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Neonatal CD71+ Erythroid Cells Do Not Modify Murine Sepsis Mortality

James L. Wynn,* Philip O. Scumpia,† Blair T. Stocks,‡ Joann Romano-Keeler,* Mhd Wael Alrifai,* Jin-Hua Liu,*, Annette S. Kim,‡ Catherine E. Alford,§ Pranathi Matta,*, Jörn-Hendrik Weitkamp,*, and Daniel J. Moore*‡∫

Sepsis is a major cause of neonatal mortality and morbidity worldwide. A recent report suggested that murine neonatal host defense against infection could be compromised by immunosuppressive CD71+ erythroid splenocytes. We examined the impact of CD71+ erythroid splenocytes on murine neonatal mortality to endotoxin challenge or polymicrobial sepsis and characterized circulating CD71+ erythroid (CD235a+) cells in human neonates. Adoptive transfer or an Ab-mediated reduction in neonatal CD71+ erythroid splenocytes did not alter murine neonatal survival to endotoxin challenge or polymicrobial sepsis challenge. Ex vivo immunosuppression of stimulated adult CD11b+ cells was not limited to neonatal splenocytes; it also occurred with adult and neonatal bone marrow. Animals treated with anti-CD71 Ab showed reduced splenic bacterial load following bacterial challenge compared with isotype-treated mice. However, adoptive transfer of enriched CD71+ erythroid splenocytes to CD71+-reduced animals did not reduce bacterial clearance. Human CD71+CD235a+ cells were common among cord blood mononuclear cells and were shown to be reticulocytes. In summary, a lack of effect on murine survival to polymicrobial sepsis following adoptive transfer or diminution of CD71+ erythroid splenocytes under these experimental conditions suggests that the impact of these cells on neonatal infection risk and progression may be limited. An unanticipated immune priming effect of anti-CD71 Ab treatment, rather than a reduction in immunosuppressive CD71+ erythroid splenocytes, was likely responsible for the reported enhanced bacterial clearance. In humans, the well-described rapid decrease in circulating reticulocytes after birth suggests that they may have a limited role in reducing inflammation secondary to microbial colonization.

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; CBMC, cord blood mononuclear cell; HKLM, heat-killed Listeria monocytogenes; LD, lethal dose; MFI, mean fluorescence intensity; nRBC, nucleated RBC.

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Neonatal sepsis remains a significant global threat and kills >1 million newborns each year (1). In developed countries, 60% of the most prematurely born neonates develop sepsis (2). Death or major disability occurs in 4 of every 10 septic neonates, even with antimicrobial treatment (3). Despite multiple attempts over the last three decades to improve survival, 10 septic neonates, even with antimicrobial treatment (3). Despite multiple attempts over the last three decades to improve survival, 10 septic neonates, even with antimicrobial treatment (3). Despite multiple attempts over the last three decades to improve survival, 10 septic neonates, even with antimicrobial treatment (3). Despite multiple attempts over the last three decades to improve survival, 10 septic neonates, even with antimicrobial treatment (3). Despite multiple attempts over the last three decades to improve survival, 10 septic neonates, even with antimicrobial treatment (3). Despite multiple attempts over the last three decades to improve survival, 10 septic neonates, even with antimicrobial treatment (3). Despite multiple attempts over the last three decades to improve survival, 10 septic neonates, even with antimicrobial treatment (3). Despite multiple attempts over the last three decades to improve survival, 10 septic neonates, even with antimicrobial treatment (3). Despite multiple attempts over the last three decades to improve survival, 10 septic neonates, even with antimicrobial treatment (3). Despite multiple attempts over the last three decades to improve survival, 10 septic neonates, even with antimicrobial treatment (3). Despite multiple attempts over the last three decades to improve survival, 10 septic neonates, even with antimicrobial treatment (3). Despite multiple attempts over the last three decades to improve survival, 10 septic neonates, even with antimicrobial treatment (3). Despite multiple attempts over the last three decades to improve survival, 10 septic neonates, even with antimicrobial treatment (3). Despite multiple attempts over the last three decades to improve survival, 10 septic neonates, even with antimicrobial treatment (3). Despite multiple attempts over the last three decades to improve survival, 10 septic neonates, even with antimicrobial treatment (3). Despite multiple attempts over the last three decades to improve survival, 10 septic neonates, even with antimicrobial treatment (3). Despite multiple attempts over the last three decades to improve survival, 10 septic neonates, even with antimicrobial treatment (3). Despite multiple attempts over the last three decades to improve survival, 10 septic neonates, even with antimicrobial treatment (3).
CD71+ cells do not modify neonatal inflammatory mortality

