Title
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Journal
The Journal of Immunology, 195(3)

ISSN
0022-1767

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Publication Date
2015-08-01

DOI
10.4049/jimmunol.1500771

Peer reviewed

HHS Public Access

Author manuscript

J Immunol. Author manuscript; available in PMC 2016 August 01.

Published in final edited form as:

J Immunol. 2015 August 1; 195(3): 1064–1070. doi:10.4049/jimmunol.1500771.

Neonatal CD71+ erythroid cells do not modify murine sepsis mortality

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Abstract

Sepsis is a major cause of neonatal mortality and morbidity worldwide. A recent report suggested 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 antibody-mediated reduction of 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 as it also occurred with adult and neonatal bone marrow. Animals treated with anti-CD71 antibody showed reduced splenic bacterial load following bacterial challenge compared to 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 the impact of these cells on neonatal infection risk and progression may be limited. An unanticipated immune priming effect of anti-CD71 antibody treatment was likely responsible for the reported enhanced bacterial clearance, rather than a reduction of immunosuppressive $CD71^+$ erythroid splenocytes. In humans, the well-described

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Disclosures:

The authors declare no competing financial interests.

rapid decrease in circulating reticulocytes after birth suggests they may have a limited role in reducing inflammation secondary to microbial colonization.

Keywords

Neonate; CD71$^+$ erythroid cells; sepsis; reticulocyte

Introduction

Neonatal sepsis remains a significant global threat and kills over 1 million newborns each year(1). In developed countries, sixty percent 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 as compared to 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, TfR), 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 of infection. CD71 is expressed on rapidly dividing cell populations including normal and malignant cells, and is markedly induced following antigen and mitogen stimulation(9). Sites of extramedullary hematopoiesis, including the neonatal liver and spleen, are necessary to support the rapid fetal and post-natal growth in the setting of significantly reduced erythroid reservoirs as compared to 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, in turn, an important consideration for experimental comparisons of the spleen between animals at different developmental ages.

Herein, we show: 1) Neither neonatal survival to endotoxin challenge nor neonatal polymicrobial sepsis survival was affected by provision of neonatal CD71$^+$ erythroid cells; 2) Antibody-mediated reduction of CD71$^+$ cells did not modify neonatal sepsis survival; 3) The *ex vivo* immunomodulatory effects mediated by murine neonatal splenocytes also occurred with hematopoietic tissue from neonatal and adult bone marrow; 4) Enhanced bacterial clearance following anti-CD71 treatment was the result of immune priming rather than the result of a reduction in immunosuppressive cells; and 5) Human neonatal CD71$^+$CD235a$^+$ 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 endotoxemia 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 they may have a limited role in reducing inflammation secondary to microbial colonization.

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 The Jackson Laboratory (Bar Harbor, ME), between 6 and 8 weeks of age and allowed a minimum of seven days 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 days (P5–7) 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). Mononuclear cells from cord blood (CBMC) and adult peripheral blood (PBMC) 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 phosphate buffered saline (PBS)(15). Spleens were harvested and single-cell suspensions were created by passing the cells through a 70μm pore size cell strainer (Falcon-type). Splenocyte and bone marrow suspensions were then subjected to red blood cell (RBC) lysis using ammonium chloride solution (KD Medical, Columbia, MD). Lysed fractions were washed twice with cold PBS, and suspended in Dulbecco's Modified Eagle's medium (DMEM, Gibco) with 10% FBS + penicillin/streptomycin. Cell viability before plating was determined manually using a hemocytometer with trypan blue exclusion. Prior to Fluorescence Activated Cell Sorting (FACS), spleens were disrupted in PBS containing 0.2M ethylenediaminetetraacetic acid (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). One million enriched $CD11b^+$ effector cells were used for co-culture 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. Isolated or enriched murine splenic leukocytes were phenotyped by cell surface staining with B220, CD71, Ter119, 7-aminoactinomycin D (7-AAD, eBioscience, BD biosciences) 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 fetal bovine serum (FBS) followed by staining with 7-AAD as viability dye (Molecular Probes), anti-CD235(GlyA)-FITC (Invitrogen) and anti-CD71-PE or -APC (BD Biosciences). For compensation we used antibody-capture beads (CompBeads, BD Biosciences). Stained cells were washed and resuspended in 100 μl FACS buffer prior to acquisition on the cytometer (FACSCanto 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 FloJo software. A minimum of 3×10^4 non-debris, live (7-AAD$^-$) cells were 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 hour, then 15% sucrose (Research Products International, Illinois) overnight, 30% sucrose for 6 hours, and blocks for sectioning were made on dry ice in embedding medium (Tissue Tek, Sakura, California). Murine tissue sections (8 μm) were stained with 4′,6-diamidino-2-phenylindole (DAPI)-gold (Molecular Probes) and anti-CD71 antibody (Abcam) and appropriate secondary antibody (Invitrogen). Tissue was examined using an Olympus IX81 microscope with a 12-bit charge-coupled device (Orca ERII, Hamamatsu) camera and images were acquired using Slidebook digital microscopy software. MFI was measured using Adobe Photoshop CS6. Cytospins were performed on sorted human cells with subsequent microscopic examination following Wright's stain or methylene blue.

