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Neutrophil and monocyte toll-like receptor 4, CD11b and reactive oxygen intermediates, and neuroimaging outcomes in preterm infants

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BACKGROUND: Activated leukocytes and infection are implicated in neonatal brain injury. Leukocyte surface receptors are increased in stroke models and may be targets for future adjunctive therapies.

METHODS: Serial blood samples were analyzed from preterm infants ($n = 51$; <32 wk gestation) on days 0, 1, 2, and 7 of life. Monocyte and neutrophil activation were evaluated via flow cytometry at baseline and following endotoxin stimulation *ex vivo* by measuring CD11b (activation), toll-like receptor 4 (TLR-4; endotoxin recognition) expression, and intracellular reactive oxygen intermediate (ROI) production (function).

RESULTS: Control preterm infants with normal neuroimaging had elevated baseline CD11b and TLR-4 expression and ROI production compared with adults as well as a robust immune response following endotoxin stimulation. Preterm infants with abnormal neuroimaging had increased neutrophil TLR-4 and ROI compared with all controls.

CONCLUSION: Preterm infants have a robust immune response compared with adults. Increased TLR-4 expression in preterm infants with abnormal neuroimaging is similar to findings in adult stroke. In addition, ROI production may cause tissue injury. The modulation of these responses may be beneficial in preterm inflammatory disorders.

Preterm infants are susceptible to inflammatory disorders resulting in multiorgan dysfunction (1). Systemic inflammation may be the final common pathway for insults caused by both hypoxia–ischemia and infection in these infants and may be associated with brain injury (2) (see **Supplementary Reference 1** online). Many studies demonstrate an association between maternal/fetal infection and periventricular leukomalacia (PVL) detected on cranial ultrasound (3) (see **Supplementary References 2–5** online), or the later development of cerebral palsy (CP) (4) (see **Supplementary References 6–10** online). Elevated cytokines have been detected histologically in the brains of preterm infants who died with white matter injury (5) (see **Supplementary References 11–14** online). Postnatal infection also contributes to the development of PVL and CP (6,7). Inhibiting inflammatory responses may decrease secondary brain injury following infection or hypoxia–ischemia (8) and recently tertiary brain injury has been described as a possible mechanism of preterm brain injury with persistent long-term inflammation (9). We were interested in the systemic inflammatory response in newborn infants at risk of brain injury by examining markers of activation of monocytes and neutrophils.

Reactive oxygen intermediate (ROI) generation is essential for neutrophil intracellular killing of invading microorganisms following phagocytosis. ROIs are a major mechanism of innate antimicrobial host defense (see **Supplementary Reference 15** online) but can cause damage by oxidizing membrane phospholipids, proteins, nucleic acids, and nucleotides (10). We studied ROI production as a marker of immune cell function. CD11b is a receptor on the cell surface that is important for neutrophil and monocyte migration to sites of infection/inflammation. Neonatal neutrophil migration is decreased at birth due to decreased total cell content of CD11b and issues related to its cell surface translocation. Although baseline CD11b expression is reported to be similar to that of adults, neonates are unable to upregulate CD11b expression to the same magnitude following lipopolysaccharide (LPS) stimulation, especially in preterm infants (11). The key receptor for recognizing endotoxin on the immune cell surface is toll-like receptor 4 (TLR-4). Healthy neonates have similar basal TLR-4 expression to adults. Both term and preterm neonates increase TLR-4 expression in response to LPS. Responses to LPS are determined by the level of TLR-4 expressed, and overexpression can lead to uncontrolled inflammation resulting in damage to healthy tissues. Shen *et al.* (12) showed a rapid increase in TLR-2 and TLR-4 expression over the first month of life but no parallel increase in LPS-induced cytokine production.

We hypothesized that markers of neutrophil and monocyte activation may be altered in preterm infants with abnormal

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neuroimaging. We examined markers of monocyte and neutrophil function and activation (CD11b (activation), TLR-4 expression (LPS recognition), and ROI production (function)) serially over the first week of life and correlated our findings with neuroimaging.

RESULTS

Patient Demographics

Fifty-one preterm infants born <32 wk gestation were included, and three infants died (normal neuroimaging $n = 40$; abnormal neuroimaging/ Death (RIP); $n = 11$). A total of 404 samples were processed. There were no differences in gender distribution, preeclampsia, prolonged rupture of membranes, maternal pyrexia, histological chorioamnionitis, surfactant treatment, gestational age, birth weight, mode of delivery, doses of antenatal steroids received, Apgar scores, cord or admission blood gas parameters, nasal continuous positive airway pressure (CPAP) hour, or duration of intubation, between preterm neonates with normal and abnormal neuroimaging (**Table 1**). There was a statistically significant difference in mortality (*n* (%)) observed between the two groups: normal vs. abnormal neuroimaging 0 (0) vs. 3 (21) ($P = 0.04$).

All infants had serial cranial ultrasounds, and 29 infants had a magnetic resonance imaging (MRI) brain at term corrected age. MRI was scored according to the Inder criteria (6) independently by a consultant Pediatric Radiologist. Twenty infants had completely normal imaging (white matter (WM) score = 5–6; gray matter (GM) score 3–5). Nine infants had evidence of WM abnormality (mild = 4: WM score 7–9; moderate = 3: WM score 10–12; severe = 2: WM score 13–15). One infant had both WM and GM abnormality (GM score 6–9; **Tables 2** and 3). Twenty-two infants had only cranial ultrasound imaging. No abnormality was detected in 14 infants. Evidence of intraventricular hamorrhage (IVH) was detected in five infants (grade 1 = 4; grade 2 = 1). Three infants had increased echogenicity in the periventricular WM.