Clearance following anti-CD71 treatment was the result of immune priming rather than the result of a reduction in immunosuppressive cells; and human neonatal CD71+CD235α cells are exquisitely sensitive to hypotonic lysis and are predominantly enucleated reticulocytes. We conclude that murine neonatal CD71+ erythrocytes have no effect on neonatal survival with endotoxia or sepsis and that there is no clinical role for targeting the subset of erythroid CD71+ cells to attenuate neonatal sepsis. Reticulocytes have been extensively characterized in human neonates and are not present in all newborns. However, when present, they dramatically decline within hours after birth, at the same time as microbial colonization dramatically increases, suggesting that they may have a limited role in reducing inflammation secondary to microbial colonization.

Materials and Methods

Mice

All studies were approved by the Institutional Animal Care and Use Committee at Vanderbilt University. Specific pathogen–free, male and female, 5–7-d-old neonatal CD71+CD235α spleen cells were purchased from the Jackson Laboratory (Bar Harbor, ME) between 6 and 8 wk of age and allowed a minimum of 7 d to equilibrate to their environment before any breeding or experimental use. Mice were maintained on breeder chow and water ad libitum. Pups aged 5–7 d were considered neonates (7, 14, 15).

Humans

All studies were approved by the Vanderbilt Institutional Review Board. Peripheral blood from healthy adult volunteers or cord blood from term healthy infants was collected fresh into blood-collection tubes containing lithium heparin (BD Biosciences). Cord blood mononuclear cells (CBMCs) and adult PBMCs were immediately isolated by Ficoll gradient separation (Ficoll-Paque Plus; GE Healthcare), as previously described (16).

Isolation of murine bone marrow and splenocytes

Both femurs and tibias from individual animals were collected and flushed with cold PBS (15). Splenocytes were harvested, and single-cell suspensions were created by passing the cells through a 70-μm pore size cell strainer (Falcon-type). Splenocyte and bone marrow suspensions were then subjected to RBC lysis using ammonium chloride solution (KD Medical, Columbia, MD). Lysed fractions were washed twice with cold PBS and suspended in DMEM (Life Technologies) with 10% FBS plus penicillin/streptomycin. Cell viability before plating was determined manually using a hemocytometer with trypan blue exclusion. Prior to FACS, spleens were disrupted in PBS containing 0.2 M EDTA (Sigma) and were not exposed to ammonium chloride.

Cell purification, immunophenotyping, and stimulation

CD11b+ effector cells were enriched from unlysed adult bone marrow using anti-CD11b magnetic beads, according to the manufacturer’s protocol (Miltenyi Biotech). One million enriched CD11b+ effector cells were used for coculture with and without stimulation via 5 μg/ml LPS (Miltenyi Biotec). One million enriched CD11b+ effector cells were given i.p. 30 min prior to LPS injection, and 3 × 10^6 FACS-enriched CD71+ erythroid neonatal splenocytes were given i.p. injection 30 min prior to sepsis generated by cecal slurry injection. Adoptive transfer of immunomodulating cells is commonly performed at the same time as the insult (17–19). We delayed endotoxin or cecal slurry injection slightly to avoid excessive volume into the limited peritoneal space of the murine neonate. In our experiments, the peritoneum was the primary site of the insult (sepsis or LPS), so we administered the adoptive cells into the peritoneum. Control mice received an identical number of enriched B220+ cells or saline via i.p. injection. For endotoxia, 3 × 10^6 FACS-enriched CD71+ erythroid neonatal splenocytes were given i.p. 30 min prior to LPS injection. Resident CD71+ erythroid splenocytes were diminished via i.p. administration of anti-CD71 mAb (150 μg/mouse; Bio X Cell, clone R17.1.3/TIB-29) daily for 2 d. This dose was chosen based on a previous report and our empiric results (8).