Experimental sepsis and endotoxemia

Mice were made septic using polymicrobial peritonitis as previously described(7). Briefly, a 6–8 week old non-pregnant female WT (C57BL/6) mouse was euthanized within 2 weeks of arrival from the vendor and the cecum was isolated. Cecal contents were expressed, weighed, suspended in 5% dextrose at a concentration of 80mg/mL, and administered via intraperitoneal (IP) injection at the desired lethal dose (LD) as indicated in each respective figure legend. Where indicated, sepsis was generated by cecal slurry administration twenty-four hours after the second dose of anti-CD71 or isotype antibody (described below). Mice were monitored after injection as previously described(7, 14, 15). Ultrapure lipopolysaccharide (LPS, *E. coli* 0111:B4, Invivogen) given via an IP 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 IP injection of 1mg LPS/g body weight twenty-four hours prior to infectious challenge(14).

Adoptive cell transfer and antibody-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 IP injection 30 minutes 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 slightly delayed endotoxin or cecal slurry injection 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 IP injection. For endotoxemia, 3×10^6 FACS-enriched $CD71^+$ erythroid neonatal splenocytes were given via an IP injection 30 minutes prior to LPS injection. Resident $CD71^+$ erythroid splenocytes were diminished via IP administration of anti-CD71 monoclonal antibody (150ug/mouse, BioXCell, clone R17 217.1.3/TIB-219) daily for 2 days. 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 [interleukin (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-15, IL-17a, G-CSF, M-CSF, GM-CSF, IFN-γ, CXCL1 (KC), CXCL10 (IP-10), CXCL5 (LIX), leukemia inhibitory factor (LIF), CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CXCL2 (MIP2), CXCL9 (MIG), CCL11 (Eotaxin), CCL5 (RANTES), TNF-α, VEGF] (Millipore) on a Luminex 100 according to the manufacturer's protocol.

Statistics

Endotoxemia or sepsis survival between groups was compared using Fisher's 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 Student's t-test. If the descriptive analyses passed normality and equal variance, then a post-hoc Tukey's multiple range test was used to compare all groups to determine if there was a difference between groups. If the descriptive analyses failed normality or tests of equal variance, a comparison using Kruskal-Wallis analysis of variance on ranks and Dunn's method was performed versus sham-treated controls. A Student's 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 splenocytes (Supplemental Figure 1A) and measured survival to endotoxin challenge (LD_{40}) as a non-infectious model of systemic inflammation. Three million $CD71^+$ erythroid neonatal splenocytes represents an increase of 60% over the endogenous $CD71^+$ erythroid neonatal splenocytes that mediate the reported immunosuppressive effect. Survival was not modified in 2 separate assessments of neonates that received enriched neonatal $CD71^+$ erythroid splenocytes (65%) as compared to neonates that received PBS (69%) (Figure 1A). Endotoxin challenge is a useful immunologic tool to generate systemic inflammation yet lacks clinical relevance and multi-organ system complexity associated with sepsis.

Therefore, we next examined the impact of adoptive transfer of neonatal $CD71^+$ erythroid splenocytes on sepsis survival using a well-characterized model of polymicrobial sepsis(7).