There were no other significant differences between the study groups with respect to chronic lung disease, patent ductus arteriosus, necrotizing enterocolitis, retinopathy of prematurity, late-onset sepsis (LOS), and number of septic episodes and antibiotic days. There were no significant differences in duration of intubation, intermittent positive pressure ventilation, nasal CPAP or nasal prong oxygen hours required, duration of free flow oxygen delivery, or maximum inspired oxygen requirements during neonatal intensive care unit stay in our study population.

Neonatal Neutrophil and Monocyte ROI Production

Preterm infants with abnormal neuroimaging produced significantly increased baseline intracellular neutrophil ROIs on day one of life compared with preterm controls ($P = 0.023$). There was a statistically significant increase in baseline ROI production in control preterms at 24–48 h ($P = 0.047$) and preterms with abnormal neuroimaging at 0–24 h of life ($P = 0.037$) compared with adults (**Figure 1a**). Irrespective of neuroimaging outcome, all preterms were LPS responsive and had increased ROI production following *ex vivo* LPS stimulation. Higher levels of ROI production were seen in both preterm groups compared with adults following stimulation which was statistically significant at 24–48 h ($P = 0.016$) and 48–72 h ($P = 0.007$) in the preterm control group and at 0–24 h ($P = 0.004$) and 24–48 h ($P = 0.044$) in the abnormal neuroimaging group (**Figure 1a**). Monocyte ROI production is significantly lower than that of neutrophils at ~20%. The baseline and poststimulation monocyte ROI level appeared greater in all neonates compared with adults throughout the first week of life and was significantly higher in the preterm control group on day 7 (baseline $P = 0.004$; LPS $P = 0.018$; **Figure 1b**).

Preterm infants born between 28 and 32 wk gestation produced significantly greater levels of neutrophil derived ROI at baseline compared with adults at 24–48 and 48–72 h of life ($P = 0.016$ and 0.038, respectively). All preterm neonates produced higher levels of polymorphonuclear leukocyte (PMN) ROI,

Table 1. Demographic variables

Characteristics	Preterm infants' neuroimaging outcome		*P* value
	Normal	Abnormal	
Neonatal			
Gestational age (weeks^{+days}) (mean ± SD)	$28^{+6} \pm 1^{+6}$	$28^{+3} \pm 1^{+6}$	0.519
Gestational age, <28/40 (*N* (%))	12 (32)	6 (43)	0.487
Gestational age, >28/40 (*N* (%))	25 (68)	8 (57)	0.487
Birth weight (grams) (mean ± SD)	1,178 ± 324	1,117 ± 305	0.542
Sex (*N* (%)) (male)	27 (73)	11 (79)	0.492
SVD (*N* (%))(mean ± SD)	10 (28)	3 (21)	0.646
Emergency CSxn (*N* (%))	26 (70)	11 (79)	0.741
Antenatal steroids, one dose (*N* (%))	8 (22)	4 (29)	0.737
Antenatal steroids, two doses (*N* (%))	28 (76)	10 (71)	0.737
Apgar score at 1 min (mean ± SD)	6 (3)	7 (2)	0.512
Apgar score at 5 min (mean ± SD)	8 (2)	9 (1)	0.092
Cord pH	7.33 ± 0.05	7.31 ± 0.09	0.395
Cord base excess	−2.7 ± 2.8	−5.6 ± 3.1	0.088
Admission gas, pH (mean ± SD)	7.27 ± 0.10	7.24 ± 0.14	0.315
Admission gas, base excess (mean ± SD)	−3.7 ± 4.1	−5.5 ± 4.1	0.196
Admission gas, lactate (mean ± SEM)	4.5 ± 1.4	6.4 ± 2.5	0.502
Chorioamnionitis (*N* (%))	7 (19)	0 (0)	0.080
Surfactant (*N* (%))	13 (35)	5 (36)	0.824
nCPAP hours (mean ± SD)	197 ± 43	162 ± 81	0.688
Hours intubated (mean ± SEM)	162 ± 63	88 ± 25	0.432
CLD (*N* (%))	8 (20)	1 (9)	0.401
NEC (*N* (%))	3 (8)	1 (9)	0.880
RIP (*N* (%))	0 (0)	3 (21)	0.040

CLD, chronic lung disease; CSxn, caesarean section; nCPAP, nasal continuous positive airway pressure; NEC, necrotizing enterocolitis; SVD, spontaneous vaginal delivery.

Table 2. Neuroimaging abnormalities

Gestational age (weeks^{+days})	Specific neuroimaging abnormality	Neuroimaging modality
25^{+6}	Echogenic PVL right frontal parietal region	CrUSS
26^{+4}	Porencephaly periventricular white matter on right	MRI
26^{+5}	Right grade 3 IVH, left grade IV IVH	MRI
27^{+3}	Extensive echogenic PVL bilaterally	CrUSS
27^{+3}	Bilateral loss white matter volume parieto-occipital region	MRI
28^{+6}	Signal abnormality in deep white matter regions bilaterally	MRI
29^{+1}	Multiple areas of signal abnormality in deep white matter bilaterally	MRI
29^{+6}	—	MRI
30^{+0}	Multiple small foci of previous hemorrhage bilaterally in PVWM	MRI
31^{+0}	Moderate dilatation of lateral ventricles due to volume loss	MRI
31^{+3}	Bilateral PVL	MRI

