Phagocytosis of neonatal pathogens by peripheral blood neutrophils and monocytes from newborn preterm and term infants

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BACKGROUND: Deficiencies in phagocytosis may contribute to the increased susceptibility of infants to early life infections. Data on phagocytosis of the major neonatal pathogens *Staphylococcus epidermidis* (SE), *Staphylococcus aureus* (SA), and *Escherichia coli* (EC) by preterm infant leukocytes are inconsistent.

METHODS: Cord and <24-h peripheral blood were collected from very preterm (<30.1 wks gestational age (GA)) and term (37–42 wks GA) infants. Monocyte and neutrophil phagocytosis of pHrodo-labeled SE, SA, and EC were analyzed using a small-volume flow cytometry assay, with simultaneous characterization of surface activation marker expression.

RESULTS: Preterm infants had lower proportions of monocytes and neutrophils capable of phagocytosis than term infants, but preterm infant phagocytes had higher phagocytic capacity. Phagocytosis was strongly correlated between cord and <24-h peripheral blood. Supplementation with exogenous complement significantly increased phagocytosis of EC but not of SE or SA. Monocyte human leukocyte antigen (HLA)-DR expression was lower in preterm infants but did not correlate with phagocytosis.

CONCLUSION: There is no defect in phagocytosis by monocytes and neutrophils from preterm compared with term infants, although preterm infants possess fewer phagocytes, possibly contributing to susceptibility to bacterial infection. Further investigation into the development of postnatal phagocytic competence is warranted.

Phagocytosis is an essential component of innate immune responses to pathogens, encompassing recognition, binding, engulfment, and degradation of particles such as bacteria. Although primarily an innate immune process, degradation and processing of microbes during phagocytosis for presentation via major histocompatibility complexes is also a crucial link to adaptive immune system responses (1). Individuals with phagocytic defects (e.g., Wiskott–Aldrich syndrome (2)) are at an increased risk of bacterial infection.

Very preterm infants (<30 wks gestational age (GA)) are extremely susceptible to bacterial infection, with risk inversely correlated with birth weight and GA (3,4). Early- and late-onset sepsis (occurring before and after 72 h, respectively) remain important causes of morbidity and mortality (4,5). The commonest pathogen group in preterm infants is coagulase-negative staphylococci, of which *Staphylococcus epidermidis* (SE) is the predominant species. Coagulase-negative staphylococci are responsible for ~50% of late-onset sepsis and 7–12% of early-onset sepsis episodes (4–6). *Escherichia coli* (EC), *Staphylococcus aureus* (SA) and Group B *Streptococcus* (GBS) account for the majority of non–coagulase-negative staphylococci infections in preterm infants (5,7,8).

Deficiencies of innate immune functions, including phagocytosis, cytokine production, and complement activity, have been reported in preterm infants and are suggested to contribute to their heightened susceptibility to bacterial infection (9,10). However, the data, particularly for deficiencies in phagocytosis, are inconsistent (11). There are no definitive comparisons of phagocytosis between adults and neonates; studies have identified positive (12,13), negative (14), and neutral (11,13) trends in functionality between the groups (15). Similarly, the reported correlation between GA and phagocytosis functionality is inconsistent (12,16). Discrepancies are also evident when comparing specific cell types, including monocytes and neutrophils (12–15).

Characterization of innate immune responses to common neonatal pathogens is essential to inform translational studies aiming to reduce the disease burden. However, immune studies of preterm cells is challenging, as blood samples from preterm infants (with a blood volume of <50 ml (17)) are limited in volume. Previous studies of preterm phagocytic responses to bacterial infection have largely used cord blood, as comparatively large volumes (5–30 ml) are readily obtained. However, characterization of postnatal peripheral blood is essential; there are few data on changes in innate

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immune function in the immediate postnatal period and it is unclear whether cord blood data are representative of peripheral blood responses.

To enable analysis of very preterm peripheral blood, we developed and optimized a small-volume, whole-blood assay utilizing a pH-sensitive dye pHrodo to detect monocyte and neutrophil phagocytic functionality. The formation of the phagosome around an ingested entity during phagocytosis relies on low pH for bactericidal functions and antigen processing and presentation (1,18). pHrodo is a highly pH-sensitive succinimidyl ester dye that fluoresces dramatically when exposed to low pH (<4.0). Phagocytosis is therefore detectable and quantifiable when the phagosome forms, and acidifies, around ingested pHrodo-labeled bacteria, minimizing high background fluorescence of cell-bound, but uningested, bacteria (18,19).

Our aims are to (i) describe this novel technique, (ii) compare monocyte and neutrophil phagocytic capacity in cord and peripheral blood, (iii) examine the effect of GA on phagocytosis by monocytes and neutrophils of common neonatal pathogens SE, SA, and EC, and (iv) investigate correlations between cellular activation marker expression and phagocytosis activity.