Provision of enriched $CD71^+$ erythroid splenocytes does not modify polymicrobial sepsis survival

$CD71^+$ erythroid splenocytes reportedly reduce the inflammatory response associated with microbial colonization but with the untoward consequence of increased risk of 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 5×10^6 highly-enriched (>90% purity) neonatal $CD71^+$ erythroid splenocytes via IP 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 mice given LPS to account for the increased severity of the septic insult. Control animals received an identical number of highly-enriched neonatal $B220^+$ splenocytes (Supplemental Figure 1B) or PBS prior to sepsis. Similar to our negative results with endotoxin challenge (Figure 1A), we found no change in sepsis survival among neonates that received enriched neonatal $CD71^+$ erythroid splenocytes as compared to PBS or neonatal $B220^+$ cells (Figure 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-a when it was measured at 2 hours after sepsis (the timing of peak plasma TNF-α using our model) in mice (n = 5 per group) that received either 5×10^6 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 either *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 antibody-mediated depletion of $CD71^+$ erythroid cells in neonatal mice was associated with a decreased splenic bacterial load as compared to mice that did not receive depleting antibody(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). We therefore examined the impact of antibody-mediated $CD71^+$ erythroid depletion on polymicrobial sepsis survival. Antibody-mediated depletion resulted in a 58% reduction in $CD71^+$ erythroid splenocyte representation (from 60 to 25% of the live splenocytes, an average absolute reduction of 3×10^6 $CD71^+$ erythroid cells) resulting in a gross change in splenic appearance (Figure 1C). Remarkably, sepsis survival for mice with diminished $CD71^+$ erythroid splenocytes was not altered as compared to neonatal mice that received isotype control antibody (Figure 1D).

Immunomodulation occurs with co-culture of adult or neonatal hematopoietic tissues

Since we did not observe an effect on mortality after inflammatory or infectious challenge with either adoptive transfer or reduction of $CD71^+$ erythroid cells, and also no difference in plasma TNF-a production *in vivo* by septic neonatal mice following adoptive transfer of $CD71^+$ erythroid cells, we therefore 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 Figure 1D). Because the neonatal spleen is largely hematopoietic, we hypothesized that the immunosuppressive effects previously observed with co-culture 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 Figure 1E). $CD11b^+$ effector cells were co-cultured in ratios with neonatal splenocytes and stimulated with heat-killed *Listeria monocytogenes* (HKLM). We confirmed that increasing the ratio of neonatal splenocytes progressively suppressed adult $CD11b^+$ effector cell TNF-α production with HKLM stimulation ($p<0.05$ by Student's t-test compared to $CD11b^+$ effector cells alone, Figure 2A). In addition to TNF-α, we also uncovered cell ratio-dependent suppression of G-CSF, CXCL5, CXCL1, CCL3, and CCL4 (Figure 2B). To directly address our hypothesis regarding an immunosuppressive effect of other hematopoietic tissues, we co-cultured 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 (Figure 2A). Interestingly, we also found cell ratio-dependent enhancement of CXCL10 and VEGF production associated with decreasing co-culture ratios of all tissues examined (Figure 2B). Taken together, these results support broad immunomodulatory effects on $CD11b^+$ effector cells occur with co-culture of actively hematopoietic tissues from neonates and adults rather than 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 previously demonstrated following antibody-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 antibody 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 reported 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 non-lethal septic challenge; 1) neonates completely replenished with $CD71^+$ erythroid cells

