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Impact of Toll-like Receptor 4 Stimulation on Human Neonatal Neutrophil Spontaneous Migration, Transcriptomics, and Cytokine Production

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Abstract

Neonates rely on their innate immune system, and neutrophils in particular, to recognize and combat life-threatening bacterial infections. Pretreatment with lipopolysaccharide (LPS), a toll-like receptor (TLR) 4 agonist, improves survival to polymicrobial sepsis in neonatal mice by enhancing neutrophil recruitment. To understand the response of human neonatal neutrophils to TLR4 stimulation, *ex vivo* spontaneous neutrophil migration, neutrophil transcriptomics, and cytokine production in the presence and absence of LPS was measured directly from whole blood of adults, term neonates, and preterm neonates. Spontaneous neutrophil migration was measured on novel microfluidic devices with time-lapse imaging for ten hours. Genome-wide neutrophil transcriptomics and plasma cytokine concentrations were also determined. Preterm neonates had significantly fewer spontaneously migrating neutrophils at baseline, and both term and preterm neonates had decreased neutrophil velocity, compared to adults. In the presence of LPS stimulation, the number of spontaneously migrating neutrophils was reduced in preterm neonates

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Conflict of Interest Disclosure

All authors have read the journal's policy on disclosure of potential conflicts of interest and have none to declare.

Authorship

SLR, JLW, LLM, DI, and SDL developed the hypothesis and designed the experiments. FE and DI provided the microfluidic spontaneous migration devices and technical support for the migration assay. SLR, RBH, TJM, JCR, JAS, MCL, RU, and HVB performed the experiments and analyzed the results. SLR, LLM, and SDL prepared the original manuscript. All authors reviewed and revised the final manuscript. No editorial support was used of the preparation of the manuscript.

compared to term neonates and adults. Neutrophil velocity was not significantly different among groups with LPS stimulation. Preterm neonates upregulated expression of genes associated with the recruitment and response of neutrophils following LPS stimulation, but failed to upregulate the expression of genes associated with antimicrobial and antiviral responses. Plasma levels of IL-1 β , IL-6, IL-8, MIP-1 α , and TNF- α increased in response to LPS stimulation in all groups, but IL-10 was increased only in term and preterm neonates. In conclusion, age-specific changes in spontaneous neutrophil migration counts are not affected by LPS despite changes in gene expression and cytokine production.

Keywords

immunology; innate immunity; inflammation; microfluidics; neutrophil migration; lipopolysaccharide

1. Introduction

Sepsis is a major cause of morbidity and mortality in neonates [1–3]. A crucial component of the neonatal response to sepsis is pathogen recognition via various innate immune cells including neutrophils [4]. We have previously demonstrated preterm neonates have diminished neutrophil chemotaxis to the bacterial chemotactic peptide, N-formylmethionine-leucyl-phenylalanine (fMLP) [5]. This finding may contribute to the increased risk of sepsis and mortality among the most vulnerable neonatal population. In neonatal mice, we have demonstrated that *in vivo* pretreatment with the toll-like receptor (TLR) 4 agonist lipopolysaccharide (LPS) improves survival to polymicrobial sepsis by enhancing neutrophil recruitment [6]. We also discovered unique cytokine and neutrophil transcriptomic profiles in umbilical cord blood compared to healthy young adult peripheral blood following *ex vivo* LPS stimulation [7]. In addition, LPS at a concentration of 1000 ng/ml has been shown to be a potent stimulus for inducing random migration in human adult neutrophils [8].

Whole blood studies are limited in the neonatal population given concerns about the volume of blood that would be required to perform a study such as this, especially among extremely low birth weight (ELBW, birth weight <1000g) neonates for whom total circulating blood volumes may be less than 100 milliliters [9]. We have overcome this challenge with the use of a novel microfluidic technology which can precisely measure neutrophil migration counts and velocities from as little as two microliters of whole blood [10]. By testing neutrophil function in the presence of the subject's own plasma, this technology mirrors a physiological environment and allows detailed comparisons of neutrophil motility in a matter of hours. In addition, these techniques do not require extensive neutrophil isolation procedures which may induce artificial stimulation [11]. Therefore, we aimed to determine the impact of TLR4 stimulation on whole blood from adults, term and preterm neonates by measuring spontaneous neutrophil migration, neutrophil transcriptomics, and cytokine production. We hypothesized that *ex vivo* exposure to LPS would improve spontaneous neutrophil migration and this could be explained by changes in neutrophil transcription. Surprisingly, *ex vivo* LPS exposure had only minimal effects on spontaneous neutrophil migration velocity and no effect on the number of migrating neutrophils. Transcriptional analysis confirmed these

findings with no changes in transcription of genes involved in the cell movement, migration, or homing of neutrophils.