CrUSS, cranial ultrasound scan; MRI, magnetic resonance imaging; PVL, periventricular leukomalacia; PVWM, periventricular white matter.

compared with adults, following *ex vivo* stimulation with LPS. This was statistically significant in the >28 wk gestation group at 24–48 and 48–72 h ($P = 0.006$ and $P = 0.002$, respectively). All infants born less than 32 wk gestation produced greater monocyte derived ROIs at (i) baseline: <28 wk on day 7 ($P = 0.04$), >28 wk on day 3 ($P = 0.017$) and day 7 of life ($P = 0.03$); and (ii) post-LPS stimulation: >28 wk ($P = 0.030$). Significantly greater levels of ROIs were produced in the >28 wk group compared with the <28 wk group at 48–72 h both at baseline ($P = 0.04$) and post-LPS stimulation ($P = 0.034$; data not shown). Preterm infants who subsequently developed necrotizing enterocolitis produced significantly greater baseline ROI on day 3 of life compared with preterm neonates who followed an uncomplicated neonatal course ($P = 0.044$). Preterm infants with LOS produced significantly higher ROI at baseline on day 7 of life ($P = 0.038$) and following LPS stimulation on day 2 of life ($P = 0.023$).

CD11b Surface Expression

Preterm neonatal PMN basal CD11b expression was increased compared with adults over the first week of life. This was statistically significant in preterm controls at 0–24 h ($P = 0.03$) and 24–48 h of life ($P = 0.05$). Preterm neonates in both groups displayed a competent immune response and upregulated PMN surface CD11b expression at all time points over the first 7 d following *ex vivo* LPS stimulation (**Figure 2a**). Whereas adult CD11b expression increased by 5.5 to 6-fold, neonates only increased their expression by on average 4-fold. This was statistically significant in preterm controls at 0–24 h of life ($P = 0.001$; **Figure 2c**). Increased basal monocyte CD11b expression was seen in preterm controls compared with adults throughout the first week, which was statistically significant on day 7 of life ($P = 0.02$). Increased CD11b expression following LPS stimulation was seen in monocytes from all preterms but all to a lesser degree than adults. This was statistically significant in the abnormal neuroimaging group at 24–48 h ($P = 0.006$) and 48–72 h ($P = 0.019$). A trend toward lower upregulation of CD11b expression was seen in the abnormal preterm neuroimaging group compared with preterm controls from birth and approached significance at 48–72 h of life ($P = 0.056$; **Figure 2b**).

The LPS induced fold increase analysis revealed a degree of LPS hyporesponsiveness in all neonates compared with adults. Adults increased CD11b expression by sevenfold following *in vitro* LPS stimulation, whereas neonates only increased CD11b expression by ~3.5–4-fold and was significant in both preterm groups on day 1 of life ($P < 0.001$ control and abnormal neuroimaging groups; **Figure 2c**). Preterm infants who developed LOS had significantly higher CD11b expression at baseline on day 2 of life ($P = 0.045$) compared with those without LOS.

Neutrophil and Monocyte TLR-4 Surface Expression

All neonates expressed significantly higher PMN TLR-4 levels at baseline and following LPS stimulation compared with adults from birth to day 7 of life (**Figure 3a**). All neonates displayed a greater LPS induced fold increase in TLR-4 expression compared with adults which was statistically significant at 0–24 h in preterm control and abnormal neuroimaging groups ($P = 0.003$; $P = 0.012$ respectively; **Figure 3c**). Baseline and post-LPS stimulated preterm neonatal monocyte TLR-4 expression was increased compared with adults throughout the first week of life. This was statistically significant at all time points in preterm controls. TLR-4 expression was significantly increased in the abnormal neuroimaging group at 0–24 h, 48–72 h, and day 7 of life (baseline) and on days 1 and 7 following LPS stimulation (**Figure 3b**). Preterm infants who developed LOS had significantly higher TLR-4 expression levels at baseline on day 2 ($P = 0.01$) and day 7 of life ($P = 0.024$) and following LPS stimulation on day 2 of life ($P = 0.009$) compared with those without LOS.

DISCUSSION

We have shown increased neutrophil ROI production in preterm infants with abnormal neuroimaging. Increased intracellular neutrophil ROIs are associated with the multiple organ dysfunction syndrome seen in adult sepsis (13) and are a marker of neutrophil activation. In addition, neutrophil ROIs are associated with increased neurotoxicity (8). Overall preterm infants also displayed a robust ROI response to LPS, which was increased compared with immunocompetent adults. All neonates had increased ROI production following *ex vivo* stimulation with LPS and demonstrated higher expression levels poststimulation than adults. Preterm infants born less than 32 wk gestation with abnormal neuroimaging had increased neutrophil ROI production on day 1 compared with control preterms. Significantly increased superoxide levels have been demonstrated in cord blood from preterm infants