by adoptive transfer or 2) neonates that did not receive cells by adoptive transfer. We found no difference in splenic bacterial load among non-lethal sepsis-challenged CD71-depleted neonates that received $CD71^+$ erythroid cells by adoptive transfer versus those that received no cells (Figure 3A), indicating that $CD71^+$ cells are not immunosuppressive in this setting. To determine whether antibody-mediated disruption of intestinal epithelium occurred we first examined healthy neonatal ileum for the expression of CD71 and found CD71 was prominent along the villi epithelium (Figure 3B). Next, we examined CD71 staining following anti-CD71 antibody exposure or isotype control antibody at 200× magnification (Figure 3C, Figure 3D) and 600× magnification (Supplemental Figure 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 as compared to animals treated with isotype control antibody. Next, we examined peritoneal washes of healthy neonates that received anti-CD71 or isotype antibody treatment alone (with no infectious challenge) and found peritoneal colonization only among the anti-CD71 treated mice (Figure 3E). To determine whether enhanced bacterial clearance occurred with bacterial challenge following anti-CD71 treatment, we examined microbial colonization of the spleen in non-lethal sepsis-challenged mice following LPS priming (used as a positive control(14)), CD71 depletion, or isotype antibody (Figure 3F). Consistent with immune priming, we found enhanced bacterial clearance with both LPS and anti-CD71 treatment as compared to 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 antibody 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 red blood cells (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 nucleated RBCs 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 robust representation of $CD71^+CD235a^+$ among CBMCs (mean 50%, range 31–69%) and minimal representation of $CD71^+CD235a^+$ among PBMCs (Figure 4A). Hypotonic lysis resulted in substantial reduction of the neonatal $CD71^+CD235a^+$ cells (85% reduction, Figure 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 (Figure 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 chronological 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 as compared to 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*. examined the fetal liver and showed a similar representation of $CD71^+$ erythroid cells to that observed in the neonatal spleen and bone marrow(26).

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 antibody-mediated $CD71^+$ erythroid cell diminution. We showed anti-CD71 treatment was associated with augmented pathogen clearance that was unaffected by replenishment of $CD71^+$ erythroid cells by adoptive transfer that demonstrates 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. Intestinal immune cell activation would, in turn, be dependent on host microbiota. Our group and others have demonstrated similar enhancements in pathogen clearance and survival associated with augmented innate immune function following minor antecedent exposure to microbial products(14, 27, 28).

Interestingly, murine intestinal epithelial inflammation associated with microbial colonization is rapidly attenuated (<6 hours after exposure) via down regulation of interleukin-1 receptor-associated kinase (IRAK)-1(29). Rapid gut colonization and early desensitization to microbiota-induced inflammation does not readily support the hypothesis that $CD71^+$ erythroid cells persist in the murine spleen for only 2–3 weeks 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 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 nRBCs may be small or even absent (mean <7% of WBC, absolute <1100/ μL) and progressively decrease over the first week of life(37). Similar decreases in the number of circulating reticulocytes (also are $CD71^+CD235a^+$) have been documented(39). The limited number or absence of $CD71^+235a^+$ 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 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 employing different inflammatory and pathogenic challenges. However, a lack of impact on murine neonatal survival to non-infectious inflammatory challenge (LPS) or septic challenge following either depletion or adoptive transfer of $CD71^+$ erythroid cells suggests the impact of these cells may be more limited than previously described. Furthermore, we have presented several lines of evidence which support 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: 1) Repletion of $CD71^+$ erythroid cells to $CD71^+$ diminished animals did not alter bacterial clearance, 2) anti-CD71 treatment was associated with reduced intestinal barrier function as evidenced by recovery of live bacteria in the peritoneum of unchallenged healthy animals, 3) Anti-CD71 treated mice demonstrated enhanced bacteria clearance similar to LPS-primed animals following non-lethal 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.

Conclusion

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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant support:

The project was supported by Vanderbilt CTSA award (UL1TR000445) from the National Center for Advancing Translational Sciences, Vanderbilt CTSA Grant UL1 RR024975-01 from the National Center for Research Resources (NCRR/NIH), the *Eunice Kennedy Shriver* National Institute Of Child Health & Human Development (NICHD) [T32HD068256 (to JRK) and HD061607 (to JHW)], National Institutes of Health (NIH) (DK090146; to DJM), GM106143 (to JLW), and by NIH MSTP (5T32GM007347-33; to BTS), and funds from the Department of Pediatrics at Vanderbilt University.

Non-standard abbreviations

CBMCs	cord blood mononuclear cells
HKLM	heat-killed *Listeria monocytogenes*
IP	intraperitoneal
IRAK	interleukin-1 receptor-associated kinase