Adaptive cell transfer and Ab-mediated depletion of CD71+ erythroid cells

Neonatal CD71+ erythroid splenocytes were enriched as described above. Neonatal mice received 3 × 10^6 FACS-enriched CD71+ erythroid neonatal splenocytes via an i.p. injection 30 min prior to sepsis generated by cecal slurry injection. Adoptive transfer of immunomodulating cells is commonly performed at the same time as the insult (17–19). We delayed endotoxin or cecal slurry injection slightly to avoid excessive volume into the limited peritoneal space of the murine neonate. In our experiments, the peritoneum was the primary site of the insult (sepsis or LPS), so we administered the adoptive cells into the peritoneum. Control mice received an identical number of enriched B220+ cells or saline via i.p. injection. For endotoxia, 3 × 10^6 FACS-enriched CD71+ erythroid neonatal splenocytes were given i.p. 30 min prior to LPS injection. Resident CD71+ erythroid splenocytes were diminished via i.p. administration of anti-CD71 mAb (150 μg/mouse; Bio X Cell, clone R17.1.3/TIB-29) daily for 2 d. This dose was chosen based on a previous report and our empiric results (8). Cell culture supernatant inflammatory mediator measurements

Cytokine/chemokine concentrations were determined on supernatants using a multiplex assay for 32 analytes [IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17A, G-CSF, M-CSF, GM-CSF, IFN-γ, CXC1L1 (KC), CXC1L10 (IP-10), CXC1L5 (LIX), IL-1β (MIP1-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL2 (MCP-2), CCL9 (MIG), CCL11 (eotaxin), CCL5 (RANTES), TNF-α, VEGF] (Millipore) on a Luminex 100, according to the manufacturer’s protocol.

Statistics

Endotoxia or sepsis survival was compared between groups using the Fisher exact test. Values were considered significant only if the two-tailed confidence level was p < 0.05. Cytokine concentrations and leukocyte phenotypes were compared using the Student t test. If the descriptive analyses passed normality and equal variance, then a post hoc Tukey multiple range test was used to compare all groups to determine whether there was a difference between groups. If the descriptive analyses failed normality or tests of equal variance, a comparison using Kruskal–Wallis ANOVA on ranks and Dunn’s method was performed versus sham-treated controls. A Student t test or a Wilcoxon signed-rank test was used to compare results from two groups. Values were considered significant if p < 0.05. Analyses were performed using Prism 6.

Results

Provision of enriched neonatal CD71+ erythroid cells does not reduce mortality to endotoxin challenge

We hypothesized that immunosuppressive neonatal CD71+ erythroid cells would reduce mortality to systemic infection. To address this hypothesis, we performed adoptive transfer of 3 × 10^6 highly enriched (>90% purity) CD71+ erythroid neonatal
spleenocytes (Supplemental Fig. 1A) and measured survival to endotoxin challenge (LD₆₀) as a noninfectious model of systemic inflammation. Three million CD7₁⁺ erythroid neonatal spleenocytes represents an increase of 60% over the endogenous CD7₁⁺ erythroid neonatal spleenocytes that mediate the reported immunosuppressive effect. Survival was not modified in two separate assessments of neonates that received enriched neonatal CD7₁⁺ erythroid spleenocytes (65%) compared with neonates that received PBS (69%) (Fig. 1A). Endotoxin challenge is a useful immunologic tool to generate systemic inflammation, yet it lacks clinical relevance and the multiorgan system complexity associated with sepsis. Therefore, we next examined the impact of adoptive transfer of neonatal CD7₁⁺ erythroid spleenocytes on sepsis survival using a well-characterized model of polymicrobial sepsis (7).