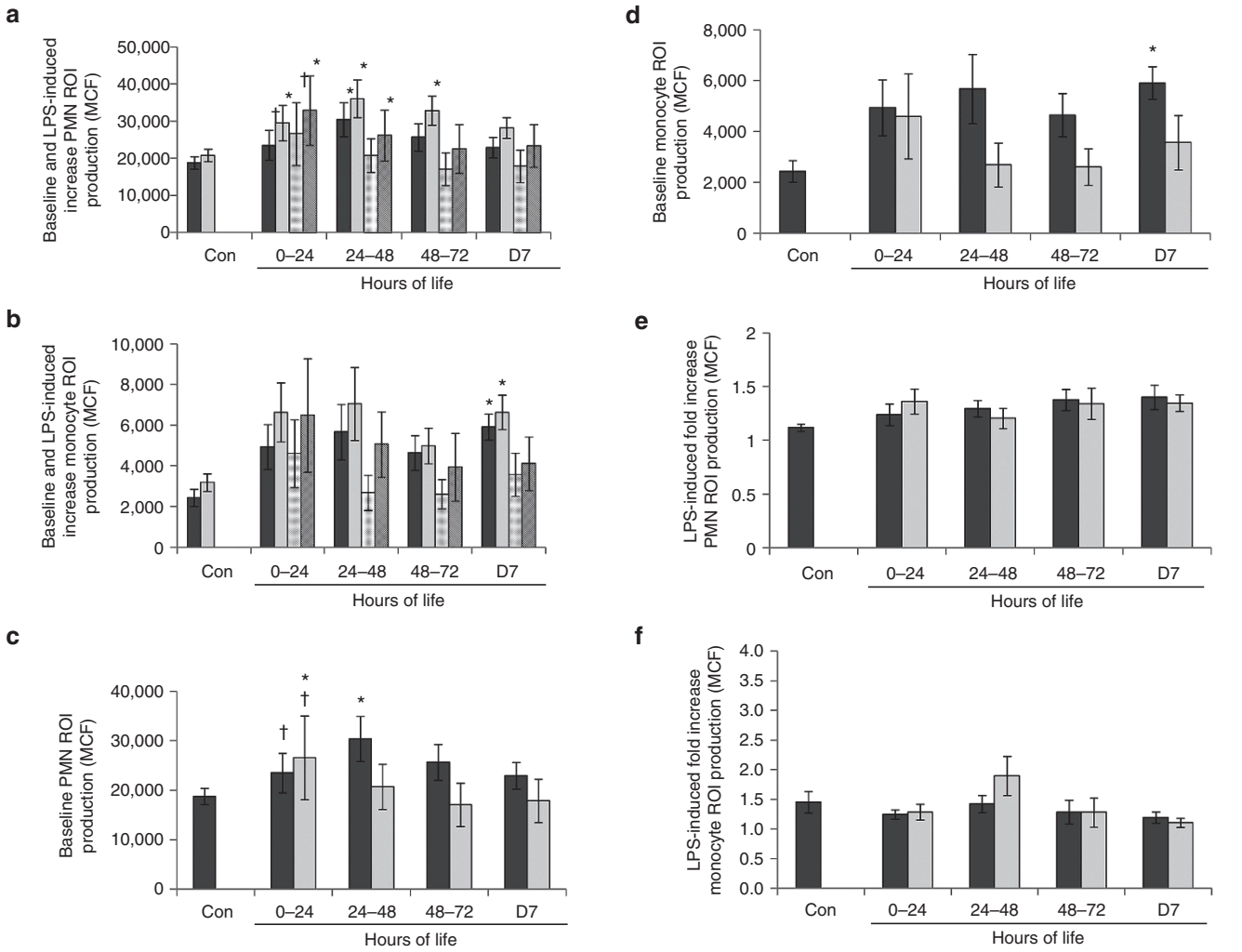

Figure 1. PMN and monocyte ROI production and neuroimaging. (**a,c**) Neutrophil and (**b,d**) monocyte ROI production assessed in cord controls (con), preterm controls with no abnormality on neuroimaging (N, $n = 40$), and preterm infants with abnormalities on neuroimaging or death (ABN, $n = 11$) at baseline and following LPS stimulation. (**e**) Neutrophil and (**f**) monocyte fold increase ROI production. *$P < 0.05$ vs. adult, †$P < 0.05$ vs. preterm controls. Results expressed as mean channel fluorescence (MCF). (**a,b**) White boxes indicate baseline expression, normal neuroimaging group; black boxes indicate LPS-induced expression, normal neuroimaging group; gray boxes indicate baseline expression, abnormal neuroimaging group; striped boxes indicate LPS-induced expression, abnormal neuroimaging group. (**c–f**) White boxes indicate normal neuroimaging group and black boxes indicate abnormal neuroimaging group. ROI, reactive oxygen intermediate.

with PVL compared with controls (14) but intracellular ROIs have not been described. In the case of maternal/fetal infection in preterm infants, LPS stimulation induces ROI and cytokine production leading to oligodendrocyte cell injury and PVL ($n = 5$) (15). Elevated oxidative products have also been demonstrated during the evolution of WM injury in the human premature infant (16). Decreased intracellular ROI production is reported in extremely preterm infants (17) (see **Supplementary References 16 and 17** online) compared with term neonates, although this is a complex area that is not well described. However, inconsistent results are reported regarding LPS responsiveness and ROI production as many different cell types and techniques have been used.

Preterm infants born less than 32 wk gestation have an altered immune phenotype over the first week of life compared with adults. Decreased CD11b expression and function are described in neonatal neutrophils (18). These neutrophil defects are more pronounced in preterm infants (11). However, these studies used cord blood neutrophils for analysis. We have shown using serial postnatal blood samples that preterm neonates have robust immune responses over the first week of life. Neutrophil and monocyte CD11b and TLR-4 expression, and ROI production were increased at baseline compared with adults. In addition, all neonates had increased CD11b and TLR-4 expression, and ROI production following *in vitro* stimulation with LPS and demonstrated higher expression levels post stimulation than adults. This may imply that neonatal neutrophils and monocytes are hyperactivated over the first week of life. Indeed, previous studies have described an increase in neutrophil CD11b expression in infants with respiratory distress syndrome (19). The presence of bacteria or cytokines prolongs the survival of neutrophils (20,21) (see **Supplementary Reference 18** online). However, neutrophils may also persist without these coexisting factors and maintain

