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Neonatal Neutrophils with Prolonged Survival Exhibit Enhanced Inflammatory and Cytotoxic Responsiveness

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ABSTRACT

Apoptosis is critical to the resolution of inflammation, as it promotes the removal of neutrophils (PMN) by the reticuloendothelial system. In contrast, PMN persistence characterizes the early stages of chronic inflammation. Adult PMN with delayed senescence retain some functionality, although this has not been described for neonatal PMN. We hypothesized that neonatal PMN with prolonged survival retain cytotoxic and inflammatory function. To test one aspect of inflammatory function, we determined surface CD11b expression on 0-h and 24-h PMN after chemotactic *formyl*-methionine-leucine-phenylalanine (fMLP) stimulation. Although fMLP induced a greater percentage up-regulation of CD11b on 0-h adult PMN, this was similar between nonapoptotic cord blood and adult PMN at 24 h. Furthermore, percentage up-regulation of CD11b was more robust for 24-h than for 0-h cord blood PMN. In contrast, there was no difference in responsiveness between 0-h and 24-h adult PMN. In studies of cytotoxic potential, we determined the expression of reactive oxygen intermediates (ROI) in phorbol 12-myristate 13-acetate–stimulated cord blood and adult PMN at 0 h and in 24-h nonapoptotic PMN, using the dihydrorhodamine 123 assay. Stimulated cord blood PMN generated more ROI than did adult PMN at both 0 h and 24 h; in addition, ROI levels in 24-h cord blood PMN were similar to those of 0-h adult PMN. We conclude that PMN with prolonged survival retain specific cytotoxic and inflammatory functions, and these are enhanced in cord blood PMN. We speculate that neonatal PMN with prolonged survival have the functional capacity to contribute to the pathogenesis of inflammatory disorders. (***Pediatr Res* 57: 424–429, 2005**)

Abbreviations

CLD, chronic lung disease
DHR, dihydrorhodamine 123
fMLP, *formyl*-methionine-leucine-phenylalanine
PE, phycoerythrin
PMA, phorbol 12-myristate 13-acetate
PMN, neutrophil(s)
ROI, reactive oxygen intermediates
7-AAD, 7-aminoactinomycin

PMN serve as the primary line of host defense during the early stages of sepsis and inflammation (1). After activation by systemic or local factors, primed endothelial cells interact with PMN through adhesion molecules that include selectins and integrins (2). These adhesive interactions initiate a process that allows the extravasation of PMN into inflamed tissue with the goal of neutralizing the inciting stimulus. In the case of inflammation mediated by bacterial or fungal microorganisms, phagocytosis and subsequent killing of these invaders occurs through mechanisms that partly involve ROI and proteases (3,4).

The concept that apoptosis of inflammatory PMN and their timely removal by resident macrophages are critical to the resolution of inflammation has been well established (5,6). During the resolution phase, PMN typically undergo the phenotypic changes associated with apoptosis, which promotes their removal by resident macrophages (7). PMN can also undergo necrosis and lysis, a process that induces cytotoxicity in surrounding tissues (8), although apoptosis is the more common and physiologically preferable route. All cells are programmed for senescence, and this program is especially prominent in PMN, cells with foreshortened half-lives. Apoptosis of PMN involves surface changes in the plasma membrane, including expression of phosphatidylserine residues and

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receptors that facilitate their phagocytosis by macrophages (6). The integrity of the cell membrane is preserved in apoptotic PMN, which potentially confers protection to the local environment from cytoplasmic proteases and enzymes. Furthermore, apoptotic PMN in adults have decreased functional capacity, which further contributes to the down-regulation of PMN stimulation during the resolution of inflammation (2,9).

The delayed removal of activated PMN appears to be a critical component in the pathogenesis of a variety of chronic inflammatory and autoimmune disorders in adults (10–13), although less is known about PMN apoptosis in the pediatric population. Infants who are delivered very early in gestation are at risk for developing the inflammatory lung disorder, bronchopulmonary dysplasia. The etiology of this CLD of premature infants is unclear and has been associated with a myriad of risk factors (14,15). Persistence of lung PMN is one characteristic associated with CLD; however, whether a delay in the apoptosis of these PMN contributes to their persistence, as has been suggested, has not been established (16,17).