**Provision of enriched CD7₁⁺ erythroid spleenocytes does not modify polymicrobial sepsis survival**

CD7₁⁺ erythroid spleenocytes reportedly reduce the inflammatory response associated with microbial colonization but with the un-toward consequence of increased risk for infection (8). To directly assess the clinically relevant possibility of infection risk, we next tested the hypothesis that provision of immunosuppressive CD7₁⁺ erythroid cells would reduce survival to sepsis by compromising the host response to infection using a well-characterized model of polymicrobial sepsis (7). We adoptively transferred 5 × 10⁶ highly enriched (>90% purity) neonatal CD7₁⁺ erythroid spleenocytes via i.p. injection prior to generating polymicrobial sepsis using the cecal slurry model (7). The number of injected CD7₁⁺ erythroid cells represented a doubling of the number of endogenous neonatal CD7₁⁺ spleenocytes and was greater that what we used in mice given LPS to account for the increased severity of the septic insult. Control animals received an identical number of highly enriched neonatal B220⁺ spleenocytes (Supplemental Fig. 1B) or PBS prior to sepsis. Similar to our negative results with endotoxin challenge (Fig. 1A), we found no change in sepsis survival among neonates that received enriched neonatal CD7₁⁺ erythroid spleenocytes compared with PBS or neonatal B220⁺ cells (Fig. 1B). To answer the question of whether adoptive transfer modifies cytokine production but not mortality, we examined early plasma cytokine production in septic neonates with or without antecedent adoptive transfer of CD7₁⁺ erythroid cells. We did not find a significant difference in the median concentration of plasma TNF-α when it was measured at 2 h after sepsis (the timing of peak plasma TNF-α using our model) in mice (n = 5/group) that received either 5 × 10⁶ CD7₁⁺ erythroid cells or PBS (67 versus 72 pg/ml, p = 0.82). These results demonstrate that, despite the reported ex vivo immunomodulatory properties associated with neonatal CD7₁⁺ erythroid cells, provision of additional immunosuppressive cells does not modify in vivo plasma concentration of TNF-α or mortality in our murine model of polymicrobial sepsis.

**Depletion of CD7₁⁺ erythroid cells does not alter neonatal polymicrobial sepsis survival**

Single pathogen challenge following Ab-mediated depletion of CD7₁⁺ erythroid cells in neonatal mice was associated with a decreased splenic bacterial load compared with mice that did not receive depleting Ab (8). However, mortality to this challenge following CD7₁⁺ erythroid reduction was not reported. Study of CD7₁-null mice is not possible because loss of CD7₁ is embryonically lethal (20). Therefore, we examined the impact of Ab-mediated CD7₁⁺ erythroid depletion on polymicrobial sepsis survival. Ab-mediated depletion resulted in a 58% reduction in CD7₁⁺ erythroid spleenocyte representation (from 60 to 25% of the live spleenocytes, an average absolute reduction of 3 × 10⁶ CD7₁⁺ erythroid cells), resulting in a gross change in splenic appearance (Fig. 1C). Remarkably, sepsis survival for mice with diminished CD7₁⁺ erythroid spleenocytes was not altered compared with neonatal mice that received isotype-control Ab (Fig. 1D).

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**FIGURE 1.** Impact of CD7₁⁺ erythroid cells on inflammatory-mediated mortality in neonates. (A) Survival after adoptive transfer with endotoxin exposure. Neonatal mice received 3 × 10⁶ highly enriched neonatal CD7₁⁺ erythroid (Ter119⁺) cells via i.p. injection 30 min prior to endotoxin challenge (LD₆₀). (B) Survival after adoptive transfer with polymicrobial sepsis. Neonatal mice received 5 × 10⁶ highly enriched neonatal spleenic CD7₁⁺ erythroid cells, B220⁺ cells, or PBS via i.p. injection 30 min prior to sepsis (LD₆₀). (C) Gross spleen appearance and splenic CD7₁⁺ erythroid cell representation by FACS in previously healthy neonatal mice following anti-CD7₁ or isotype Ab treatment. Data are mean + SD. (D) Impact of anti-CD7₁ treatment or isotype-control Ab on sepsis survival following low (top; LD₃₀) and high (bottom; LD₆₀) mortality challenges. *p < 0.05, t test.
**Immunomodulation occurs with coculture of adult or neonatal hematopoietic tissues**

Because we did not observe an effect on mortality after inflammatory or infectious challenge with either adoptive transfer or reduction of CD71+ erythroid cells and no difference in plasma TNF-α production in vivo by septic neonatal mice following adoptive transfer of CD71+ erythroid cells, we determined whether there was immunosuppression ex vivo. Importantly, CD71+ erythroid cells represent a large portion of hematopoietic tissues, including murine fetal liver, neonatal spleen and bone marrow, and adult bone marrow (9–11) (Supplemental Fig. 1D). Because the neonatal spleen is largely hematopoietic, we hypothesized that the immunosuppressive effects previously observed with coculture of neonatal splenocytes would also be present in other hematopoietic tissues from adults and neonates. To address this hypothesis, we highly enriched CD11b+ effector cells via magnetic bead–based positive selection from adult whole bone marrow (Supplemental Fig. 1E). CD11b+ effector cells were cocultured with neonatal splenocytes and stimulated with HKLM. We confirmed that increasing the ratio of neonatal splenocytes progressively suppressed adult CD11b+ effector cell TNF-α production with HKLM stimulation (p < 0.05, Student t test, compared with CD11b+ effector cells alone, Fig. 2A). In addition to TNF-α, we uncovered cell ratio–dependent suppression of G-CSF, CXCL5, CXCL1, CCL3, and CCL4 (Fig. 2B). To directly address our hypothesis regarding an immunosuppressive effect of other hematopoietic tissues, we cocultured enriched HKLM-stimulated adult CD11b+ effector cells with neonatal or adult bone marrow and measured supernatant inflammatory mediators. We found similar cell ratio–based alterations in TNF-α production for neonatal and adult bone marrow (Fig. 2A). Interestingly, we also found cell ratio–dependent enhancement of CXCL10 and VEGF production associated with decreasing coculture ratios of all tissues examined (Fig. 2B). Taken together, these results support that broad immunomodulatory effects on CD11b+ effector cells occur with coculture of actively hematopoietic tissues from neonates and adults, rather than as a unique property of neonatal splenocytes.