2. Materials and Methods

2.1. Study design

This prospective observational study was conducted between September 2016 and August 2017 at UF Health Shands Children's Hospital, a 202-bed tertiary medical center including a Level IV Neonatal Intensive Care Unit (NICU). The study was approved by the Institutional Review Board prior to initiation. All neonates admitted to the Newborn Nursery and NICU were screened for inclusion. Neonates born between 24 and 42 weeks gestational age were eligible for participation. Neonates were excluded from enrollment if any major congenital anomaly was known or suspected. Neonates were withdrawn from the study if the sample collection did not coincide temporally with a clinically indicated blood draw. Written informed consent was obtained from the parents or guardians of neonatal subjects prior to sample or data collection. Consent was provided only to review the neonatal medical record. Healthy adults aged 21 – 45 years were also recruited for participation. Adult subjects were excluded from enrollment if any of the following was reported: pre-existing organ dysfunction, positive for human immunodeficiency virus, recent use of oncolytics, current use of steroids, or history of autoimmune disease. Written informed consent was obtained from all adult subjects prior to sample collection.

2.2. Blood sample collection

Whole blood samples were collected in tubes anticoagulated with lithium heparin (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA) from young adults (21–45 years old), term neonates (gestational age 37–42 weeks), and preterm neonates (gestational age 24–36 weeks). For adults, a four milliliter blood sample was collected by venipuncture. For neonates, a single 700 microliter blood sample was collected at the time of a clinically indicated blood draw. Blood was collected from term neonates in the Newborn Nursery prior to discharge between 24 and 36 hours of life and at the same time as the state's newborn screening panel. Blood was collected for preterm neonates in the NICU on day four of life in order to reduce the impact of potentially immunomodulating medications that were administered to the mother prior to delivery and to allow the family time to consider study participation.

2.3. Patient data collection

Neonatal data including demographics, clinical variables, and outcomes were recorded from the electronic medical records of the birth hospitalization. Prolonged premature rupture of membranes (PROM) was defined as any rupture of membranes that persisted for more than 24 hours and occurred prior to the onset of labor. Apgar scores were recorded at one and five minutes following birth. Neonatal antibiotics, corticosteroids, and indomethacin were recorded if administered any time prior to study sample collection. Use of mechanical ventilation was recorded based on the respiratory status of the neonate at the time of study sample collection.

2.4. Spontaneous neutrophil migration assay

Microfluidic devices with a single whole blood loading chamber (WBLC), erythrocyte filters, migration channels, and posts were utilized as previously described and validated [10] (Fig. 1a). Erythrocyte filters block the dispersion of erythrocytes into the migration channel, whereas spontaneously migrating neutrophils can travel through without a decrease in speed. Erythrocyte filters additionally prevent the entrance of other, non-neutrophil leukocytes into the migration channels with neutrophils consisting of greater than 96% of cells entering the channels. Posts located at the end of the migration channels allow neutrophils to change direction and continue spontaneous movement. Microfluidic spontaneous migration devices were filled with either Hank's Balanced Salt Solution (HBSS) with bovine albumin [final concentration 0.2 mg/ml] (Sigma-Aldrich, St. Louis, MO, USA) alone or a mix of HBSS with albumin and lipopolysaccharide from *Escherichia coli* 026:B6 [final concentration 1000 ng/ml] (Sigma-Aldrich, St. Louis, MO, USA). The device was then placed in a desiccator under vacuum for 10 minutes to ensure complete filling of the device. Following device filling, two microliters of whole blood stained with Hoechst Stain Solution (Sigma-Aldrich, St. Louis, MO, USA) were pipetted into the WBLC. Time-lapse imaging was performed every 30 seconds for 10 hours on a Nikon Bio Station IMqTM microscope inside of a biochamber maintained at 37° C and 5% CO₂. Neutrophil migration counts were calculated using Nikon Imaging Software ElementsTM High Content Analysis (Nikon Instruments Inc., Melville, NY, USA). Neutrophil velocities were calculated using ImageJ software (NIH, Bethesda, MD, USA) [12].