We previously determined that neonatal PMN have a delay in their onset of spontaneous and Fas-mediated apoptosis *in vitro* (18). PMN that survive conditions associated with spontaneous apoptosis retain some degree of functionality (9), although whether this applies to neonatal PMN has not been described. We reasoned that retained functional capacity in surviving neonatal PMN might not only delay their clearance by the reticuloendothelial system, but could augment the inflammatory response by promoting PMN–endothelial interactions as well as the incitement of injury to surrounding tissues. In this article, we present the results of studies designed to test our hypothesis that surviving, nonapoptotic neonatal PMN retain their inflammatory and cytotoxic potential.

METHODS

PMN isolation and culture. Samples from the umbilical veins of term placentas after uncomplicated cesarean section or from the peripheral venous blood of healthy adult volunteers were collected into heparinized syringes and processed immediately. Samples were obtained following informed consent in accordance with the guidelines of the Institutional Review Board for Human Studies at the University of Florida.

Dextran-sedimented leukocytes were subjected to density centrifugation, and the resultant was subjected to hypotonic lysis of contaminating erythrocytes, as previously described (19). Isolated PMN (10^6 cells/mL) suspended in RPMI 1640/2% FCS (Mediatech Inc., Herndon, VA) were cultured in polypropylene tubes at 37°C, 5% CO_2, for up to 24 h.

Up-regulation of surface CD11b on PMN after chemotactic stimulation. After culture, PMN aliquots were washed with Ca- and Mg-free PBS, then incubated with either PBS alone (control) or in the presence of 10 nM fMLP (Sigma Chemical Co., St. Louis, MO) for 15 min at 37°C. Cells were then washed in PBS and stained with an anti-CD11b MAb conjugated to PE (IgG_{2a}, clone D12) or a PE-conjugated isotype control IgG Ab (both from BD Immunocytometry Systems, San Jose, CA), then washed with PBS. To differentiate between nonapoptotic, early apoptotic, and late apoptotic/necrotic populations, PMN stained with anti-CD11b MAb, or its isotype control, were subsequently treated with annexin V, which binds exposed phosphatidylserine residues on apoptotic cells (20), and 7-AAD, a stain that identifies nonviable, necrotic cells in combination with annexin V (21,22). Briefly, 10^6 PMN were suspended in 1 mL binding buffer (BD PharMingen, San Diego, CA) and 100 μL aliquots were removed for staining with 5 μL of annexin V-FITC (BD PharMingen) and 15 μL of 100 μg/mL 7-AAD (Sigma Chemical Co.). Cells were incubated for 15 min in the dark at room temperature (RT), followed by the addition of 400 μL of 1× binding buffer. Compensation of FITC and PE was performed using CaliBRITE beads (BD Immunocytometry). Using multicolor flow cytometric analysis (BD FACScan; BD Biosciences, Lexington, KY), surface expression of CD11b on unstimulated and fMLP-stimulated PMN, in both the apoptotic/non-necrotic and nonapoptotic populations, was determined by measuring the mean fluorescence of at least 5000 gated events in the FL1-FL2 channels, after exclusion of the 7-AAD–positive (late apoptosis/necrotic) populations. PMN populations were identified and gated based on specific forward- and side-scatter characteristics.

Enrichment of the nonapoptotic PMN fraction. Nonapoptotic PMN were separated from apoptotic PMN in 24-h cultures using immunomagnetic techniques, which we modified for this study (9,23). Briefly, cell aliquots were removed from culture and combined to obtain approximately 20–25 × 10^6 PMN. After a PBS wash, PMN were suspended in 0.1 mL binding buffer, then stained with 20 μL annexin V-PE (both from BD PharMingen) for 15 min at RT. PMN washed in binding buffer were then resuspended in 1 mL of the buffer. The cell suspension was stained with 100 μL of an anti-PE selection cocktail for 15 min at RT, followed by an incubation with 50 μL of magnetic nanoparticles (EasySep, StemCell Technologies, Vancouver, BC, Canada) for 10 min at RT. After the addition of 1.5 mL binding buffer, the PMN-magnetic particle suspension was transferred to a polystyrene tube, which was then placed in a magnet (EasySep), as per the manufacturer's instructions. The purity of the nonapoptotic (annexin V-negative) PMN suspensions was >90% as determined by flow cytometric analysis of eluted PMN by annexin V-PE staining. The resultant annexin V–negative enriched PMN were then incubated with PMA or PBS and stained using the DHR assay, as described below.