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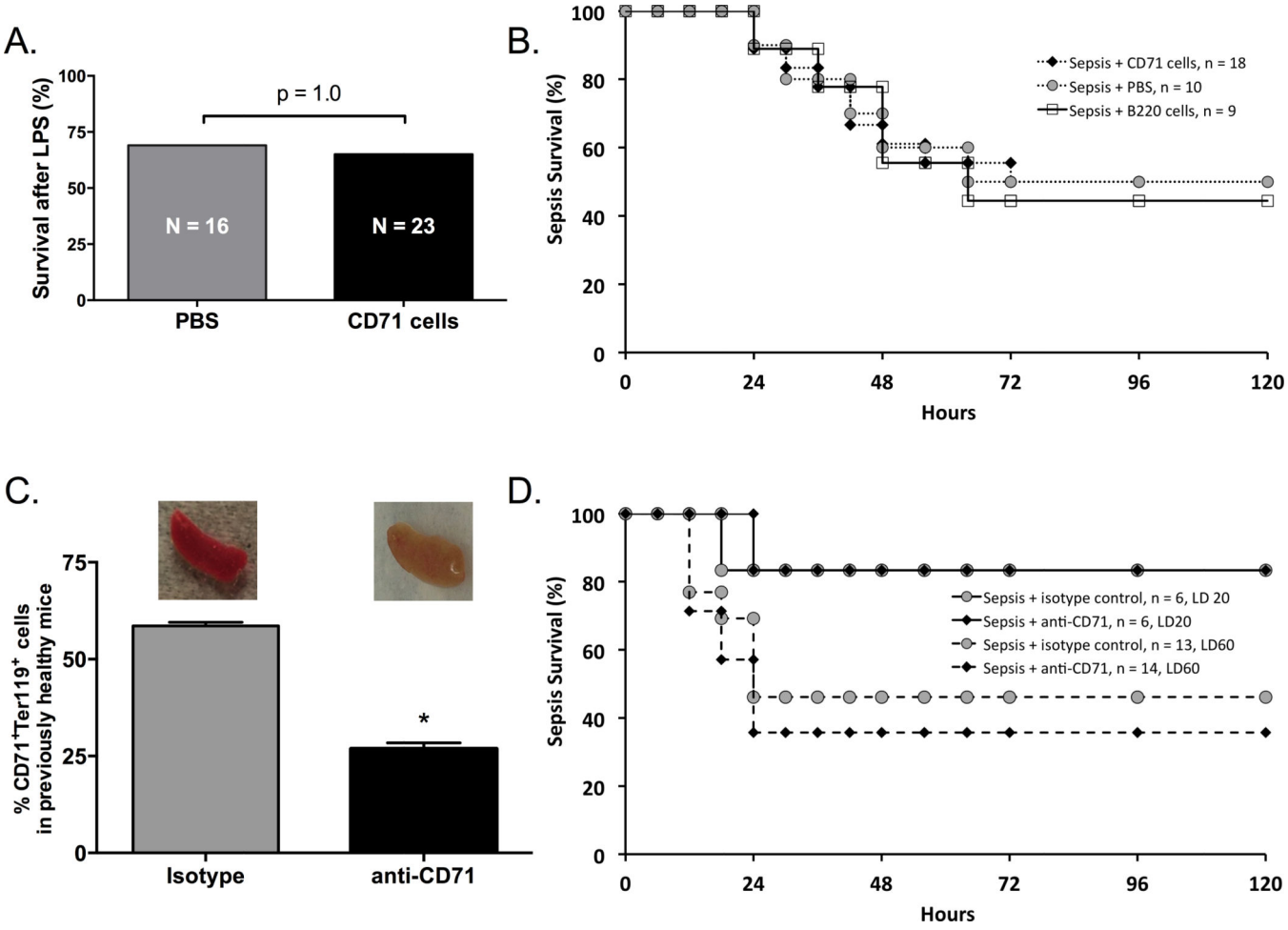

Figure 1. Impact of CD71$^+$ erythroid cells on inflammatory-mediated mortality in neonates

A. Survival after adoptive transfer with endotoxin exposure. Neonatal mice received 3×10^6 highly enriched neonatal CD71$^+$ erythroid (Ter119$^+$) cells via IP injection 30 minutes prior to endotoxin challenge (LD_{40}). **B.** Survival after adoptive transfer with polymicrobial sepsis. Neonatal mice received 5×10^6 highly enriched neonatal splenic CD71$^+$ erythroid cells, B220$^+$ cells, or PBS via IP injection 30 minutes prior to sepsis (LD_{60}). **C.** Gross spleen appearance and splenic CD71$^+$ erythroid cell representation by FACS in previously healthy neonatal mice following anti-CD71 or isotype antibody treatment. Mean with std dev shown. *-$p<0.05$ by T-test. **D.** Impact of anti-CD71 treatment or isotype control antibody on sepsis survival following low (top, LD_{20}) and high (bottom, LD_{60}) mortality challenges.