RESULTS

Detection of Phagocytosis With pHrodo-Labeled Bacteria Using Whole Blood

The phagocytosis assay conditions used for neonatal samples were initially optimized for time, dose, and minimum blood volume requirement using peripheral blood from healthy adult volunteers (data not shown). Incubation of preterm infant whole blood with pHrodo-labeled bacteria, under optimal conditions, resulted in a marked shift in fluorescence of phagocytic cells clearly detectable above the background of unstimulated blood (**Figure 1b**,**1d**). Both phagocyte populations (monocytes and neutrophils) were readily identified in preterm infant samples using a combination of anti-CD14 staining and side-scatter properties (**Figure 1a**). The percentage of pHrodo$^+$ cells and the associated median pHrodo fluorescence intensity (MFI) of phagocytic cells were readily determined. As expected, nonphagocytic lymphocytes did not fluoresce (**Figure 1c**). Blocking of phagocytosis in adult blood by preincubation with 20 μg/ml cytochalasin D (CD) was observed although complete abrogation of phagocytosis was not achieved (data not shown). Confocal microscopy of adult blood with pHrodo-labeled SE, with or without the addition of CD showed low levels of fluorescence of bacteria associated

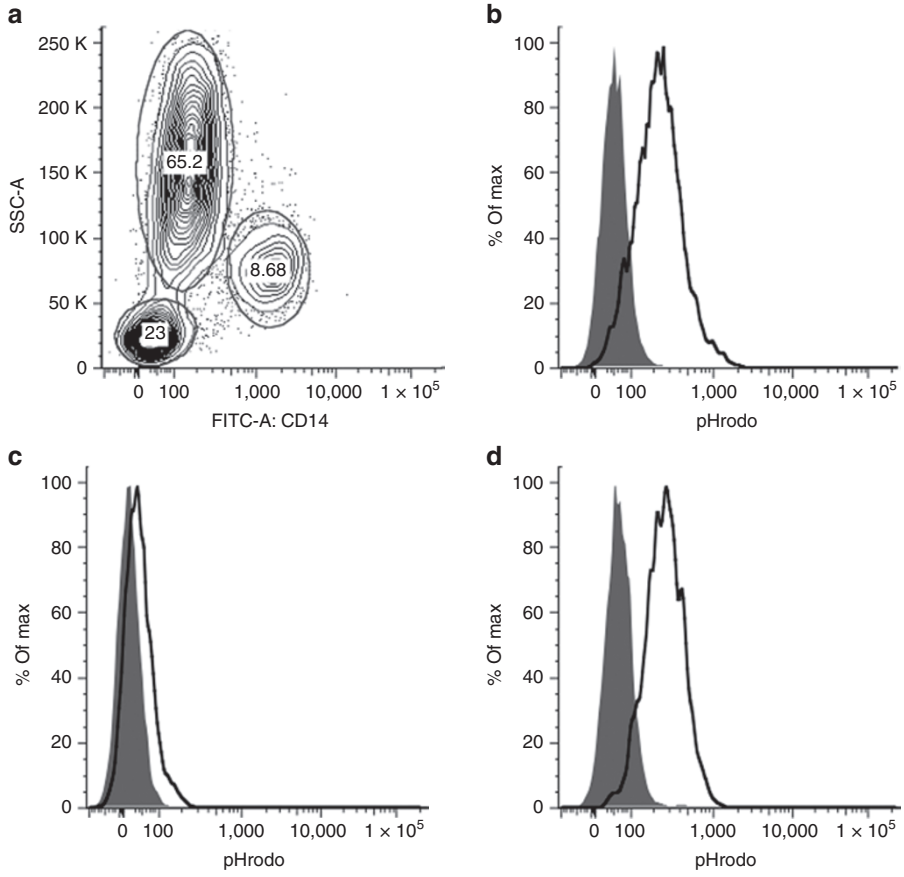

Figure 1. Analysis of phagocytosis using pHrodo-labeled bacteria. (**a**) Representative plot of an infant peripheral blood sample collected under the optimized phagocytosis protocol, showing inclusion gates and percentages for monocytes, neutrophils, and lymphocytes based on side-scatter area (SSC-A) and CD14 staining. (**b**–**d**) Representative histograms from (**a**) showing pHrodo fluorescence in the presence (solid black line) or absence (shaded grey) of pHrodo-labeled SE by neutrophils, lymphocytes, or monocytes, respectively.

with CD-treated cells in comparison with bright fluorescence following phagocytic engulfment (data not shown). Assay optimization was conducted using in-house pHrodo-labeled SE; however, conditions were maintained for commercially purchased pHrodo-labeled SA and EC to ensure comparability during analysis.