Measurement of ROI elaboration. This group of studies was designed to measure PMN elaboration of ROI, one marker of cytotoxic function. We used the flow cytometric DHR assay to determine ROI elaboration in 0-h PMN and in 24-h PMN enriched for the nonapoptotic fraction (as outlined above). The DHR assay is based on the oxidation of nonfluorescent DHR to the green-fluorescent compound, rhodamine 123, in the presence of ROI, particularly superoxide anion and hydrogen peroxide (24,25). Briefly, PMN at 0 h and 24 h (enriched annexin V–negative populations) were washed in PBS and resuspended in 0.2 mL PBS (5×10^6 cells/mL). Cells were incubated either in the presence of 10 nM PMA (Sigma Chemical Co.) to stimulate ROI production, or in PBS alone, for 15 min at 37°C. The reaction was stopped by washing treated PMN with ice-cold PBS. PMN in 0.2 mL PBS were stained with 10 μL DHR (Sigma Chemical Co., 500 ng/mL final) and incubated for 10 min at 37°C. After incubation, 500 μL ice-cold PBS were added to the stained cells, which were then kept on ice. The degree of rhodamine fluorescence emitted by stained PMN was immediately analyzed by flow cytometry on at least 5000 gated events, using the FL1 channel.

Data analysis. Data were analyzed by *t* test or one-tailed ANOVA, as appropriate, using SigmaStat for Windows software (Version 2.03, SPSS, Inc., Chicago, IL). A p value <0.05 was considered to be statistically significant. Data are presented as the mean ± SD.

RESULTS

Functional CD11b expression is maintained on PMN with prolonged survival. At 0 h, the majority (>96%) of PMN in cultures derived from adults or umbilical cord blood were nonapoptotic, as determined by negativity for annexin V staining (Fig. 1); the proportion of necrotic PMN was <1% in either population. A greater proportion of cord blood PMN were nonapoptotic (annexin V-negative) after 24 h of culture (35 ± 3%) compared with adult PMN (14 ± 4%, $p < 0.001$), similar to differences we previously reported (18). At 24 h, the proportion of necrotic (annexin V–positive/7-AAD–positive) PMN were similar in cord blood (6 ± 5%) and adult (7 ± 2%, $p = 0.35$) cultures.

We observed that surface levels of CD11b were higher on cord blood PMN at 0 h in the baseline, unstimulated state (mean fluorescence, 718 ± 12) than in adult PMN (594 ± 82, $p < 0.05$) (Fig. 2, *A* and *B*). However, chemotactic stimulation with fMLP increased surface expression of CD11b to a greater degree in adult PMN than in cord blood PMN (Fig. 3), results that are similar to those we and others have observed (19,26,27).

In 24-h surviving, nonapoptotic (annexin V–negative) cord blood and adult PMN (Fig. 2, *A* and *B*), baseline surface levels of CD11b were lower than those measured at baseline at 0 h