**CD71+ cells are prominent in neonatal ileum, and anti-CD71 treatment diminishes intestinal barrier function**

Enhanced bacterial clearance was demonstrated following Ab-mediated reduction of CD71+ erythroid cells (8). This effect might be the result of reducing CD71+ immunosuppressive cells or an unrelated effect of exposure to the Ab used in the reduction procedure. It is noteworthy that anti-CD71 treatment only activated intestinal immune cells, and their activation was dependent upon intestinal microbiota. These results suggest that the observed effect could result from a breach of intestinal barrier function after anti-CD71 treatment. Because CD71 is robustly expressed on gut epithelium (21), anti-CD71 treatment prior to pathogenic challenge would not uniquely target splenic CD71+ erythroid cells and could alter intestinal barrier function. Therefore, we hypothesized that enhanced bacterial clearance after anti-CD71 treatment was the result of immune priming by leaked microbiota rather than the absence of CD71+ erythroid cells (14, 22). To differentiate between immune priming and the absence of immunosuppression, we examined the splenic bacterial load in two groups of anti-CD71–treated neonates following a nonlethal septic challenge: neonates completely replenished with CD71+ erythroid cells by adoptive transfer and neonates that did not receive cells by adoptive transfer. We found no difference in splenic bacterial load among nonlethal sepsis–challenged CD71-depleted neonates that received CD71+ erythroid cells by adoptive transfer versus those that received no cells (Fig. 3A), indicating that CD71+ cells are not immunosuppressive in this setting. To determine whether Ab-mediated disruption of intestinal epithelium occurred, we first examined healthy neonatal ileum for the expression of CD71 and found that CD71 was prominent along the villi epithelium (Fig. 3B). Next, we examined CD71 staining following anti-CD71 Ab exposure or isotype-control Ab at ×200 magnification (Fig. 3C, 3D) and ×600 magnification (Supplemental Fig. 1C). We found a reduction in the MFI for CD71 in the intestine of anti-CD71–treated animals and the presence of skip lesions along the villi compared with animals treated with isotype-control Ab. Next, we examined peritoneal washes of healthy neonates that received anti-CD71 or isotype Ab treatment alone (with no infectious challenge) and found peritoneal colonization only among the anti-CD71–treated mice (Fig. 3E). To determine whether enhanced bacterial clearance occurred with bacterial challenge following anti-CD71 treatment, we examined microbial colonization of the spleen in nonlethal sepsis–challenged mice following LPS priming [used as a positive control (14)], CD71 depletion, or isotype Ab treatment (Fig. 3F). Consistent with immune priming, we found enhanced bacterial clearance with both LPS and anti-CD71 treatment compared with isotype-control treatment. Therefore, anti-CD71 treatment results in decreased bacterial burden in the spleen; this decrease is not reversed by the restoration of CD71+ cells by adoptive transfer, indicating that these cells are not responsible for the observed effect of the Ab treatment on bacterial burden.

**Human CD71+ erythroid cells in cord blood are exquisitely sensitive to hypotonic lysis**

Investigations in mice may provide key mechanistic insights, but translation to clinical benefit requires overlap of murine findings.