Phagocytosis of Important Neonatal Pathogens by Infant Peripheral Blood

Phagocytosis of SE, SA, and EC by <24 h peripheral blood cells was assessed and compared between preterm and term infants (Figure 2). Monocytes and neutrophils from all infants were able to phagocytose all three bacterial species, although SE was preferentially phagocytosed over SA and EC. Term infant blood samples had a significantly higher proportion of both monocytes and neutrophils capable of SE and EC ingestion than preterm infants (SE: 66 vs. 45%, $P = 0.0235$; and 54 vs. 36%, $P = 0.0207$, EC: 15 vs. 6%, $P = 0.0001$; and 12 vs. 3%, $P < 0.0001$ for monocytes and neutrophils, respectively). However, the level of SA and EC uptake (MFI) by preterm infant monocytes and neutrophils capable of phagocytosis was significantly higher than that by term infants (SA: 222 vs. 184, $P = 0.0013$; and 204 vs. 160, $P = 0.0002$, EC: 208 vs. 172, $P = 0.0104$; and 171 vs. 140, $P = 0.0003$ for monocytes and neutrophils, respectively). Similar trends were observed for SE (314 vs. 259, $P = 0.5861$ and 212 vs. 179, $P = 0.0462$, for monocytes and neutrophils, respectively; Figure 2).

Cord Blood Phagocytosis Responses Are a Good Surrogate for <24 h Peripheral Blood Responses

While examination of peripheral blood is ideal for determining postnatal phagocytic responses, cord blood is routinely used as a surrogate for many preterm infant studies. We therefore investigated whether cord blood phagocytic responses are representative of peripheral blood responses of 1-day-old neonates. The proportion of phagocytic monocytes and neutrophils in cord and <24 h infant blood were significantly correlated ($P < 0.0001$ using nonparametric Spearman correlation) directly and strongly for all bacteria tested (SE: $r = 0.76$ and 0.83; SA: $r = 0.68$ and 0.76; and EC: $r = 0.70$ and 0.72 for monocytes and neutrophils, respectively; Figure 3). The degree of bacterial uptake (MFI) by monocytes and neutrophils also correlated between cord and <24 h samples for all bacterial species, although less strongly than for the percentage of positive cells (SE: $r = 0.77$ and 0.76; SA: $r = 0.67$ and 0.78; and EC: $r = 0.58$ and 0.70 for monocytes and neutrophils, respectively; $P < 0.0001$ all comparisons, data not shown).

Cellular Activation Status Does Not Correlate With the Levels of Phagocytosis in Preterm and Term Infants

Expression levels of activation markers on phagocytes were determined to establish if the phagocytic differences observed between preterm and term infants were due to differences in cellular activation. Monocyte expression of human leukocyte antigen (HLA)-DR and CD86, and neutrophil expression of CD64, were compared between preterm and term infants using <24 h blood samples. Monocyte expression of CD86 was comparable between preterm and term infants, as was neutrophil expression of CD64, with no significant difference between the groups for either marker. Of note, expression of the monocyte lineage and activation marker HLA-DR was more than two-fold higher on monocytes from term compared with preterm infants (MFI of 5,584 vs. 2,500, $P = 0.0003$; Figure 4). The effect of differential HLA-DR expression was investigated by