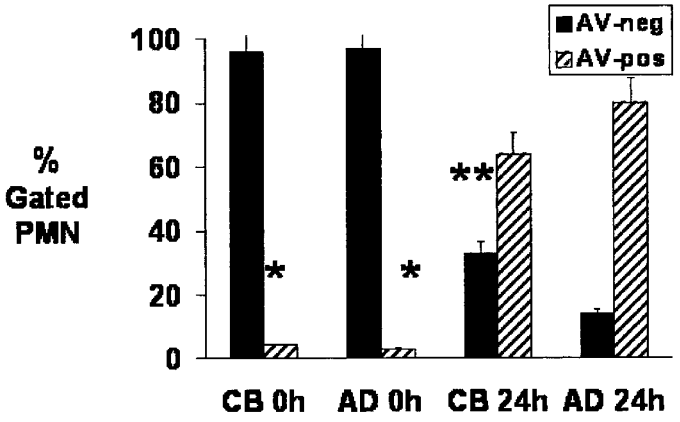

Figure 1. Surviving and apoptotic cord blood and adult PMN at 0 h and 24 h. To differentiate between surviving, nonapoptotic PMN, viable apoptotic PMN, and necrotic, nonviable PMN, cord blood (*CB*) and adult (*AD*) cells at 0 h and after 24 h of culture were stained with annexin V–FITC and 7-AAD, and gated PMN populations were analyzed by multicolor flow cytometry. The annexin V–negative (*AV-neg*) populations represent viable, nonapoptotic PMN; the annexin V–positive (*AV-pos*) populations represent the apoptotic, non-necrotic PMN (AV-pos/7-AAD-neg) population. Results represent data from experiments performed in duplicate ($n = 5$). *$p < 0.001$, 0-h AV-neg PMN (CB, AD) *vs* 24-h AV-neg PMN (CB, AD). **$p < 0.001$, CB 24-h AV-neg PMN *vs* AD 24-h AV-neg PMN.

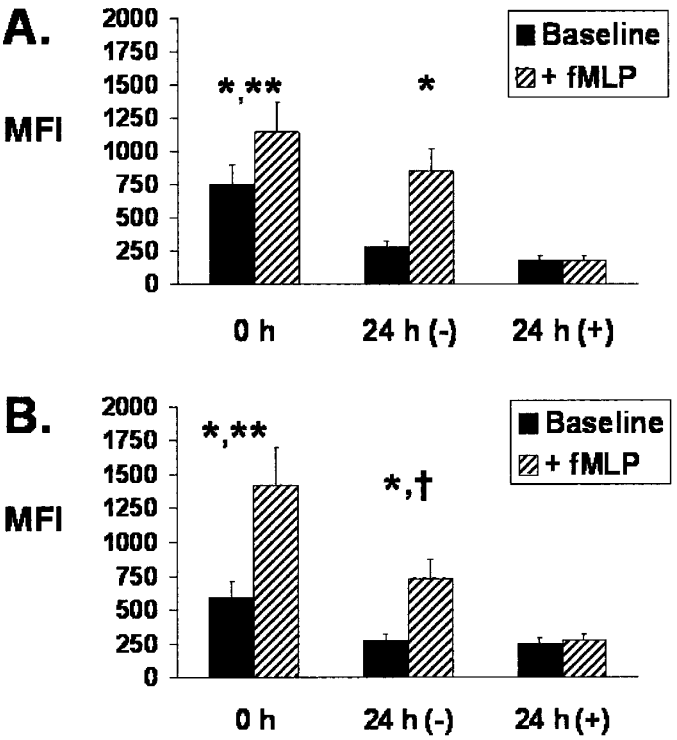

Figure 2. Surface CD11b expression on 0 h and 24 h PMN after chemotactic stimulation. At 0 and 24 h, CB (*A*) and AD (*B*) PMN were incubated with PBS alone (baseline) or fMLP (10 nM) for 15 min. After washing, cells were stained with annexin V-FITC, CD11b-PE, and 7-AAD. Gated PMN populations were analyzed by multicolor flow cytometry. Shown is the mean fluorescence intensity (*MFI*) of surface PMN CD11b at baseline or after chemotactic stimulation (+fMLP) at 0 h, at 24 h in nonapoptotic annexin V–negative PMN [24 h(−)], and at 24 h in annexin V–positive (apoptotic/non-necrotic) [24 h(+)] PMN. Results represent data from experiments performed in duplicate ($n = 8$). *$p < 0.001$, baseline *vs* stimulated CD11b levels; **$p < 0.05$, baseline CD11b on 0 h PMN *vs* baseline CD11b levels on 24 h(−) PMN and 24 h(+) PMN; †$p < 0.05$, stimulated CD11b levels on 24 h(−) PMN *vs* stimulated CD11b levels on 0 h and 24 h(+) PMN.

