sup>, Ly6G<sup>-</sup>, F480<sup>+</sup>) in the peritoneum via flow cytometry 1 d after sepsis, whereas neonates had significantly decreased numbers ( $n = 7-8$ ) ( $p = 0.001$ , Tukey's multiple comparisons). Data shown are from two or more independent experiments. **(B)** IPA functional pathway analysis revealed that elderly mice have significantly more upregulation of pathways involved in the recruitment of neutrophils, myeloid cells, and phagocytes, whereas neonates have minimal upregulation of these same pathways (\*Z score  $> 2$ ). Heat maps show the gene expression of the functional category Immune Cell Trafficking-Recruitment 24 h following sepsis. Orange represents pathways with an overexpression of genes leading to the activation of the pathway, whereas white represents genes that are neither upregulated nor downregulated to significance level of  $p < 0.001$ .

spite significant differences in baseline neutrophil and lymphocyte differential counts ( $p < 0.05$ ) (Supplemental Fig. 1A). Therefore, it was concluded that the leukocyte transcriptome from healthy elderly and young adult mice could be combined and used as a single control group for comparison with the septic elderly and young adult mice. However, when we compared the transcriptome from healthy neonatal mice with healthy adult mice, we found that there were 5798 probe sets representing 3987 genes differentially expressed between neonatal and adult mice that were significant at  $p < 0.001$ . In addition, the overall pattern of gene expression was significantly different as determined by leave-one-out cross-validation (Fig. 5A). This difference was unlikely to be caused

by the murine WBC differential counts, as neonatal WBC differential counts were not significantly different from in young adult or elderly mice. Additionally, when examining the differential make-up of the circulating leukocytes in response to sepsis, it is easy to see that the transcriptomic differences cannot be easily explained by differences or similarities in the prevalent type of circulating leukocyte after injury (Supplemental Fig. 1B).

We examined individual genes that were significantly different between neonatal and healthy adult mice at  $p < 0.001$ . We found that healthy neonatal mice have decreased expression of genes involved in adaptive immunity, including *MhcII*, *Cd40*, and *Btla*, as well as genes involved in inflammation, such as *Il1B* and *Arg2*,

**A ROS Production****B Free Radical Scavenging**

**FIGURE 4.** Neonatal neutrophils and macrophages have decreased ROS production compared with adult mice. **(A)** Neonatal mouse neutrophils (CD11b<sup>+</sup>, Ly6G<sup>+</sup>) and macrophages (CD11b<sup>+</sup>, Ly6G<sup>-</sup>, F480<sup>+</sup>) 1 d after sepsis had decreased ability to produce ROS in response to PMA stimulation compared with adult mice 1 d after sepsis ( $p < 0.0001$ ; two-way ANOVA). **(B)** Functional pathway analysis showed that elderly mice had increased upregulation of genes involved in the synthesis, production, and metabolism of ROS compared with neonatal and/or young adult mice, which is consistent with in vivo results. Heat maps show the gene expression of the functional category Free Radical Scavenging 2 h after sepsis in neonatal, young adult, and elderly mice. Orange represents pathways with an overexpression of genes leading to the activation of the pathway, and white represents genes that are neither upregulated nor downregulated to significance level of  $p < 0.001$ . Graph shows the various subcategories and their corresponding significance level (Z score) of upregulation or downregulation.  $*p < 0.001$ .