2.5. Neutrophil transcriptome assay

Whole blood samples were aliquoted and mixed 1:1 with HBSS with bovine albumin [final concentration 0.2 mg/ml] alone or HBSS with albumin and LPS from *Escherichia coli* 026:B6 [final concentration 100 ng/ml] (Sigma-Aldrich, St. Louis, MO, USA). Aliquots were then incubated on a rocker for two hours at 37° C and 5% CO₂. Following incubation, neutrophils were captured from whole blood using positive selection with anti-CD66b monoclonal antibody, clone 80H3 (BioRad, Hercules, CA, USA) coated microfluidic devices as previously described and validated [5, 7, 11, 13]. The microfluidic devices have greater than 95% neutrophil purity and capture a fixed number of neutrophils, thus the total number of neutrophils captured were equivalent among groups regardless of any differences in total or differential counts. Captured neutrophils were lysed *en bloc* using RLT buffer (Qiagen, Valencia, CA, USA). The cell lysate was passed through a QiaShredder column, and RNA was extracted from lysates using QIAGEN RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA integrity was assessed with an Agilent 2100 Bioanalyzer (Santa Clara, CA, USA). RNA was labeled using Nugen Ovation Pico WTA System V2 (NuGEN Technologies, Inc, San Carlos, CA, USA). Resulting cDNA was labeled and fragmented using Nugen Encore Biotin Module, hybridized onto GeneChip Human Transcriptome Array 2.0 (Affymetrix, Santa Clara, CA, USA) and processed following manufacturer's instructions. Genome-wide expression was normalized using RMA as implemented in Partek Genomics Suite, Version 6.6 (Partek Inc, St. Louis, Mo).

2.6. Genomic analysis

Genome-wide expression was compared among six groups (unstimulated and stimulated adults, unstimulated and stimulated term neonates, unstimulated and stimulated preterm neonates) using both unsupervised and supervised analyses. In the unsupervised analysis, probe sets with a coefficient of variation (CoV) greater than 50% were identified. In the supervised analysis, significant probe sets were identified using an f-test at $p < 0.001$. Genome-wide expression was also compared among unstimulated groups and stimulated groups separately, using an f-test at $p < 0.001$. Finally, individual groups (adults, term neonates, preterm neonates) were compared with and without stimulation using a paired t-test at $p < 0.001$. Leave-one-out cross-validation was used to compute the correct classification rate for each sample. The classification rate was compared to chance based on 1000 random permutations. Genomic analysis was performed using BRB ArrayTools™ Version:4.5.1-Stable Release. Hierarchical clustering was generated with DNA-Chip Analyzer (dChip) software. Principal Component Analysis (PCA) was generated with Partek® Genomics Suite® Version:6.6. Once significant probe sets were identified, Gene Ontology™ analysis was conducted as implemented in BRB ArrayTools™. Fold changes of significant genes were calculated between stimulated and unstimulated groups.

2.7. Functional pathway analysis

Functional pathway analysis was performed on probe sets that were differentially expressed between groups based on the f-test at $p < 0.001$ and had greater than a 1.5 fold-change using Ingenuity® Pathway Analysis (IPA®) software (Ingenuity Systems, Redwood City, CA, USA). IPA® predicts canonical pathways and biological functions based on the expression values of gene groups and prior knowledge of expected effects of the gene products. Predictions with a negative log p-value greater than 1.3 and a z-score greater than 2 or less than -2 were considered significant.