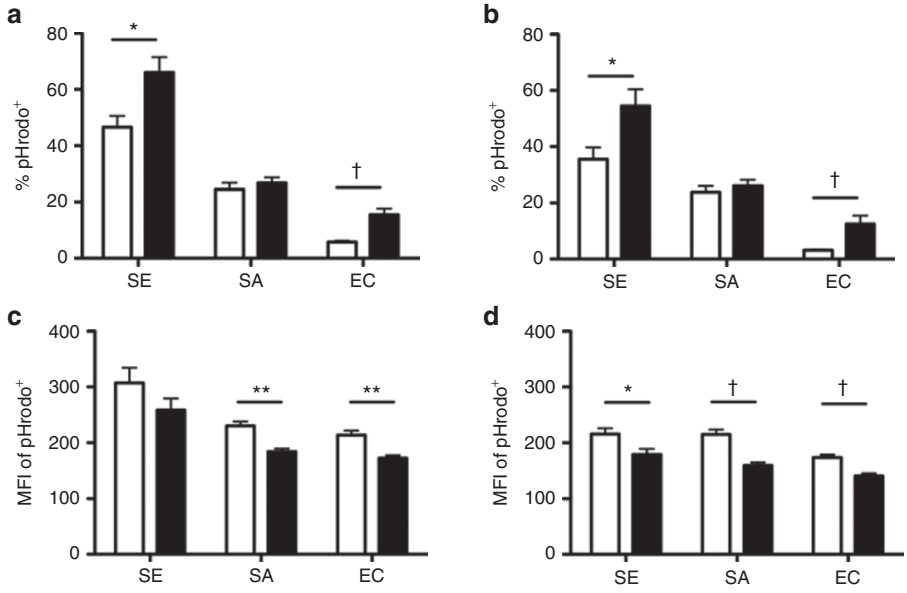

Figure 2. Phagocytosis of SE, SA, and EC by term and preterm infants. Peripheral (<24 h postnatal) blood samples from very preterm (<30.1 wks GA, white bars, $n = 57–58$) and term (37–42 wks GA, black bars, $n = 20$) infants were incubated with 1×10^8 bacteria/ml before analysis by flow cytometry. The percentages of pHrodo$^+$ monocytes (**a**) and neutrophils (**b**) and MFI of pHrodo + monocytes (**c**) and neutrophils (**d**) were compared. *$P < 0.05$; **$P < 0.005$; †$P < 0.0001$. EC, *Escherichia coli*; MFI, median pHrodo fluorescence intensity; SA, *Staphylococcus aureus*; SE, *Staphylococcus epidermidis*.

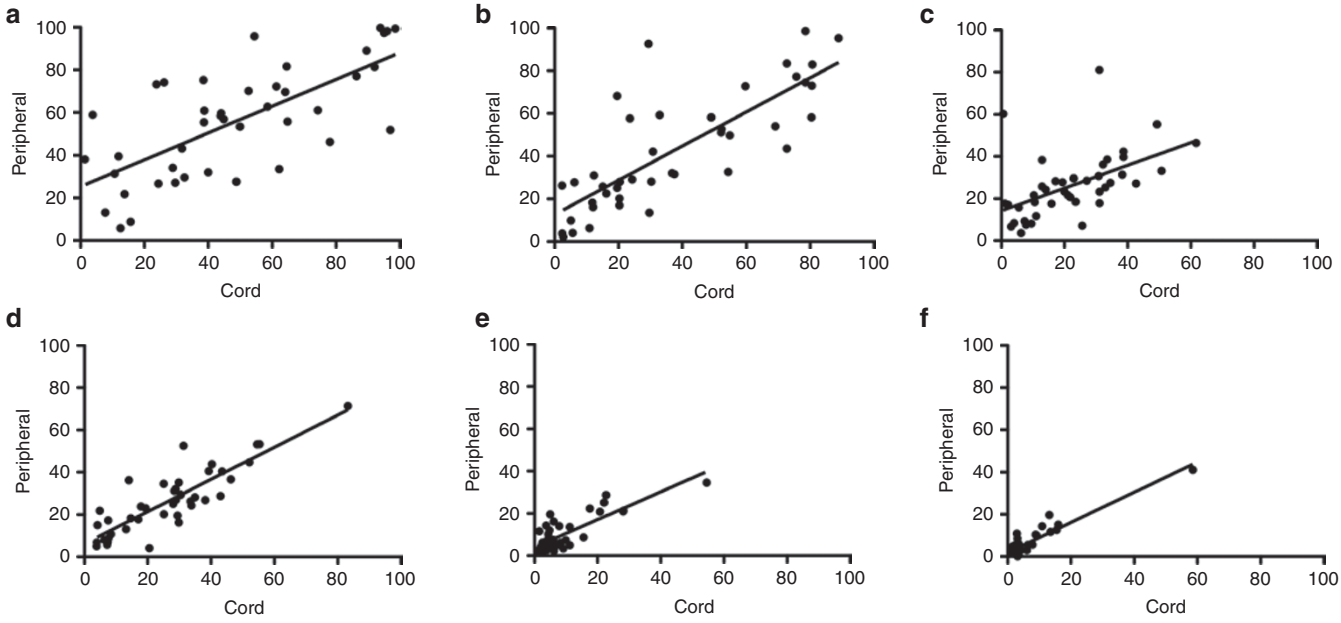

Figure 3. Correlation of infant (<30–42 wks GA) cord and peripheral blood phagocytosis of SE ($n = 42$), SA ($n = 41$) and EC ($n = 42$) by monocytes (panels **a**, **c**, and **e**, respectively) and neutrophils (**b**, **d**, and **f**, respectively). Data shown are percentages of pHrodo$^+$ phagocytes. The degree of correlation was determined using the nonparametric Spearman correlation with r values as follows: **a** = 0.76, **b** = 0.83, **c**: $r = 0.68$, **d**: $r = 0.76$, **e**: $r = 0.70$, **f**: $r = 0.72$. $P <$ 0.0001 for all correlations. EC, *Escherichia coli*; SA, *Staphylococcus aureus*; SE, *Staphylococcus epidermidis*.