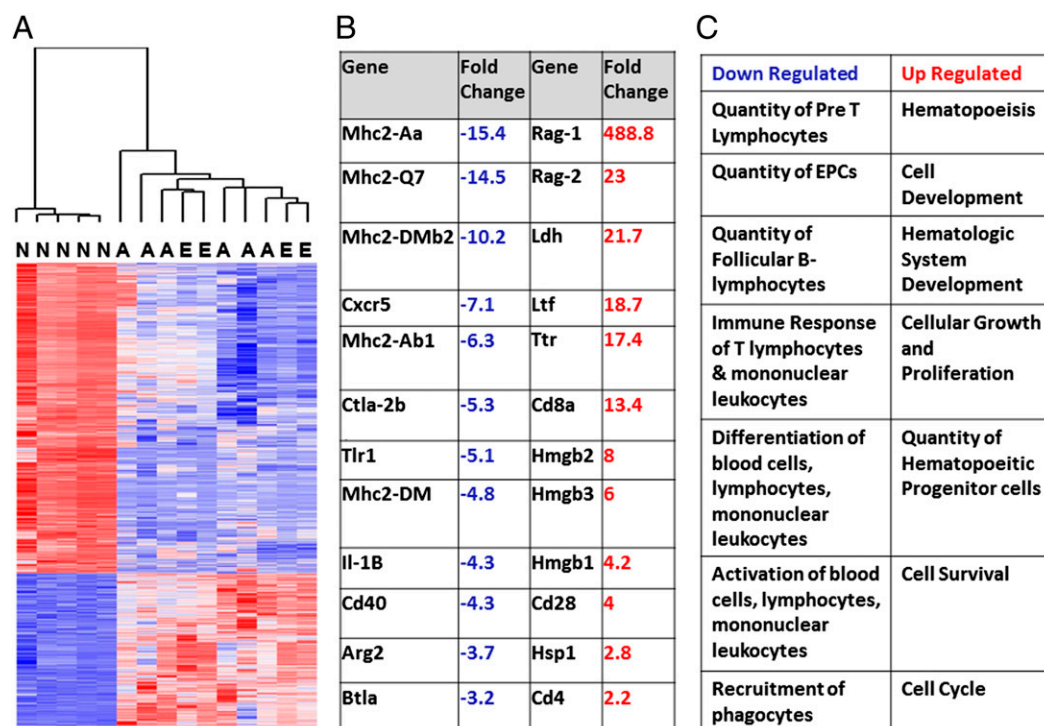
which were significantly downregulated compared with adult controls (Fig. 5B). The two most highly expressed genes in the naive neonatal mouse compared with adults were *Rag1* and *Rag2*, which are only expressed in developing lymphocytes, and are indicative that the neonatal adaptive immune system is most likely in an active state of development.

We then performed functional analysis on the leukocyte transcriptome from healthy mice using IPA and examined the most highly expressed categories of genes. We found that leukocytes from healthy neonatal mice had overrepresented pathways involving hematopoiesis, hematologic systems development, cellular growth, and proliferation, as well as cellular development, which were all significantly upregulated compared with naive adults (Z score  $> 2$ ) (Fig. 5C). We also found that neonatal mice had

significant downregulation of genes in pathways involved with the quantities, differentiation, and activation of immune cells (Z  $< -2$ ) (Fig. 5C).

#### *Phenotypic changes are manifested at the level of the blood leukocyte transcriptome*

We compared the genomic responses between neonatal, young adult, elderly, and control mice at each individual time point. There were 10,876, 7,231, and 11,539 probe sets, representing 7,012, 4,990, and 7,479 unique genes whose expression changed between neonates, young adults, and elderly mice at 2 h, 1 d, and 3 d, respectively ( $p < 0.001$ ). Besides having significant differences in baseline gene expression compared with their adult counterparts (Fig. 5), neonatal mice failed to upregulate many genes important



**FIGURE 5.** The neonatal transcriptome is fundamentally different at baseline compared with the murine adult. **(A)** Heat map shows the gene expression patterns of naive neonatal (N), young adult (A), and elderly (E) control mice. Gene expression patterns from young adult and elderly mice could not be differentiated genomically at baseline by an unsupervised analysis. There were 5798 probe sets (representing 3987 genes) differentially expressed between neonatal and adult mice (including young adult and elderly) that were significant at  $p < 0.001$ . The overall pattern of gene expression was significantly different as determined by leave-one-out cross-validation. **(B)** Fold changes of selected immune-related genes from neonatal control mice compared with adult control mice. Neonatal mice have increased suppression of genes involved in adaptive immunity, including MHC II, and decreased expression of genes involving innate immunity and inflammation ( $p < 0.001$ ). **(C)** Categories of functional pathways containing genes that are either upregulated or downregulated in naive neonatal control mice as compared with adult control mice from IPA.

in both the innate and adaptive immune responses and remained in a predominantly downregulated state throughout the entire time course (Supplemental Fig. 2, Supplemental Table I). The same was true of elderly mice; however, it occurs in a delayed fashion and to a lesser extent.

When reviewing the global gene expression patterns of the neonatal response to polymicrobial sepsis over time compared with the adult response, one can see that, at 2 h following sepsis, neonatal mice only upregulate a very small portion of their genome compared with the patterns of adult mice over time, with only 1183 probe sets representing 884 unique genes that are significantly altered following sepsis to >4893 and 4520 unique genes that are changed in the young adult and elderly populations, respectively (Supplemental Fig. 2). Over the time course of sepsis, neonatal mice remain in a mostly unstimulated state, appearing more similar to control mice with minimal upregulation of genes important in the immune response associated with reduced survival (Supplemental Table I). Thus, the neonatal response to sepsis is markedly attenuated and remains unaffected over time compared with both young adult and elderly animals. These results mirrored the results from the IPA functional pathway analysis in which we found that elderly mice had increased upregulation of genes involved in the synthesis, production, and metabolism of ROS compared with neonatal or young adult mice (Fig. 4B).