![FIGURE 2. Dose-dependent supernatant cytokine inhibition and promotion ex vivo. (A) Supernatant TNF-α measurements from coculture of enriched and HKLM-stimulated adult CD11b+ effector cells with the indicated hematopoietic tissues. Data are mean ± SD. (B) Fold change in enhanced and suppressed supernatant inflammatory mediators following 1:1 coculture of the indicated tissues with enriched CD11b+ effector cells stimulated with HKLM. *p < 0.05, t test, versus CD11b+ effector cells alone.](http://www.jimmunol.org/.../10.4049/jimmunol.1600567)
in humans. The mononuclear fraction from healthy human cord blood contains a large population of nucleated RBCs (nRBCs) and reticulocytes (both express CD71 and CD235a) (8, 23, 24). Similar to the immunosuppressive function by neonatal murine CD71+ erythroid splenocytes, human cord blood CD71+ erythroid cells can inhibit TNF-α production ex vivo (8). To determine whether the human cells were nRBCs or reticulocytes, we first examined PBMCs isolated from the peripheral blood of healthy adult donors (n = 5) and CBMCs from term newborns (n = 6). As expected, we confirmed the robust representation of CD71+CD235a+ cells among CBMCs (mean 50%, range 31–69%) and the minimal representation of CD71+CD235a+ cells among PBMCs (Fig. 4A). Hypotonic lysis resulted in a substantial reduction in neonatal CD71+CD235a+ cells (85% reduction, Fig. 4B). These results are in stark contrast to the stability of murine neonatal CD71+ erythroid splenocytes to hypotonic lysis (19% reduction, data not shown) and suggest that these cells are predominantly reticulocytes (23, 24). FACS enrichment of the neonatal human CD71+CD235a+ cells with cytospin examination after Wright’s stain or methylene blue confirmed that these cells were predominantly enucleated reticulocytes (Fig. 4A).

**Discussion**

Developmental age significantly impacts the host immune response to sepsis (5). Specifically, the structure, cellular composition, and function of the murine spleen are heavily influenced by chrono-

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**FIGURE 3.** Anti-CD71 treatment is associated with enhanced bacterial clearance due to immune priming. (A) Splenic bacterial load 24 h after nonlethal sepsis challenge (0.6 mg of cecal slurry/g body weight given 24 h after the last Ab dose) in CD71-depleted neonates that received either 5 × 10^6 highly enriched neonatal splenic CD71+ erythroid cells by adoptive transfer or no cells. (B) CD71 expression in healthy neonatal ileum (original magnification ×200). (C) Isotype Ab–treated neonatal ileum and anti-CD71–treated ileum 24 h after the last dose of Ab (original magnification ×200). (D) Reduction in CD71 MFI in ileum of anti-CD71–treated and isotype control–treated neonates 24 h after the last dose of Ab. (E) CFU recovered from peritoneal washes of healthy neonates 24 h after last dose of isotype Ab or anti-CD71 treatment alone. (F) Splenic bacterial load 24 h after nonlethal sepsis challenge (0.6 mg of cecal slurry/g body weight given 24 h after the last Ab dose) among neonates that received treatment with anti-CD71 Ab or isotype Ab or priming with a single dose of LPS (1 μg/g, 24 h prior to challenge). *p < 0.05, ANOVA.

**FIGURE 4.** CD71+CD235a+ cells are prominent in human neonatal cord blood and are predominantly enucleated reticulocytes. (A) Representative FACS plots for post-Ficoll mononuclear fraction of healthy adult peripheral blood, healthy term neonate cord blood, healthy term neonate cord blood after hypotonic lysis, enrichment of neonatal cord blood CD71+CD235a+ cells, H&E stain of enriched CD71+CD235a+ cells (top), methylene blue stain of enriched CD71+CD235a+ cells (bottom, inset showing appearance of nucleated cell). Top, original magnification ×400; bottom, original magnification ×1000. (B) Effect of hypotonic lysis on CD71+CD235a+ cells from healthy adult peripheral blood and healthy neonatal cord blood after Ficoll-based isolation of mononuclear cells. *p < 0.05 by t test.
logical age (12–14). In contrast to the immune function of the spleen in the healthy adult, the spleen is normally a major site of erythropoiesis during fetal and neonatal life. Extramedullary erythropoiesis is required in neonates to support the extremely rapid growth rate in the setting of significantly reduced erythroid reservoirs compared with the adult (10). Accordingly, the murine neonatal spleen, bone marrow, and liver are known to harbor large numbers of erythroid progenitors for several weeks after birth (25). Fraser et al. (26) examined the fetal liver and showed a representation of CD71+ erythroid cells similar to that observed in the neonatal spleen and bone marrow.