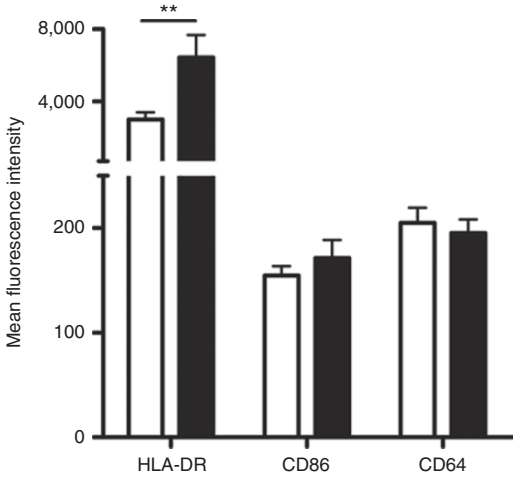

Figure 4. Expression levels of monocyte and neutrophil activation markers. Peripheral blood from preterm ($n = 57$; white bars) and term ($n = 19$; black bars) infants was incubated with anti-HLA-DR, anti-CD86, and anti-CD64 antibodies before analysis by flow cytometry. Data plotted are the median fluorescence intensity (MFI) of HLA-DR and CD86 expression by monocytes (identified as Lin-2$^+$HLA-DR$^+$) and CD64 expression by neutrophils (identified as SSC^{hi}Lin2$^-$). $^{**}P = 0.0003$. HLA, human leukocyte antigen.

correlating expression levels with phagocytosis (both proportion of phagocytic cells and MFI). No significant correlation of either measure of phagocytosis with HLA-DR expression was observed (data not shown).

Addition of Exogenous RbC Selectively Enhances Phagocytosis in Preterm Infants

Hypocomplementemia is suggested to be critical in preterm infant phagocytic function (20). We therefore investigated the role of complement by adding whole blood to pHrodo-labeled bacteria with or without rabbit complement (RbC) and comparing the nonopsonic phagocytic capability of each sample and the potentially additive effect of exogenous complement. The proportion of cells phagocytosing EC was significantly increased in both term and preterm neutrophils and preterm monocytes, whereas phagocytosis of SE and SA was not altered for either cell type with the addition of RbC (**Table 1**).

DISCUSSION

The simultaneous assessment of multiple bacterial species and cell types and the various outputs for phagocytosis described is the first definitive description of the differences in preterm and term infant phagocytic capabilities. Preterm infants have fewer peripheral blood monocytes and neutrophils capable of bacterial phagocytosis than term infants, but their remaining phagocytes possess higher capacity for bacterial uptake than those from term infants. These differences were independent of phagocyte activation and, for SE and SA, not influenced by exogenous complement. We also demonstrate that cord blood provides a reasonable surrogate for <24 h peripheral blood from preterm and term infants.

The development of an assay to simultaneously detect phagocytosis by monocytes and neutrophils in a minimal whole blood volume was essential. This is a broadly relevant issue as current data on neonatal phagocytosis, largely derived from cord blood are conflicting and their relevance to the age at which neonatal sepsis occurs *in vivo* is unclear. Previous studies have reported both positive (16) and negative (12) correlations between GA and phagocytosis. Furthermore, studies comparing phagocytosis activity of monocytes and neutrophils from adults and

Table 1. Phagocytosis with and without exogenous rabbit complement (RbC)

	Monocytes				Neutrophils			
	Preterm		Term		Preterm		Term	
%	WB	+RbC	WB	+RbC	WB	+RbC	WB	+RbC
SE	46±5	40±5	66±5	53±6	36±5	33±5	54±6	46±5
SA	24±3	25±3	27±2	24±2	22±2	27±3	26±2	28±3
EC	6±1	11±1[a]	16±2	15±2	3±1	12±2[b]	12±3	18±2[c]
MFI								
SE	314±39	275±27	259±20	220±12	212±12	207±10	179±10	169±8
SA	223±9	224±10	184±5	184±6	205±9	211±10	160±5	165±7
EC	208±9	204±11	173±5	169±5	171±7	181±9	141±5	145±4

EC, *Escherichia coli*; MFI, median pHrodo fluorescence intensity; SA, *Staphylococcus aureus*; SE, *Staphylococcus epidermidis*; WB, whole blood.

[a]$P = 0.0004$. [b]$P < 0.0001$. [c]$P = 0.0058$.

Whole blood samples were added to 10^8 pHrodo-labeled bacteria/ml combined with or without RbC. Samples were analyzed by flow cytometry and the percentage of pHrodo$^+$ cells and MFI of pHrodo$^+$ cells was determined. Data shown are mean ± SEM.

neonates have reported diminished (14), enhanced (12,13), or equivalent capability (11,13). These inconsistent findings are likely to reflect methodological differences, as sample type (e.g., purified cells or whole blood), preparations and species of bacteria, together with the use of humoral factors (e.g., complement) vary considerably between studies (11). Although previous studies have utilized pHrodo as a tool for assessing phagocytosis, none have extended its use to simultaneous analysis of multiple cell types, or to whole blood assays (18,21).