Functional pathway analysis 24 h after sepsis revealed that neonatal and elderly mice fail to significantly upregulate immune-related functional pathways 24 h after sepsis. In fact, the functional analysis at this time point revealed that neonatal mice have significantly downregulated genes in pathways having to do with proliferation of immune cells; quantity of T lymphocytes; devel-

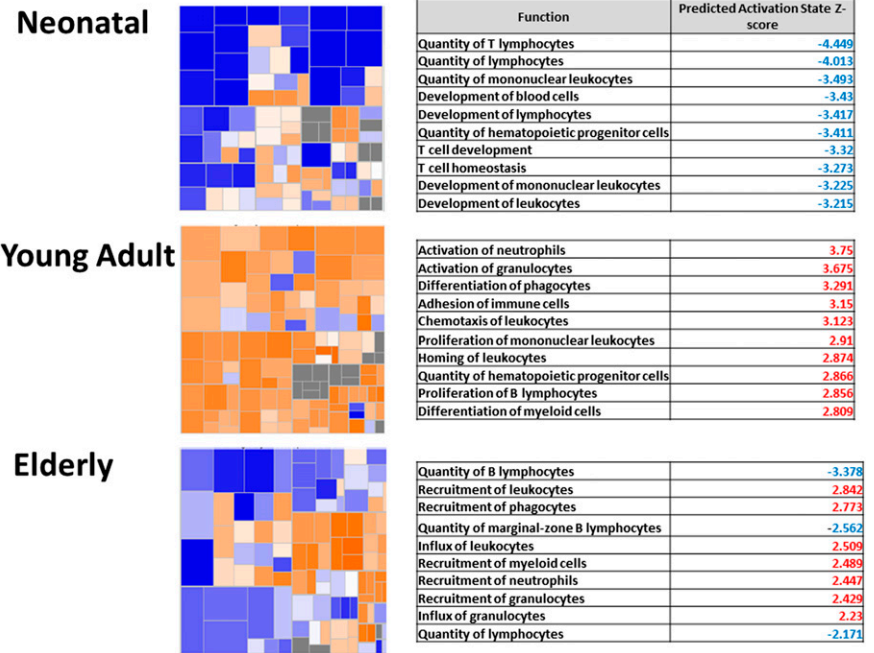
opment of blood cells, leukocytes, and mononuclear leukocytes; and the proliferation of immune-related cells (Z score < -2) (Fig. 6). Alternatively, young adult mice upregulate pathways involved in activation of leukocytes, cell movement and migration of cells, chemotaxis, phagocytosis, and cell homing (Z score > 2), which are not significantly upregulated or downregulated in either neonates or elderly mice (Fig. 6). Although elderly mice do upregulate some immune-related pathways such as recruitment and quantities of phagocytes and neutrophils, they do not upregulate or have downregulated pathways involving immune cell activation, chemotaxis, and leukocyte homing.

This functional genomic data at the level of the leukocyte transcriptome mirror the results we found when performing functional experiments in vivo. First, we examined the absolute numbers of CD11b<sup>+</sup>Ly6G<sup>+</sup>neutrophils and CD11b<sup>+</sup>Ly6G<sup>+</sup>F4/80<sup>+</sup> macrophages found in the peritoneum 1 d after sepsis and found that elderly mice had an increased ability to recruit innate immune effector cells to the peritoneum, whereas neonatal mice had a significantly decreased ability to recruit these same cells ( $p < 0.0001$ ,  $p < 0.05$ , one-way ANOVA) (Fig. 3A). Likewise, IPA functional pathway analysis revealed that, indeed, elderly mice have significantly more upregulation of pathways involved in the recruitment of neutrophils, myeloid cells, and phagocytes (Z-score > 2), whereas neonates have minimal upregulation of these same pathways (Fig. 3B).

Additionally, elderly mice have gene expression changes consistent with the downregulation of pathways involved with the quantity and function of the adaptive immune system, with specific increased downregulation of humoral immune-related pathways involving decreased quantities of B lymphocytes, IgG, IgM, and Ig (Z score < -2).



**FIGURE 6.** Neonatal and elderly mice do not upregulate important immune-related pathways 24 h following sepsis. Heat maps show the gene expression of the functional category Hematological Systems Development and Function 24 h following sepsis. Orange represents pathways with an overexpression of genes leading to the activation of the pathway, whereas blue represents pathways with an overexpression of genes whose activation will lead to downregulation of the pathway. Neonatal mice have a large proportion of their pathways that are predominantly downregulated 24 h after sepsis, and the majority of young adult pathways are upregulated. Elderly mice fall in between. The corresponding tables show the top 10 pathways and their corresponding Z score within the functional category 24 h after sepsis. Red indicates that the pathway is significantly up-regulated, whereas blue indicates that the pathway is significantly downregulated.