It was reported that CD71+ erythroid cells are enriched in the murine spleen to quench inflammation secondary to microbial colonization (8). This conclusion was supported, in part, by microbiota-dependent immune cell activation that occurred exclusively in the intestine following Ab-mediated CD71+ erythroid cell diminution. We showed that anti-CD71 treatment was associated with augmented pathogen clearance that was unaffected by replenishment of CD71+ erythroid cells by adoptive transfer, which demonstrates that the beneficial effect was not due to a decrease in immunosuppression. Anti-CD71 treatment led to recoverable microbiota in the peritoneum, which would be expected to activate local intestinal immune cells. In turn, intestinal immune cell activation would be dependent on host microbiota. Our group (14) and other investigators (27, 28) demonstrated similar enhancements in pathogen clearance and survival associated with augmented innate immune function following minor antecedent exposure to microbial products.

Interestingly, murine intestinal epithelial inflammation associated with microbial colonization is rapidly attenuated (<6 h after exposure) via downregulation of IL-1R–associated kinase-l (29). Rapid gut colonization and early desensitization to microbiota–induced inflammation do not readily support the hypothesis that CD71+ erythroid cells persist in the murine spleen for only 2–3 wk after birth to quench inflammation secondary to colonization. In fact, human neonates are colonized with microbiota during delivery and undergo a continuous and dynamic colonization process for the first several years of life (30–32).

Of note, nRBCs are CD71+CD235a+ and may be absent or represent a very small portion of cord or peripheral blood from healthy human neonates, and they are significantly increased by intrauterine stress (hypoxia, infection [IL-6], or intrauterine growth restriction) (33–38). Day-of-birth peripheral blood nRBC reference ranges from 14,319 term infants revealed that nRBCs may be small or even absent (mean <7% of WBCs, absolute <1100/µl) and progressively decrease over the first week of life (37). Similar decreases in the number of circulating reticulocytes (also CD71+CD235a+) were documented (39). The limited number or absence of CD71+CD235a+ cells (nRBCs and reticulocytes) from peripheral blood samples on the first day of life, coupled with a decreasing trend for these cells during the first days of life (which is a period of robust microbial colonization), does not preclude the potential action of quenching inflammation due to microbial colonization, but it strongly suggests that there are other, more likely, mechanisms that account for the presence of these cells.

We acknowledge the potential limitations surrounding a comparison of the host response between preclinical models using different inflammatory and pathogenic challenges. However, a lack of impact on murine neonatal survival to noninfectious inflammatory challenge (LPS) or septic challenge following either depletion or adoptive transfer of CD71+ erythroid cells suggests that the impact of these cells may be more limited than previously described. Furthermore, we presented several lines of evidence supporting that enhanced bacterial clearance in neonatal mice following anti-CD71 treatment was the result of immune priming after exposure to microbiota rather than the result of a reduction in immunosuppressive cells: depletion of CD71+ erythroid cells to CD71−+ diminished animals did not alter bacterial clearance; anti-CD71 treatment was associated with reduced intestinal barrier function, as evidenced by recovery of live bacteria in the peritoneum of unchallenged healthy animals; and anti-CD71–treated mice demonstrated enhanced bacteria clearance similar to LPS–primed animals following nonlethal sepsis challenge. Although the potential confounders of murine modeling can be debated, the identification of enucleated reticulocytes as the cell type enriched in human neonatal cord blood, in conjunction with the absence or rapid decline in reticulocytes after birth during a time of robust microbial colonization, does not readily support the hypothesis that CD71+ cells are enriched to quench intestinal inflammation associated with microbial colonization.

Developmental age strongly impacts the cellular composition of the murine spleen. Neither provision nor reduction of enriched CD71+ erythroid cells had an effect on neonatal mortality with polymicrobial sepsis. Ex vivo immunosuppression of stimulated adult CD11b+ cells by neonatal splenocytes also occurred with adult and neonatal bone marrow. Enhanced bacterial clearance after anti-CD71 treatment was the result of immune priming following a minor disruption in gut integrity, rather than a reduction in immunosuppressive cells. The paucity or absence of reticulocytes in peripheral blood at birth does not readily support an anti-inflammatory role in humans.

Disclosures
The authors have no financial conflicts of interest.

References