We found that preterm infants possess a decreased proportion of whole-blood phagocytes capable of phagocytosing the common neonatal pathogens SE, EC and, to a lesser extent, SA compared with term infants. The paucity of current studies comparing phagocytosis functionality between term and preterm infants and the variations in methodologies employed make it difficult to compare our data to published observations. However, the decreases observed in the proportion of phagocytic cells in preterm compared with term infants are supported by previous investigations of neutrophil phagocytosis of EC and SA (22,23). The higher phagocytic potential in preterm infants observed in our study has also been previously reported for phagocytosis of EC by monocytes and GBS by neutrophils (12,24). By contrast, other studies and our own previous data have described reduced or equivalent phagocytic potential of neutrophils and monocytes from preterm and term infants for EC, SE, or GBS (22,24,25). These discrepant data are most likely due to methodological differences in assessing phagocytosis.

The key observation of preferential phagocytosis of bacteria, particularly SE, by monocytes compared with neutrophils has not been previously reported. These findings, if corroborated, may guide future interventions that target specific cell types that are most important in phagocytosing specific pathogens. In keeping with this approach, we have previously shown that monocytes are the key cytokine-producing cell type when exposed to SE or GBS and that phagocytosis is essential for stimulating cytokine production in these cells (24).

The phagocytic responses of neutrophils and monocytes from cord and <24 h peripheral samples were strongly correlated and highly comparable, indicating that phagocytosis functionality is maintained over this relatively short yet clinically relevant time frame.

Cord blood is routinely used as a noninvasive, easily accessible neonatal sample as a surrogate for peripheral samples in immunological studies (24,26,27). However, the correlation between phagocytosis in cord and peripheral blood has not been reported previously. Phagocytosis of EC by monocytes from cord blood and day 3 peripheral blood has been investigated but no statistical comparisons were reported (28). Our data are therefore important in verifying cord blood as a suitable and convenient surrogate to explore early peripheral responses in infants. Our optimized, low sample volume protocol allows investigation of phagocytosis of peripheral samples in the first weeks of life, the time of greatest risk for early-onset sepsis and late-onset sepsis (3).

Variations in phagocytosis levels in response to different bacteria were as anticipated. Many bacteria have developed immune evasion mechanisms, such as polysaccharide capsules, surface expression of protein A causing incorrect orientation of opsonizing antibodies and the antiphagocytic effect of lipopolysaccharide (29–31). These different mechanisms may account for the differential phagocytosis of SE, SA, and EC, indicating pathogen-specific phagocytic capabilities of preterm and term infants. Neutrophils have previously been shown to phagocytose unopsonized SE more readily than EC, as we observed (32). The more effective phagocytosis of SE over SA and EC, although counter-intuitive, suggests that there is no defect in preterm phagocytosis of the most common pathogen in this population. Instead, the pathogenicity of SE appears to be reliant on evasion of the postengulfment killing mechanism of phagocytes. One possible caveat to these findings is the necessary use of heat-killed bacterial preparations, and commercially prepared SA and EC stocks that are non-neonatal sepsis isolates, which may influence interactions

with infant phagocytes. We have previously shown that heat-killed SE activates a reduced set of innate immune responses in comparison to live SE, at least in terms of cytokine release (33). Future studies could examine the real-time phagocytosis of live, neonatal bacterial isolates by infant monocytes and neutrophils (18). However, this would first require modification and optimization of the methodologies used, in order to ensure they are compatible with the very small postnatal blood volumes available from extremely preterm infants.

The activation markers HLA-DR, CD64, and CD86 have previously been associated with early diagnosis and/or prognosis of sepsis and bacterial infection (34–36). Although this is the focus of much of the investigation into HLA-DR expression, this is the first study to correlate expression with phagocytosis. Of note, although expression levels were significantly lower in preterm infants, as previously described (36,37), there was no correlation of expression with reduced phagocytosis, suggesting that the prognostic utility of HLA-DR expression is independent of phagocytosis functionality. The difference in HLA-DR expression and any effect on phagocytosis may be transient, as others have reported equivalent expression levels between preterm and term infants up to 7–9 d of age (38). Similarly, expression of CD64 on neutrophils and CD86 on monocytes was not correlated with phagocytic activity. Levels were comparable between preterm and term infants, as previously described for CD64 (35,39), although decreased CD86 has been reported in preterm neonates (37). These results are perhaps not surprising, given that all preterm infants included in this study were healthy and had not been exposed to chorioamnionitis *in utero*. Expression of these activation markers is independent of phagocytosis, suggesting the altered phagocytic capability of preterm and term infants is not due to activation state of their phagocytic cells.