(cord blood PMN: 189 ± 66 *versus* 749 ± 267, $p < 0.001$; adult PMN: 269 ± 38 *versus* 594 ± 82, $p < 0.001$), and baseline surface expression of CD11b was higher on nonapoptotic adult PMN than on nonapoptotic cord blood PMN ($p < 0.05$). Stimulation with fMLP of surviving, nonapoptotic cord blood PMN induced a greater degree of up-regulation of surface CD11b levels from baseline values (202 ± 110%) than the degree of stimulation observed in 0-h cord blood PMN (63 ± 34%, $p < 0.05$; Fig. 3). In contrast, in adult samples, stimulation with fMLP induced up-regulation of CD11b to a similar degree at 0 h and in surviving 24-h PMN (164 ± 57% *versus* 166 ± 62%). Stimulation of nonapoptotic PMN with fMLP induced the up-regulation of surface CD11b to levels that were 46% and 51% of levels on stimulated 0-h cord blood and adult PMN, respectively. Surface CD11b levels on stimulated nonapoptotic cord blood (517 ± 13) and adult (731 ± 258) PMN were not statistically different from each other ($p = 0.24$).

Baseline surface expression of CD11b on the 24-h apoptotic, annexin V–positive populations was not different between adult (249 ± 57) and cord blood PMN (188 ± 91, $p = 0.22$; Fig. 2). Unlike the responses of nonapoptotic PMN, surface expression of CD11b on annexin V–positive, apoptotic cord blood or adult PMN did not increase over their respective baseline levels following stimulation with fMLP (Fig. 2, *A* and *B*; Fig. 3).

PMN with prolonged survival generate ROI. Using the DHR assay and flow cytometric analyses, we examined the elaboration of ROI at baseline and after PMA stimulation in 0-h adult and cord blood PMN, and at 24 h in PMN cultures enriched for the annexin V–negative fraction. At 0 h, PMN from both adult and cord blood sources showed minimal elaboration of ROI under baseline conditions (Fig. 4). In contrast, stimulation of these PMN with the phorbol ester PMA induced generation of ROI in both cell populations, with a greater relative expression of ROI in cord blood PMN samples (mean fluorescence, 876 ± 320 *versus* 600 ± 139 in adult PMN; $p < 0.05$). In studies of 24-h nonapoptotic PMN, PMA-stimulated cord blood PMN samples also had greater expression of ROI (458 ± 224) than did stimulated nonapoptotic adult PMN (280 ± 105, $p < 0.05$). In addition, although the expression of ROI was lower on 24-h nonapoptotic PMN compared with 0-h PMN, ROI expression on 24-h nonapoptotic cord blood PMN was not different from ROI expression on freshly isolated 0-h adult PMN ($p = 0.22$).

DISCUSSION

Our goal in the present study was to determine whether surviving neonatal PMN retain their potential for inflammatory and cytotoxic activity. In a set of *in vitro* experiments, we determined that, compared with adult PMN, nonapoptotic cord blood PMN that survived 24 h of culture had an enhanced response to stimulation as evidenced by their capacity to up-regulate CD11b and to elaborate ROI.

In studies focusing on inflammatory function, we observed that cord blood and adult PMN had a lower surface expression