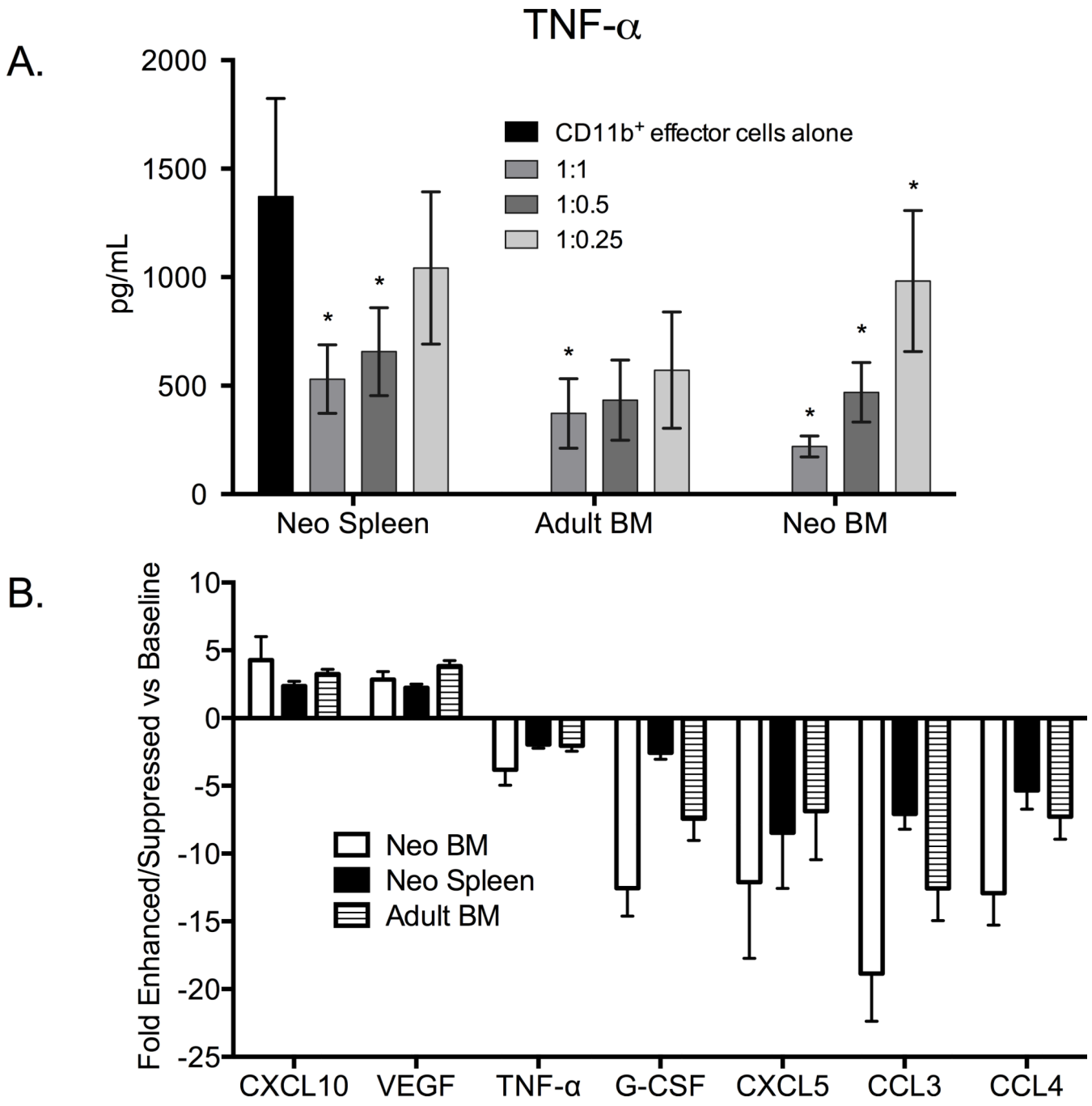

Figure 2. Dose-dependent supernatant cytokine inhibition and promotion *ex vivo*

A. Supernatant TNF-a measurements from co-culture of enriched and heat-killed *Listeria monocytogenes* stimulated adult CD11b$^+$ effector cells with indicated hematopoietic tissues in the indicated ratios. *-$p<0.05$ by T-test compared to CD11b$^+$ effector cells alone, Mean with std dev shown. **B.** Fold change of enhanced and suppressed supernatant inflammatory mediators following 1:1 co-culture of indicated tissues with enriched CD11b$^+$ effector cells stimulated with heat-killed *Listeria monocytogenes*.