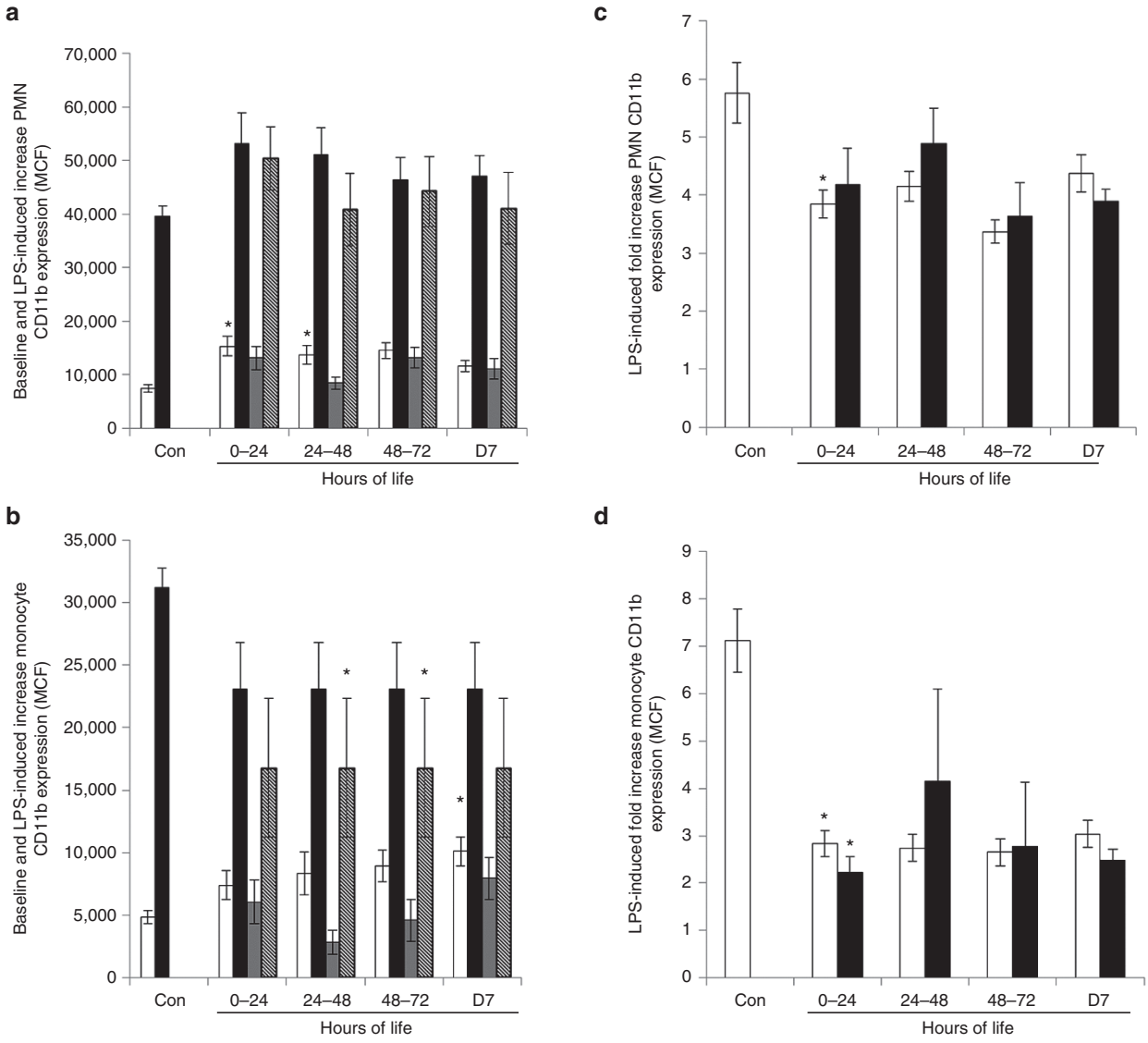

Figure 2. PMN and monocyte CD11b expression and neuroimaging. (**a**) Neutrophil and (**b**) monocyte CD11b expression assessed in preterm controls with no abnormality on neuroimaging (N, $n = 40$) and preterm infants with abnormalities on neuroimaging or death (ABN, $n = 11$) at baseline and following LPS stimulation. Fold increase CD11b expression by (**c**) neutrophils and (**d**) monocytes.*$P < 0.05$ vs. adult. Results expressed as mean channel fluorescence (MCF). (**a,b**) White boxes indicate baseline expression, normal neuroimaging group; black boxes indicate LPS-induced expression, normal neuroimaging group; gray boxes indicate baseline expression, abnormal neuroimaging group; striped boxes indicate LPS-induced expression, abnormal neuroimaging group. (**c,d**) White boxes indicate normal neuroimaging group; black boxes indicate abnormal neuroimaging group.

their inflammatory functions (20). These nonapoptotic neutrophils retain integrin-mediated adherence (22) and upregulate CD11b expression in response to stimulation (23).

Elevated neutrophil TLR-4 levels are associated with chorioamnionitis, and impaired lung development, and alterations in lung fibronectin are described in mice with elevated TLR-4 expression following intra-amniotic injection of LPS. Shen *et al.* (12) showed monocyte TLR-4 expression in preterm infants was lower than term but rapidly increased although LPS induced cytokines did not increase in parallel.