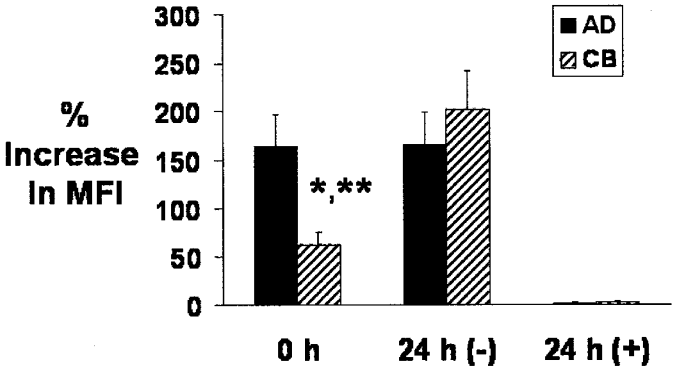

Figure 3. Up-regulation of CD11b on 0 h and 24 h PMN after chemotactic stimulation. Shown is the percentage up-regulation (increase) of surface CD11b levels (mean fluorescence intensity, *MFI*) after stimulation with fMLP (10 nM) in 0 h PMN, in surviving, nonapoptotic PMN [24 h(−)], and in apoptotic PMN [24 h(+)] from cord blood (*CB*) and from adults (*AD*). Results represent data from experiments performed in duplicate ($n = 8$). *$p < 0.05$, percentage up-regulation of CD11b on 0 h CB PMN *vs* 0 h AD PMN; **$p < 0.05$, percentage up-regulation of CD11b on 0 h CB PMN *vs* 24 h(−) CB PMN.

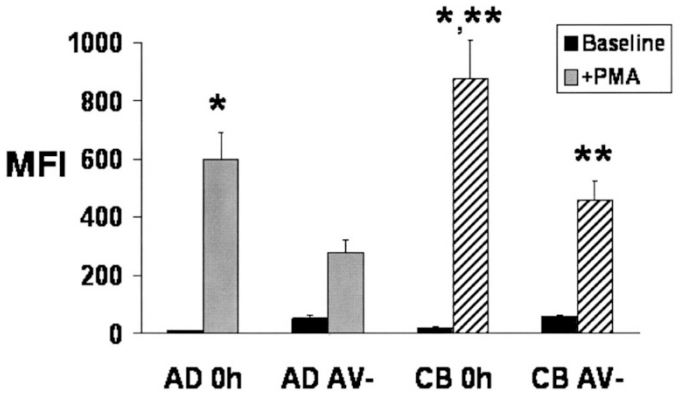

Figure 4. PMA-stimulated elaboration of ROI on surviving and apoptotic PMN. 0 h PMN and nonapoptotic, annexin V–negative (AV−) PMN from adult (*AD*) and cord blood (*CB*) were incubated with PBS alone (baseline) or with PMA (10 nM). After incubation, cells were stained with DHR and gated PMN populations were analyzed by flow cytometry in the FL1 channel. Shown here is the mean fluorescence intensity (*MFI*) of 0 h PMN and surviving (AV−) 24-h PMN at baseline or after PMA stimulation. Results represent data from experiments performed in duplicate ($n = 9$). *$p < 0.001$, PMA-stimulated ROI levels in 0-h PMN *vs* 24-h AV− PMN; **$p < 0.05$, PMA-stimulated ROI levels in CB PMN (0 h, 24 h AV−) *vs* AD PMN (0 h, 24 h AV−).

of CD11b after 24 h of culture compared with 0-h levels. Surviving 24-h adult PMN retained their capacity to up-regulate CD11b in response to chemotactic stimulation, confirming observations made by Dransfield *et al.* (9). In contrast, although 0-h cord blood PMN had impaired up-regulation of CD11b, as previously reported (28), stimulated surviving 24-h cord blood and adult PMN increased surface CD11b levels to a similar degree. The preservation of this function in surviving PMN has implications for their inflammatory potential. Up-regulation of surface CD11b in response to stimulation is critical to PMN-endothelial adhesion and is an important initial step during inflammation (29). This adherence is partly mediated through the interactions of PMN $\beta 2$ integrins, including CD11b, with endothelial ligands such as ICAM-1, the expression of which are increased on activated endothelium. Studies have shown a preponderance of ICAM-1 on lung vascular surfaces and in soluble form in the lung effluent and in the sera of premature neonates at risk for developing CLD (30). Pertinently, adult PMN with prolonged survival retain their capacity to adhere to fibrinogen, a CD11b-mediated event (9). Our observations of similar CD11b expression on surviving adult and cord blood PMN suggests that surviving cord blood PMN also retain an adhesive capacity, although this remains to be tested. Such functional interactions of surviving neonatal PMN with vascular endothelium would likely contribute to the persistence of the inflammatory response.