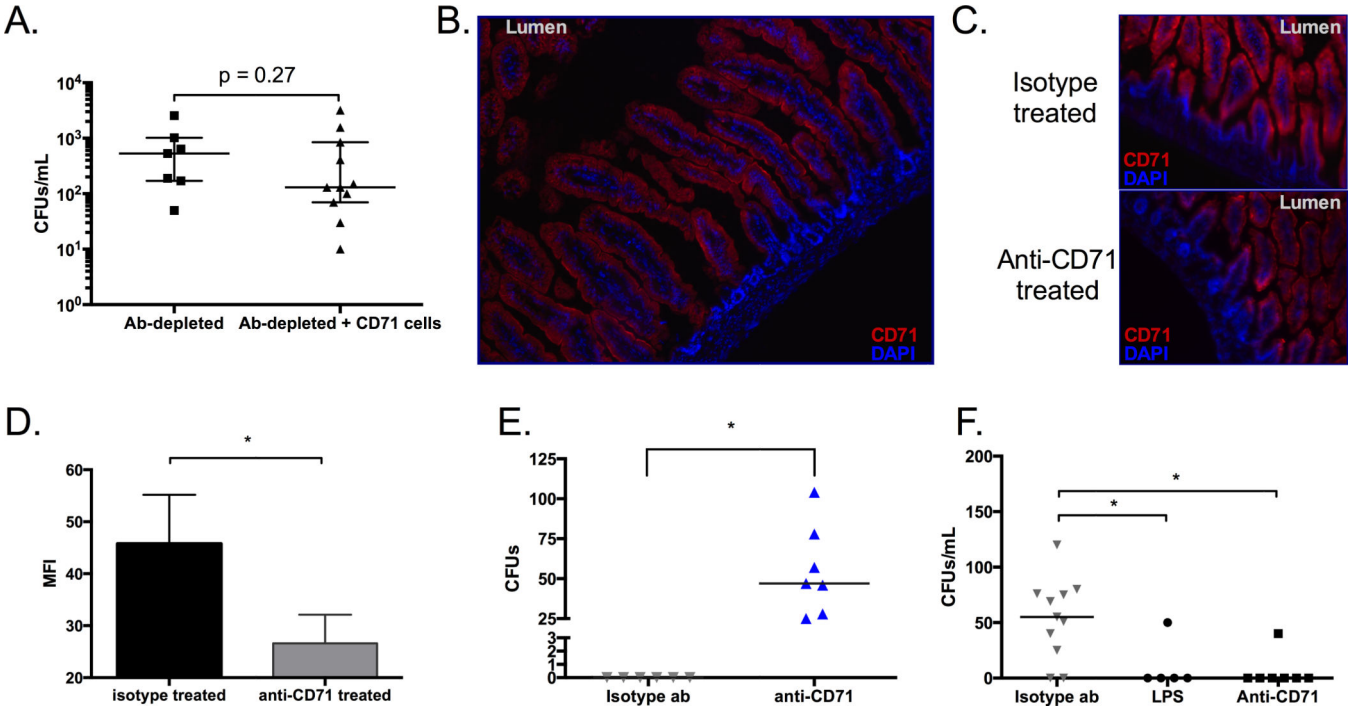

Figure 3. Anti-CD71 treatment is associated with enhanced bacterial clearance due to immune priming