In contrast, preterm neutrophils and monocytes retained their LPS responsiveness with respect to TLR-4 expression and ROI production. Animal models demonstrate an upregulation of TLR-4 on cerebral tissues following hyperoxic resuscitation at birth. In addition, activation of TLR-4 on microglial cells causes oligodendrocyte injury which occurs in PVL (24). This form of brain injury is prevalent in preterm infants. There is much discrepancy in the literature regarding the immune function of preterm infants during the neonatal period. We demonstrated that preterm infants born less than 32 wk gestation have a robust immune response in the first week of life, compared with adult controls. The role of immune function and infection in neonatal neurodevelopmental outcome requires further study.

Inconsistent results are reported regarding neonatal endotoxin (LPS) responsiveness and ROI production (11). Decreased monocyte cord blood ROI production is reported in extremely preterm infants (17) (see **Supplementary**

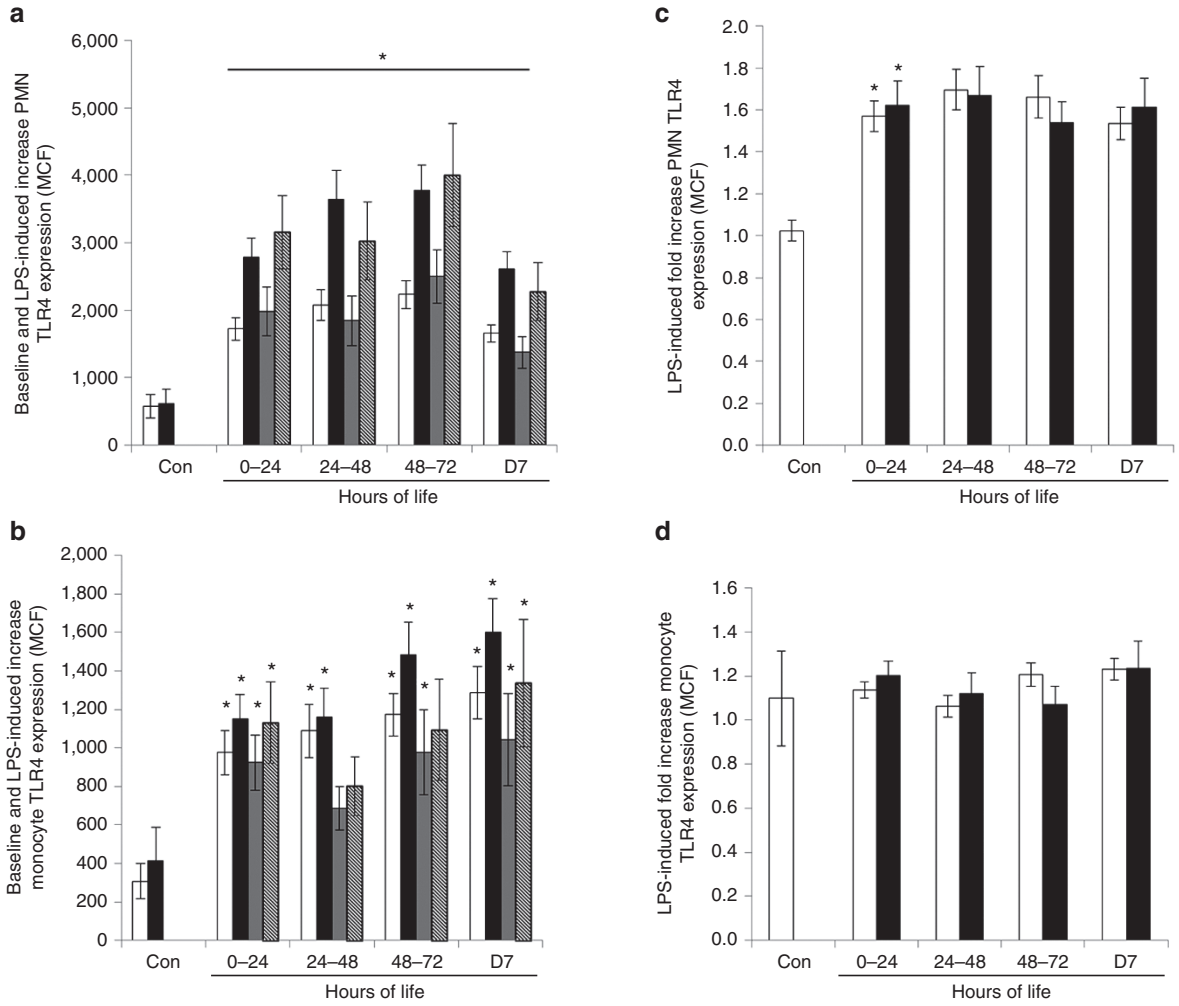

Figure 3. PMN and monocyte TLR-4 expression and neuroimaging. (**a**) Neutrophil and (**b**) monocyte TLR-4 expression assessed in preterm controls with no abnormality on neuroimaging (N, $n = 40$) and preterm infants with abnormalities on neuroimaging or death (ABN, $n = 11$) at baseline and following LPS stimulation. Fold increase TLR-4 expression in (**c**) neutrophils and (**d**) monocytes.*$P < 0.05$ vs. adult. Results expressed as mean channel fluorescence (MCF). (**a,b**) White boxes indicate baseline expression, normal neuroimaging group; black boxes indicate LPS-induced expression, normal neuroimaging group; gray boxes indicate baseline expression, abnormal neuroimaging group; striped boxes indicate LPS-induced expression, abnormal neuroimaging group. (**c,d**) White boxes indicate normal neuroimaging group; black boxes indicate abnormal neuroimaging group.