The role of complement in phagocytosis is critical, opsonizing bacteria to aid engulfment and removal of infection (14). Newborns, particularly preterm infants, have decreased levels and function of complement pathways (9,10,40). Phagocytosis of GBS, SE, and EC by isolated phagocytes has been shown to increase with, or be complement-dependent (25,41,42). However, in the presence of native complement in whole blood, we have shown that complement from preterm infants is not deficient or functionally defective for the uptake of SE and SA. We have also previously reported similar observations for GBS phagocytosis (24). The important observation that restoration of the proportion of preterm infant cells capable of phagocytosing EC to term infant levels with the addition of rabbit complement suggests that EC phagocytosis is indeed complement-dependent. Of note, neutrophils appear to be more sensitive to complement than monocytes for phagocytosis of EC, with an increase in both term and preterm infant phagocytic cells observed with the addition of exogenous complement. Our findings indicate that a complement deficiency or malfunction in preterm infants is not responsible for the differences in phagocytosis of SE or SA between preterm and term infants, but does contribute to decreased phagocytosis of EC.

In conclusion, we have identified a decrease in number but an increase in functionality of phagocytic cells in preterm compared with term infant whole blood. These differences and the magnitude of phagocytosis measured are comparable in cord and <24 h peripheral blood, confirming cord as a reasonable surrogate for peripheral samples immediately after birth. Deficiencies in phagocyte activation in preterm infants were not responsible for the disparities between term and preterm phagocytosis; however, complement plays a role in the proportion of cells capable of ingesting EC. Preferential phagocytosis of SE over SA and EC suggest pathogen-specific phagocytosis functionality. The immune defects underlying the increased susceptibility of preterm infants to SE may therefore reside in facets of the innate immune response other than phagocytosis. These findings warrant additional investigation, particularly at later time-points to identify further differences in the development of phagocytosis functionality between preterm and term infants, and whether these contribute to the heightened susceptibility to infection in preterm infants.

METHODS

Blood Sampling and Preparation

The King Edward Memorial Hospital (Perth, Australia) Ethics Committee approved the study protocol and written, informed consent was obtained (814/EW). Cord blood was collected into preheparinized syringes as soon as possible (within 4 h) postnatally from cord vessels, and where necessary, from vessels feeding into cord vessels, immediately adjacent to the base of the cord on the fetal placental surface. Collection sites were alcohol swabbed before sampling to minimize possible maternal blood contamination. The majority of preterm cord samples (20 out of 26) were collected <1 h after delivery (median collection time 14 min), four were collected between 1 and 2 h after delivery (median collection time of 100 min) and only two were collected 3 h postdelivery. All term infant cord samples were collected <2 h after delivery (median collection time of 74 min). Infant <24 h peripheral venous blood samples were collected into heparinized tubes within 24 h of birth. To exclude possible confounding by concurrent infection, we excluded infants with histological evidence of chorioamnionitis or with subsequent early-onset sepsis. The demographics of each donor group were: 58 preterm infants (27 female) median 27.6 wks GA (range: 23.4–30.1) and 20 term infants (11 female) median 40 wks GA (range: 37.4–42).

Heparinized blood samples from healthy adults were used to optimize experimental conditions and minimize the blood volume for subsequent infant samples. Additionally, heparinized adult blood samples were left for up to 48 h at room temperature (RT) after collection, before the phagocytosis assay, to determine the reliability of neutrophil and monocyte phagocytosis when measurement was delayed. The stability of the pHrodo signal after phagocytosis in monocytes and neutrophils was determined by storing adult samples, postassay, at 4 °C in the dark for 1 or 3 d before flow cytometry to determine the reliability of batch analysis. No significant differences in fluorescence intensities were observed between batches (data not shown).

pHrodo Labeling of Bacteria

The SE isolate (wild-type strain 1457) was originally from a patient with an infected central venous catheter. Stocks were grown to mid-log phase (OD_{600}: 0.7–0.8) in Heart Infusion Broth (Oxoid, Adelaide, Australia) and heat-killed at 80 °C for 15 min. Postkilling nonviability of the SE was confirmed by culture. Total bacterial counts were made using a Helber Bacteria Counting Chamber (ProSciTech, Thuringowa, Australia). Bacteria were washed in pyrogen-free phosphate-buffered saline (PBS) and counted before labeling with pHrodo dye (Invitrogen, Mulgrave, Australia), in keeping with the manufacturer's protocol. Briefly, 2.5×10^9 bacterial cells were equilibrated to pH 8.5 by washing

in 100 mmol/l sodium bicarbonate. Cells were resuspended in 750 µl sodium bicarbonate and incubated with 0.5 mmol/l pHrodo dye for 45 min at RT. Several wash steps with methanol and PBS removed excess dye. Labeled bacteria were resuspended in H_2O and either used immediately or lyophilized and frozen (−20 °C for future reconstitution). Bacteria were vortexed and sonicated to ensure single cell suspensions before counting. The pH response range of the dye was routinely verified by comparing fluorescence in PBS of pH 7.4 and pH 4.0.

Commercial stocks of prelabeled, lyophilized SA and EC strain K12 (both from Invitrogen) were reconstituted in H_2O before counting and aliquots were lyophilized and stored at −20 °C. SA, EC, and SE stocks were stored at a concentration of 2.5×10^9/ml.

