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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 on 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. The Journal of Immunology, 2015, 195: 1064–1070.

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, neonatal sepsis management remains limited to antimicrobial treatment and supportive care (4). Developmental age strongly influences innate and adaptive immune function and the host immune response to sepsis (5, 6). In particular, neonatal mice and humans exhibit greater mortality and an attenuated inflammatory response to sepsis compared with adults (7). However, neonatal-specific mechanistic investigations into the pathophysiology of sepsis have lagged behind those of adults.

Recently, murine neonates were reported to harbor an immunosuppressive CD71+ (also known as transferrin receptor) erythroid (Ter119+) population of splenocytes, which were absent in adult spleens (8). Evidence was presented that suggested these cells provided the beneficial effect of reducing the inflammatory response associated with early life microbial colonization via arginase-2 but at the untoward expense of an increased risk for infection. CD71 is expressed on rapidly dividing cell populations, including normal and malignant cells, and is markedly induced following Ag and mitogen stimulation (9). Sites of extramedullary hematopoiesis, including the neonatal liver and spleen, are necessary to support the rapid fetal and postnatal growth in the setting of significantly reduced erythroid reservoirs compared with adults (10). As such, the CD71+ erythroid population represents a large portion of murine fetal liver, neonatal spleen, and adult bone marrow (9–11). Predictably, adult and neonatal spleens in both rodents and humans are different in cellular composition and function (12–14), which is an important consideration for experimental comparisons of the spleen between animals at different developmental ages.

In this article we show that neonatal survival to endotoxin challenge and neonatal polymicrobial sepsis survival were not affected by provision of neonatal CD71+ erythroid cells; an Ab-mediated reduction in CD71+ cells did not modify neonatal sepsis survival; the ex vivo immunomodulatory effects mediated by murine neonatal splenocytes also occurred with hematopoietic tissue from neonatal and adult bone marrow; enhanced bacterial
cleared following anti-CD71 treatment was the result of immune priming rather than the result of a reduction in immunosuppressive cells; and human neonatal CD71+CD235a- cells are excessively 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 C57BL/6 mice were purchased from 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 + 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 Biotec). One million enriched CD11b+ effector cells were used for coculture with and without stimulation via 5 × 10^6 heat-killed Listeria monocytogenes (HKLM; InvivoGen). Murine neonatal CD71+ erythroid splenocytes were targeted and enriched using FACS on a BD FACSAria III. All FACS Abs were obtained from either eBioscience or BD Biosciences. Isolated or enriched murine splenic leukocytes were phenotyped by cell surface staining with B220, CD71, Ter119, and 7-aminoactinomycin D (7-AAD) in FACS buffer (PBS with 3% FBS with no azide) on a BD Fortessa. Human PBMCs were processed for same-day flow cytometry by washing with FACS buffer containing 20% heat-inactivated FBS, followed by staining with 7-AAD as viability dye (Molecular Probes), anti-CD235a(GlyA)-FITC (Invitrogen), and anti-CD71-PE or anti-CD71- allophycocyanin (BD Biosciences). For compensation, we used Ab-capture beads (CompBeads; BD Biosciences). Stained cells were washed and resuspended in 100 μl FACS buffer prior to acquisition on the cytometer (FACS Canto II, Becton Dickinson). To remove erythrocytes after initial data collection, samples were treated with Pharm Lyse buffer (BD Biosciences) and washed. FACS samples were analyzed using FlowJo software. A minimum of 3 × 10^4 nondebris, live (7-AAD-) cells was used for analysis.

Immunofluorescence and cytospin staining

Neonatal small intestine was collected, and tissues were placed in 10% formalin (Fisher Scientific) at 4°C for 1 h, 15% sucrose (Research Products International, Mount Prospect, IL) overnight, and 30% sucrose for 6 h; blocks for sectioning were made on dry ice in embedding medium (Tissue-Tek; Sakura, Torrance, CA). Murine tissue sections (8 μm) were stained with DAPI-gold (Molecular Probes), anti-CD71 Ab (Abcam), and appropriate secondary Ab (Invitrogen). Tissue was examined using an Olympus IX81 microscope with a 12-bit charge-coupled device (Ora ER11; Hamamatsu) camera, and images were acquired using SlideBook digital microscopy software. Mean fluorescence intensity (MFI) was measured using Adobe Photoshop CS6. Cytospins were performed on sorted human cells with subsequent microscopic examination following Weight’s stain or methylene blue.

Experimental sepsis and endotoxemia

Mice were made septic using polymicrobial peritonitis, as previously described (7). Briefly, a 6–8-wk-old nonpregnant female wild-type (C57BL/6) mouse was euthanized within 2 wk of arrival from the vendor, and the cecum was isolated. Cecal contents were expressed, weighed, suspended in 5% dextrose at a concentration of 80 mg/ml, and administered via i.p. injection at the desired lethal dose (LD), as indicated in each figure legend. Where indicated, sepsis was generated by cecal slurry administration 24 h after the second dose of anti-CD71 or isotype Ab (described below). Mice were monitored after injection, as previously described (7, 14, 15). Ultrapure LPS (Escherichia coli 0111:B4; InvivoGen), given via an i.p. injection, was used to generate endotoxemia. The LPS dose used (20 μg/g body weight) was determined empirically and reproducibly generated 35–40% mortality in neonatal pups. For LPS-priming experiments, mice received an i.p. injection of 1 μg LPS/g body weight 24 h prior to infectious challenge (14).

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 endotox 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 endotoxemia, 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 217.1.3/ITB-219) daily for 2 d. This dose was chosen based on a previous report and our empirical 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-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17a, G-CSF, M-CSF, GM-CSF, IFN-γ, CXCL1 (KC), CXCL10 (IP-10), CXCL5 (LIX), CXCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL2 (MIP2), CXCL9 (MIG), CCL11 (eotaxin), CCL5 (RANTES), TNFα, VEGF) (Millipore) on a Luminex 100, according to the manufacturer’s protocol.

Statistics

Endotoxemia 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 inflammation. To address this hypothesis, we performed adoptive transfer of 3 × 10^6 highly enriched (>90% purity) CD71+ erythroid neonatal
CD71+ erythroid splenocytes reportedly reduce the inflammatory response associated with microbial colonization but with the untoward 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 $5 \times 10^6$ highly enriched neonatal CD71+ erythroid splenocytes 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+ splenocytes and was greater that what we used in our murine model of polymicrobial sepsis.

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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 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 treatment compared with isotype-control Ab. Next, we examined peritoneal washes of healthy neonates that received anti-CD71 or isotype Ab treatment alone (with no infection 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.
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 chronolog-
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-1 (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 deletion 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: repletion 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.

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