Table 3. MRI scoring

Gestational age (weeks^{+days})	WM signal abnormality	PVWM volume loss	Cystic abnormalities	Ventricular dilatation	Thinning of corpus callosum	Gyral maturation	Subarachnoid space size	Total score	
								WM (15)	GM (7)
29^{+6}	2	1	1	2	1	1	2	7	3
26^{+5}	1	3	1	3	3	1	3	11	4
31^{+0}	1	3	1	3	3	1	1	11	2
26^{+4}	2	2	3	2	1	1	2	10	3
30^{+0}	3	1	1	1	1	1	1	7	2
31^{+3}	3	3	3	3	3	1	2	15	3
27^{+3}	2	3	3	2	3	3	3	13	6
29^{+1}	3	1	1	1	1	2	2	7	4
28^{+6}	3	1	1	1	1	1	1	7	2

(WM score: normal 5–6; mild 7–9; moderate 10–12; severe abnormality 13–15. GM score: normal 3–5; abnormal 6–9).
GM, gray matter; MRI, magnetic resonance imaging; WM, white matter; PVWM, periventricular white matter.

References 16 and 17 online) compared with term neonates. However, the majority of studies were performed on umbilical cord blood rather than postnatal neonatal samples. Umbilical cord blood has decreased endotoxin responsiveness and may not reflect the postnatal neonatal immune responses (25) (see **Supplementary References 19 and 20** online). In another study of postnatal sampling of preterm infants, neutrophil ROI production was increased in preterm neonates following *Escherichia coli* stimulation (26), and ROI production was reduced with decreasing gestation age. This study included only 15 infants <32 wk at each time point over the first week of life and was consistent with our findings. In contrast to our study, other groups assessed cytokine levels from cord blood samples or dried blood spots and many at only one time point during the neonatal period.

Persistent inflammation, with prolonged neutrophil survival, is a critical component in the pathogenesis of chronic inflammatory disorders in adults (27) (see **Supplementary References 21–23** online) and neonates (28) (see **Supplementary References 24–26** online). Accumulation of neutrophils in tissues mediates injury via their inflammatory and cytotoxic functions in addition to the recruitment and activation of further neutrophils from the circulation (29) (see **Supplementary References 27–29** online). Persistent inflammation has been associated with the development of CP and may be a possible therapeutic target once clearly delineated (30) (see **Supplementary Reference 30** online).

Activated neutrophils and monocytes may be a target for treatment of inflammatory disease in preterm infants. Both vitamin A and pentoxyfylline (phosphodiesterase inhibitor) decrease neonatal neutrophil ROI production and have a good safety record in preterm infants (see **Supplementary Reference 31** online). Similarly, allopurinol decreases free radicals although a randomized controlled trial did not show a decrease in PVL (31). Experimental inhibition of ROIs by blocking NADPH oxidase with apocynin or edavarone (a free radical scavenger) may be possible therapeutic agents and the latter has improved outcome in adult ischaemic brain injury (32).

The tendency of extremely low birth weight premature infants to respond in a more vigorous fashion to inflammatory stimuli than term infants can in part explain their vulnerability to multiple organ damage including the brain, lung, intestine, and eye (33). In conclusion, we demonstrated robust systemic preterm monocyte and neutrophil ROI production even in infants <28 wk gestation. This source of oxidative damage may play a major role in neonatal inflammatory disorders especially in the first few days of life (34) (see **Supplementary Reference 32** online). Decreased antioxidant defenses in preterm infants make them particularly susceptible to end-organ dysfunction (10) (see **Supplementary Reference 32** online). The increased ROI response with no LPS-induced upregulation of CD1b and TLR-4 may imply that the ROI response is not mediated at the receptor level. Immunomodulation of excessive systemic neutrophil and monocyte activation may have therapeutic potential.

METHODS

Reagents

The following reagents were used: lipopolysaccharide from *E. coli* serotype 0111:B4 (LPS), fetal calf serum (FCS), dihydrorhodamine^{123} (DHR), and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Aldrich (Arklow, Ireland). Phycoerythrin labeled CD11b and BD FACS lysing solution were purchased from BD Biosciences (Oxford, UK). Alexa Fluor 647 antihuman TLR-4 was purchased from eBiosciences (Hatfield, UK). BD FACS lysing solution was purchased from BD Biosciences. Phosphate-buffered saline (PBS) was purchased from Oxoid, Thermo Fisher Scientific (Cambridge, UK). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin solution, and L-glutamate were purchased from GibcoBRL Life Technologies/Invitrogen (Dublin, Ireland).

Patient Groups

Ethical committee approval was received from a tertiary referral, university-affiliated maternity hospital (National Maternity Hospital, Holles Street) with >9,500 deliveries per annum for the study period March 2010 to March 2011. Fully informed written consent was obtained from the subjects and parents of all infants enrolled in this study in the following groups: (i) adults: healthy adult men and nonpregnant women, aged 25–51 years and (ii) preterm infants: postnatal samples from infants born less than 32 wk gestation. Infants with congenital abnormalities or evidence of maternal substance abuse were excluded. Adults were included as internal controls to ensure consistent responses in the *in vitro* model. They were used as a regular positive (LPS-induced) and negative controls (spontaneous).

A convenience sample of infants was prospectively enrolled and all samples were analyzed by FOH. Clinical details including maternal positron emission tomography, histological chorioamnionitis, antenatal steroid administration, Apgar scores, RDS, and ventilation days were recorded. A complete course of antenatal steroids was defined as betamethasone 12.5 mg given twice, 12 h apart, and a single dose was termed partial antenatal steroid treatment. Neonatal outcomes were recorded as follows: RDS (35); chronic lung disease; necrotizing enterocolitis (36), LOS, IVH (37), and patent ductus arteriosus (38).