Neonatal PMN have a variety of functional impairments (26, 27,31,32) that might be related to incomplete terminal differentiation, as suggested by observations that segmented PMN in the bone marrow are functionally immature compared with those in the circulation (33). Cord blood has been observed to contain a mixed population of immature and mature PMN, and lower expression of CD11b was associated with decreased PMN maturity (34). Thus, the subset of cord blood PMN resistant to apoptosis might represent a population that attains an "adult phenotype" under conditions that promote apoptosis in more mature PMN. Our observation of the enhanced up-regulation of CD11b on stimulated 24-h cord blood PMN to the degree achieved by adult PMN lends support to this possibility.

To begin to define the cytotoxic potential of surviving neonatal PMN, we designed studies to determine their elaboration of ROI. We determined that 0-h cord blood PMN had a greater elaboration of ROI in response to phorbol ester stimulation compared with adult PMN, an observation also made by other investigators (32,35,36). After 24 h of culture, ROI elaboration was proportionately decreased in nonapoptotic PMN from both groups. However, nonapoptotic cord blood PMN maintained an enhanced responsiveness to stimulation relative to that of adult PMN, and, in fact, elaborated ROI at levels similar to those of 0 h adult PMN, an observation that also promotes the notion of an ongoing functional maturation of cord blood PMN. The elaboration of ROI by PMN is an important and necessary element in the elimination of pathogens during the clearance of infection (37) and is thus a beneficial aspect of normal PMN function. Conversely, the continued elaboration of ROI by lung PMN in the absence of a requirement for bactericidal activity has implications for damaging effects to the lung parenchyma (38–41). The significant degree of ROI elaboration by surviving neonatal PMN that we observed suggests that these cells are potent inducers of tissue injury.

PMN have been implicated as critical perpetrators of chronic inflammation in a variety of disorders, including CLD, and is a topic that has been elegantly reviewed (6). The persistence of lung PMN is a well-accepted characteristic of inflammatory lung diseases in both adults and neonates (38, 42, 43). Long-standing observations have confirmed a contributory role of oxygen radicals to the etiology of chronic lung inflammation in both the adult and neonatal populations (6,38–40). Furthermore, oxygen radicals have been shown to mediate DNA damage (44), an important mechanism of lung injury (45,46). PMN are not the sole source of ROI; other leukocytes, as well as the lung microenvironment itself, can potentially elaborate

these mediators (38,47). However, PMN are a major source of oxygen radicals (48,49), and evidence has shown a link between the depletion of PMN and a diminished development of inflammation in a variety of animal models (42,50,51).

Several recent studies have shown delayed apoptosis of lung PMN in premature neonates at risk for developing CLD (16,17), although the involved mechanisms remain to be defined. In previous *in vitro* studies, we observed that neonatal PMN had an intrinsic delay in both spontaneous and Fas-mediated apoptosis (18), which was associated with decreased activity of caspase-3 (52), a critical downstream mediator of PMN apoptosis (53). Delayed apoptosis and the subsequent survival of PMN leads to their persistence in tissues, because PMN removal by the reticuloendothelial system depends on the recognition of, and ingestion of, apoptotic PMN by resident macrophages (5–7). Our present studies confirm previous observations of preserved function in adult PMN with prolonged survival (9,54). In addition, however, we provide evidence that surviving cord blood PMN have an enhanced responsiveness to inflammatory stimuli. The intrinsic survival tendency of these PMN, combined with their functional robustness, indicate their potential contribution to the pathogenesis of neonatal inflammation. Certainly, other inflammatory cells, such as alveolar macrophages, or the cellular components of the microenvironment itself, likely play an important contributory role as well. Studies are currently underway to further define the functional capacity of surviving PMN and to investigate their role in these complex cellular interactions. In addition, a more complete understanding of the age-related factors that regulate PMN apoptosis and survival may be important in developing novel therapeutic approaches to neonatal inflammation (41,55).

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