Three days following sepsis, functional analysis in surviving animals reveals that both neonatal and young adult mice have gene expression patterns consistent with the downregulation of immune-related pathways, including T cell development and homeostasis and quantity and differentiation of leukocytes ( $Z$  score  $< 2$ ), whereas the gene expression of elderly mice 3 d after sepsis is more consistent with the upregulation of pathways involved with cell movement and homing, recruitment of myeloid cells, granulocytes, neutrophil transcription, proliferation of blood cells, and the quantities of leukocytes and HSCs ( $Z$  score  $> 2$ ); hence, it may be the continued expression of inflammatory mediators that ultimately leads to their increased mortality.

Discussion

We can confirm using a murine model of polymicrobial sepsis that both the neonate and the elderly fare poorly when compared with the young adult. To our knowledge, this is the first report comparing the functional and genomic responses to the exact same model of sepsis in the neonate and the elderly, two populations who experience increased mortality. At the most fundamental level of the leukocyte transcriptome, the genomic response to polymicrobial sepsis is markedly different between the aged and the neonatal mouse, despite a similar increased mortality response.

In this study, we have demonstrated that the genomic expression of leukocytes from healthy, neonatal mice is fundamentally different from that of adult mice, thus likely a strong contributing factor to the neonate's subsequent dysfunctional response to polymicrobial sepsis. We have also shown that, despite aged mice having increased mortality from sepsis, there are minimal gene expression differences at baseline that can help distinguish them, thus indicating that there is something inherent within the elderly murine host response to sepsis that is responsible for their increased mortality. Regardless, both the neonatal and the elderly murine genomic response to sepsis are strikingly different from the young adult mouse, and this response is associated with adverse outcomes. This difference in genomic response is not a result of the differences in the WBC differential counts (Supplemental Fig. 1). In naive animals, elderly mice have a higher percentage of neutrophils and a lower percentage of lymphocytes than young adult

mice; however, there are no differences between neonatal mice and young or elderly mice, which is where there are significant genomic differences. Additionally, the differences in baseline neutrophil and lymphocyte percentages between elderly and young adult mice are significant ( $p < 0.05$ ) (Supplemental Fig. 1A), but these two groups of mice were unable to be distinguished from each other genomically (Fig. 5).

Mortality from sepsis remains a significant health care problem. For the neonatal population, the mortality from sepsis is highest in very low birth weight infants who were born prematurely and has remained relatively stable throughout the years, despite improvements in neonatal intensive care and evolution of ventilatory strategies (4, 8). It is well known that immunity in neonates is distinct from that in adults, and it has been shown that, in mice, the adaptive immune system does not play a protective role in neonatal sepsis, and, instead, neonatal mice rely on the innate immune system for protection from severe infection (12). The neonatal innate immune response has been found to be dysfunctional or immature as studies have found murine neonates to have impaired phagocytic ability of innate immune effector cells and poor production of neutrophil extracellular traps compared with adults (12, 16, 17). Additionally, genomic studies that examine the response to sepsis in neonates through school-aged children have found that neonates have impaired innate immunity compared with older children (18).

For the elderly population, the incidence of sepsis continues to increase, right along with the increase in the aging population, and mortality remains high (3). For years, it was thought that the elderly's defense against pathogens was compromised mainly because of changes in the adaptive immune system mediated by defective T and B lymphocytes; however, it has since been discovered that aging has a profound impact on innate immunity as well (19), with a chronic proinflammatory state as well as a predilection toward myelopoiesis. One can easily see the common thread underlying the immune-related deficits in both the neonatal and elderly populations, and, similar to the neonatal murine population, it has been shown that elderly mice have a higher mortality than young adult mice following polymicrobial sepsis (14, 19, 20), and current evidence for the mechanism of this increased mortality is limited.