Phagocytosis Assay

Whole blood (25 µl) was transferred to wells of polypropylene 96-well round-bottomed plates (Corning Inc., Corning, NY) with complete medium (RPMI, Gibco, Life Technology, Paisley, Scotland) with 5% heat-inactivated fetal calf serum (MultiSer Biosciences, Castle Hill, Australia), 10 mmol/l HEPES, 1 mmol/l sodium pyruvate and 550 µmol/l β-mercaptoethanol (all Invitrogen) to a final volume of 120 µl. Samples were incubated either with or without 10% (v/v) baby RbC PelFreez, Rogers, AR).

Samples were incubated at 37 °C with EC, SA, or SE at 10^8 bacteria/ml for 15 min, before transfer to ice to halt phagocytosis. Cytochalasin D (20 µg/ml; Sigma-Aldrich, Sydney, Australia) was added to some experiments using adult blood, 10 min before addition of bacteria to inhibit phagocytosis. The multiplicity of infection value was ~50:1 (bacteria:phagocyte) based on the median of accepted ranges of phagocytes (total neutrophils plus monocytes) in neonates (12×10^9/l (43)). Samples were then incubated with 1 µl of antihuman CD14–fluorescein isothiocyanate (FITC) (BD, North Ryde, Australia) on ice for 15 min. Cells were washed twice with 2 ml ice-cold PBS and red blood cells were lysed by incubation with 1 ml FACSLyse (BD) for 15 min at RT. Cells were washed once in PBS, resuspended in Stabilizing Fixative (BD) and stored at 4 °C in the dark before analysis. Monocytes were subsequently identified during FACS analysis as $SSC^{int}CD14^+$ cells, and neutrophils as $SSC^{hi}CD14^-$ cells. Validation checks using adult blood samples demonstrated the pHrodo signal in fixed neutrophils and monocytes was stable for at least 3 d at 4 °C (data not shown), supporting batch acquisition of sample data by FACS.

Microscopy Confirming Phagocytosis

Adult mononuclear cells were coincubated with an multiplicity of infection of 100:1 pHrodo-labeled SE for 1 h at 37 °C with or without pretreatment with 20 µg/ml CD for 10 min. Cells were harvested, washed in PBS and cyto-centrifuged onto microscopy slides. Nuclear staining was performed using Hoechst 33342 (Invitrogen) and mounted using low-fade mounting media. Fluorescent and transmission imaging used a Bio-Rad MRC 1000 confocal laser-scanning microscope (Bio-Rad Laboratories Pty., Ltd., Gladesville, Australia) with PlanApo objectives.

Activation Marker Expression Analysis

Separate aliquots of blood (150 µl) were incubated with the following antibodies; Lin-2 FITC and HLA-DR PECy7 together with either CD86 APC and CD64 PE or matched isotype controls (all from BD) for 15 min at RT. Cells were lysed with 2 ml FACSLyse (BD) for 15 min at RT, then washed and resuspended in stabilizing fixative (BD) and stored at 4 °C in the dark before analysis.

Flow Cytometry

Assay optimization experiments were analyzed using a BD FACSCalibur dual-laser flow cytometer (BD) and validation experiments were acquired with a BD FACSCanto II triple-laser machine (BD). Two-color analysis of green and red fluorescence detected CD14-FITC positive monocytes and pHrodo positive phagocytes, respectively. Detection of FITC fluorescence was achieved on both flow cytometers using a 530/30 nm band-pass filter while pHrodo fluorescence was detected through a 670 nm long-pass filter. No compensation for the two parameters was required. Equivalent fluorescence intensities for both machines were established using SPHERO Rainbow calibration beads (SpheroTech Inc., Lake Forest, IL) and checked weekly. Untreated whole blood samples served to determine the cut-off for fluorescence for phagocytosis and 2,000 monocytes were collected from each sample.

Activation marker assessment experiments were analyzed on the same FACSCanto II flow cytometer using the following band-pass filters for detection; FITC: 530/30 nm, PECy7: 780/60 nm, APC: 660/20 nm, and PE: 585/42 nm. Compensation controls were included for each fluorophore and the matrix was calculated and applied using Flowjo software (Tree Star, Ashland, OR). Monocytes (>2,000) were identified as $Lin\text{-}2^+HLA\text{-}DR^+$ and neutrophils as $Lin\text{-}2^-SSC^{hi}$. Matched isotype controls for CD64 and CD86 were included to determine the gate identifying positive expression. All data were analyzed with Flowjo software.

Statistical Analysis

Two-group comparisons were made by Mann-Whitney *U* and Spearman's test was used for analysis of correlations. Analyses were conducted using Prism 5 for Mac (GraphPad, La Jolla, CA). Data presented as bar graphs are shown as mean ± SEM. Differences were considered significant at $P < 0.05$.

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