Neuroimaging

All preterm infants had serial cranial ultrasounds, performed by a consultant pediatric radiologist who was blinded to blood results and clinical outcome (V.D.), at 0–24 h, 24–72 h, day 7, 1 mo, and day of discharge. MRI of the brain was performed at term equivalent in all infants ≤30 wk gestation or 30–32 wk with an abnormality on cranial ultrasound. All scans were scored and reported independently by a single pediatric radiologist according to the Inder standardized scoring system (6) which employs eight 3-point scales including components of both WM and GM abnormality. On completion of the study infants were retrospectively divided into subgroups according to findings on neuroimaging and gestational age as follows: (i) normal neuroimaging (preterm control): infants with no abnormalities on imaging studies (serial cranial ultrasound scans and/or MRI brain) or evidence of grade 1–2 IVH; (ii) abnormal neuroimaging (preterm AN): infants with grade 3–4 IVH; increased echogenicity of the periventricular WM on two or more cranial ultrasound scans or on MRI brain at term corrected; evidence of cystic or noncystic PVL or infants who died in the postnatal period prior to discharge from the neonatal intensive care unit.

Blood Sampling

Neonatal blood sampling at 0–24, 24–48, 48–72 h and day 7 of life was paired with routine phlebotomy. Arterial samples were taken when peripheral or umbilical arterial catheters were *in situ*. Otherwise, peripheral venous samples were obtained. Five hundred microliters was obtained at each time point and was collected in serum blood bottles. Samples were transported to the laboratory for quantification of cell surface antigen expression and ROI production which commenced within 90 min of sample collection in all cases. Whole blood was incubated for 1 h in 37 °C with proinflammatory

agent LPS 1 μg/ml to mimic an inflammatory response *in vitro* (39). Samples were analyzed using an Accuri C6 flow cytometer with a CFlow Plus software. Leukocyte populations were selected based on their scatter profiles, forward scatter and side scatter. Whole blood neutrophil and monocyte population gates were confirmed by cell sorting in Flow Cytometer gates. This was achieved by using the Flow Cytometer and cell sorter. Sorted cells were then collected and fixed on a slide, stained, and analyzed under a microscope (**Supplementary Figure S1** online). Cell morphology was validated by a consultant hematologist and a histologist in the hospital. A camera was mounted on the microscope, and pictures of the cells were taken. The analysis of CD11b and TLR4 expression in addition to ROIs were performed on neutrophil and monocyte populations. CD11b was labeled with phycoerythrin which is excited by a 488 nm wavelength laser. TLR4 was labeled with Alexa Fluor 647 which is excited by a 633 nm laser. This facilitated the quantification of CD11b and TLR4 expression in the same sample aliquot. DHR was used to stain for ROI production and is excited by a 500 nm laser. CD11b and ROI signal were collected on the photomultiplier 2 (FL-2-A) using a 585/40 filter. TLR4 signal was filtered with a 675/25 filter and collected on the PMT4 (FL-4–A).

Quantification of Intracellular ROI Production

Generation of ROIs was evaluated by flow cytometry using the technique of Smith and Wiedemann (40). Whole blood (50 μl) was incubated with or without LPS (1 μl) at 37 °C for 1 hour. All samples were subsequently incubated with DHR (100 μmol/l) at 37 °C for 10 min before stimulation with 1 μl (16 μmol/l) of PMA for 20 min at 37 °C. The reaction was then halted by placing samples on ice. Samples were analyzed using an Accuri C6 flow cytometer with CFlow Plus software from BD Biosciences. Leukocyte populations were selected based on their scatter profiles; forward scatter and side scatter. Neutrophil ROI fluorescence intensity was collected on the PMT2 (FL-2-A) using a 585/40 filter and expressed as mean channel fluorescence. Each sample was acquired over 2 min at medium speed. DHR has been shown to detect mainly intracellular H_2O_2 and OH radical production (40).

Quantification of Cell Surface Antigen Expression

The expression of CD11b and TLR-4 antigens on the surface of neutrophils and monocytes was measured by flow cytometry. Whole blood (50 μl) was treated with 5 μl of phycoerythrin-CD11b and 2.5 μl antihuman TLR-4 antibody and left at 4 °C for 20 min. FACS was added and incubated for 10 min at room temperature. The sample was centrifuged at 3,000 rpm for 5 min at 4 °C. The pellet was suspended twice with DMEM 500 μl and stored on ice before analysis by flow cytometry. The fluorescence intensity is denoted by mean channel fluorescence, which is the average intensity of fluorescence emitted by all cells chosen for measurement and is comparable to the relative number of receptors present on the surface of each cell. The flow cytometer used was Accuri C6. Each sample was acquired over 2 min at medium speed, and a minimum of 5,000 events were collected and analyzed. All measurements were performed under the same instrument settings (39).

Statistics

Statistical analysis was carried out using ANOVA using PASW statistical package version 18, IBM (Armonk, NY). Equal variance was assumed and Tukey's *post hoc* multiple comparisons was used. Chi square statistic and independent samples *t*-test were carried out for analysis of demographics. Two-way ANOVA was used in the comparison baseline and LPS induced CD11b, TLR-4 expression, and ROI production between neonates and adults. Significance was assumed for values of $P < 0.05$. Results are expressed as mean ± SEM unless otherwise indicated.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/pr

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