There have been conflicting reports implicating the role of serum cytokines, with some studies citing increased levels of serum cytokines as being responsible for the elderly's higher mortality following sepsis (3), whereas others found a lack of cytokine upregulation following infection (21, 22). Also, it is known that elderly mice have a predisposition toward myeloid responses following an infectious insult (23); however, these myeloid cells have been shown to exhibit phenotypic differences from those found in young adult mice (24–26). This was shown in our functional analysis of the elderly response to sepsis when 24 h after sepsis elderly mice exhibited significant upregulation of pathways involving the recruitment and quantities of myeloid cells; however, they did not significantly upregulate or had downregulation of pathways leading to their chemotaxis, homing, or activation, all of which were highly expressed in the young adult mice ( $Z$  score  $> 2$ ). Similarly, we have shown in vivo that elderly mice preserve the ability to recruit myeloid cells to the peritoneum after sepsis; however, they fail to activate those cells, leading to the inability to clear bacteria from the site of infection, most likely contributing to their increased mortality.

From a functional standpoint, at baseline, neonatal mice upregulate such systems as Hematologic Systems Development, Cellular Development, Growth and Proliferation, Hematopoiesis, and Tissue Morphology, as would be expected ( $Z$  score  $> 2$ ). They have downregulation of systems involved with cell death and organismal survival along with decreased quantities of both B and T lymphocytes ( $Z$  score  $< -2$ ). This baseline difference is not surprising as neonates are in a transition period from a state in utero, where they need a less reactive immune system to not mount a response against the mother's Ags, to an independent, fully functional immune system after they are born. Some evidence of this is in murine mice aged 5–7 d, in which the splenic architecture is different from that of the adult spleen. We have found that the neonatal spleen had poor marginal zone development and increased numbers of myeloid cells, and it resembles more of a hematopoietic organ as it does in fetal life rather than a clearance organ as it is in adult life (11). The age that the “switch” from a more neonatal type spleen to a more adult type spleen occurs is currently unknown and will be key in developing appropriate therapies to modulate the neonatal immune system so that it responds appropriately to an infectious insult.

Although murine neonates do appear to upregulate some important inflammatory genes after sepsis (i.e., CXCL10, TNF- $\alpha$ -related genes), they do so to a much lesser extent than adult mice (Supplemental Table I). These findings were also evident when we performed ex vivo whole blood stimulation of human adult and neonatal cord blood with endotoxin. We found that the neonatal cord blood inflammatory cytokine and chemokine responses were markedly attenuated compared with stimulated adult blood ( $p < 0.001$ ) (Fig. 2B). These findings are also in accordance with human genomic data published that showed that, compared with school-aged children, neonates had a larger number of downregulated genes following sepsis compared with healthy neonatal controls (18). Similarly, 2 h after polymicrobial sepsis, 63% of the genome is downregulated and significantly different from adults ( $p < 0.05$ ) in neonatal mice. Additionally, although some individual genes may be upregulated in response to sepsis, both functional in vivo and genomic analysis reveals that, overall, neonates do not generate an immune response great enough to significantly upregulate important immune-related pathways after sepsis, leading to decreased immune cell recruitment, activation, and function. In fact, a majority of their immune-related pathways remain in a significantly downregulated state (Fig. 6).

The elderly mice also exhibit a suboptimal response to CS sepsis, although the pattern is quite different from the suboptimal response found in the neonatal population. Like neonates, the overall genomic response is attenuated compared with young adult mice. However, although elderly mice do preserve the ability to recruit myeloid cells to the peritoneum, they do not fully activate these cells and they remain partially dysfunctional. Additionally, elderly mice do not appear to be as able to return toward baseline levels of gene expression as is observed in young adult mice and have a significant number of inflammatory genes whose expression remains increased to a greater extent than young adult mice 3 d after sepsis (Supplemental Table I). This elderly murine response with an inability to return to baseline is similar to published data from the Glue Grant “Inflammation and Host Response to Injury Large Scale Collaborative Research Program,” in which we reported that patients with complicated outcomes exhibited prolonged, altered gene expression and a failure to return to baseline gene expression levels within 28 d (27).

In summary, the murine response to polymicrobial sepsis varies greatly over the spectrum of age from the neonatal period through young adulthood, and into the elderly population. Both neonatal and elderly mice have increased mortality following a murine model of polymicrobial sepsis; however, the underlying mechanisms for this increased mortality appear to be fundamentally distinct when examining their transcriptomic responses to sepsis. The neonatal transcriptome appears to be distinct from the adult transcriptome not only at baseline, where adaptive immune gene expression is suppressed, but also in response to polymicrobial sepsis, where their innate immune responses are markedly attenuated. Elderly mice exhibit a dysfunctional response to sepsis as well, also characterized by attenuated innate inflammatory response with continued systemic inflammation and adaptive immune suppression, with the inability to return toward baseline homeostasis. Considering these genomic differences, sepsis therapies aimed at the neonatal and elderly populations will have to overcome many hurdles.

## Disclosures

The authors have no financial conflicts of interest.

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