A. Splenic bacterial load 24 hours after non-lethal sepsis-challenge (0.6mg of cecal slurry/g body weight given twenty-four hours after last antibody 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 (200×). **C.** Top-isotype antibody-treated neonatal ileum (200×) twenty-four hours after last dose of antibody, Bottom-anti-CD71-treated ileum (200×) twenty-four hours after last dose of antibody. **D.** Reduction in CD71 MFI in ileum of anti-CD71 treated versus isotype control-treated neonates twenty-four hours after last dose of antibody. **E.** Colony forming units recovered from peritoneal washes of healthy neonates twenty-four hours after last dose of isotype antibody or anti-CD71 treatment alone. **F.** Splenic bacterial load twenty-four hours after non-lethal sepsis-challenge (0.6mg of cecal slurry/g body weight given twenty-four hours after last antibody dose) among neonates that received treatment with anti-CD71 antibody, isotype antibody, or priming with a single dose LPS (1μg/g, twenty-four hours prior to challenge), *p<0.05 by ANOVA.

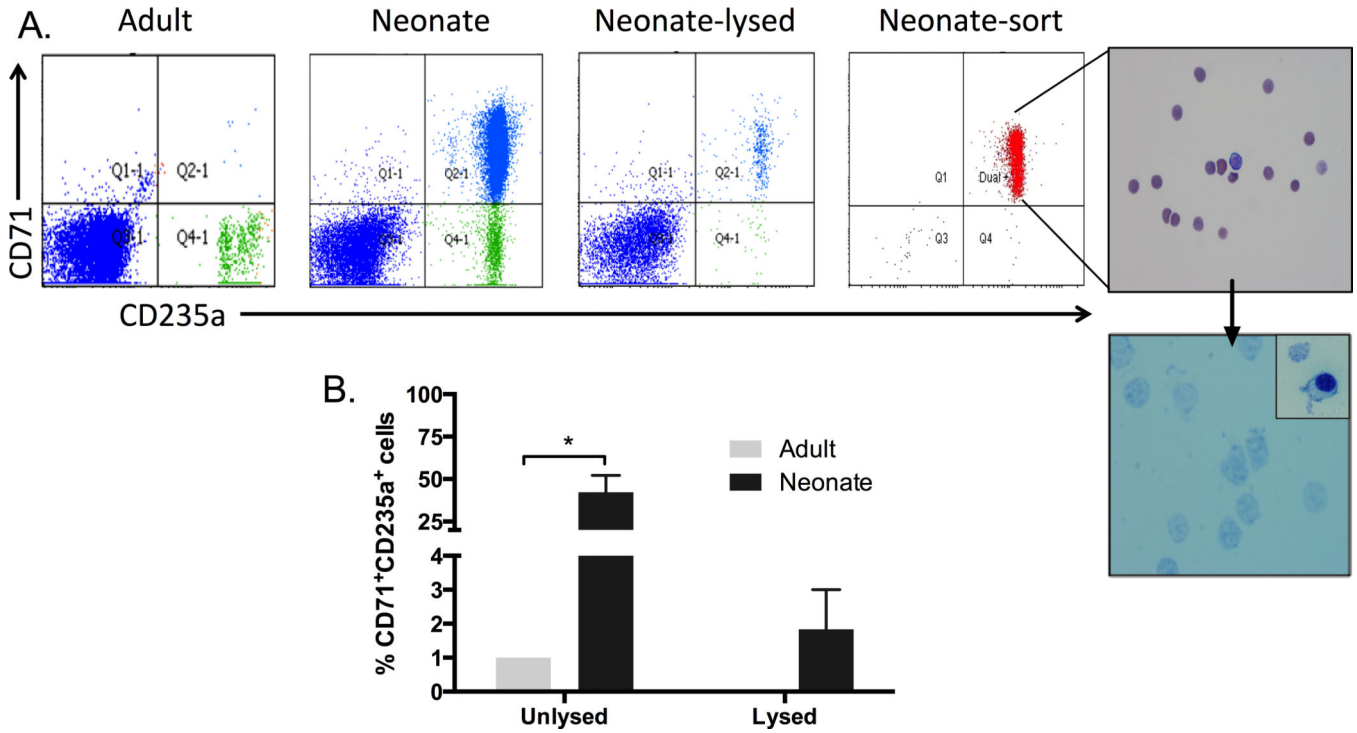

Figure 4. CD71$^+$CD235a$^+$ cells are prominent in human neonatal cord blood and are predominantly enucleated reticulocytes

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$^+$235a$^+$ 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). **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.