2.8. Cytokine production assay

Whole blood samples were aliquoted and mixed 1:1 with HBSS with bovine albumin [final concentration 0.2 mg/ml] alone or HBSS with albumin and LPS from *Escherichia coli* 026:B6 [final concentration 100 ng/ml] (Sigma-Aldrich, St. Louis, MO, USA). Aliquots were then incubated on a rocker for two hours at 37° C and 5% CO₂. Following incubation, whole blood was centrifuged at 1800 × g for 10 minutes at 4° C, and cell-free supernatant was collected and stored at -80° C until batch processing to reduce the degree of intra-assay variability. The concentrations of interleukin (IL)-1β, IL-6, IL-8 (CXCL8), IL-10, IL-12 p70, interferon-γ (IFNγ), tumor necrosis factor-α (TNFα), macrophage inflammatory protein-1α (MIP-1α, CCL3), monocyte chemotactic protein-1 (MCP-1, CCL2), and interferon gamma-induced protein-10 (IP-10, CXCL10) were determined using Milliplex® Multiplex kits (EMD Millipore, Billerica, MA, USA) on the Luminex® MAGpix Multiplex reader. Cytokine assay sensitivities (minimum detectable concentrations, pg/mL) are provided in Supplemental Table 1.

2.9. Statistical analysis

Data are presented as either frequency and percentage, median and range, or mean and standard deviation. Analysis of variance (ANOVA), Student's t-tests, and Tukey's honest significant difference (HSD) tests were used to compare continuous parametric variables as appropriate. Wilcoxon tests and Kruskal-Wallis tests were used to compare continuous nonparametric variables. Fischer's exact test was used for comparison of categorical variables. All significance tests were two-sided, with p-values less than 0.05 considered statistically significant. Statistical analyses were performed with R statistical software package (Vienna, Austria), SAS software (Cary, NC, USA), and Prism GraphPad Software (La Jolla, CA, USA).

3. Results

3.1. Patient data

Patient data regarding demographics, clinical variables, and outcomes are provided in Table 1. Maternal age and rectovaginal Group B Streptococcus (GBS) colonization were similar between mothers of term and preterm neonates. There was no evidence of clinical chorioamnionitis in either group. Mothers of preterm neonates had a greater incidence of prolonged PROM and were more likely to receive antepartum antibiotics, corticosteroids, and magnesium. Preterm neonates were more likely to be born via cesarean section compared to term neonates. Presence and duration of labor could not be accurately extracted from the neonatal medical record and access to the maternal medical record was not granted. As expected, preterm neonates had lower gestational age, birth weight, and Apgar scores at one and five minutes compared to term neonates. Preterm neonates were also more likely to receive mechanical ventilation, antibiotics, and indomethacin.

3.2. Spontaneous neutrophil migration

Unstimulated preterm neonatal neutrophils migrated in significantly fewer numbers compared to unstimulated term neonatal ($p=0.0034$) or adult ($p=0.0280$) neutrophils based on a time series random intercept model (Fig. 1b). Spontaneous neutrophil migration velocities were significantly reduced in both unstimulated term and preterm neonates compared to unstimulated adults (unstimulated preterm 13.57, unstimulated term 15.33, unstimulated adult 21.48 $\mu\text{m}/\text{min}$; $p=0.0007$) (Fig. 1c). The velocity of unstimulated preterm neonatal neutrophils was comparable to that of term neonates. With *ex vivo* LPS stimulation, preterm neonatal neutrophils migrated in significantly fewer numbers compared to LPS-stimulated term neonatal ($p=0.0332$) or adult ($p=0.0151$) neutrophils (Fig. 1d). Whereas unstimulated neutrophil velocities from term and preterm neonates were reduced compared to young adults, there was no significant difference in neutrophil velocities among LPS-stimulated groups (stimulated preterm: 17.47, stimulated term: 17.06, stimulated adult: 19.18 $\mu\text{m}/\text{min}$; $p=0.3607$) (Fig. 1e).

3.3. Neutrophil transcriptomics

An unsupervised analysis of gene expression from the six groups (unstimulated and stimulated adults, unstimulated and stimulated term, unstimulated and stimulated preterm)

identified 4013 probe sets, representing 2594 genes, differentially expressed with a CoV greater than 50%. When these 4013 probe sets were hierarchically clustered, LPS-stimulated groups were distinguishable from unstimulated groups, and adults and neonates further separated into distinct groups. The unsupervised clustering did not distinguish gene expression patterns between unstimulated term and preterm neonates.

Using a supervised analysis, the expression of 4582 probe sets, representing 3977 genes, significantly differed among the six groups ($p < 0.001$). When these 4582 probe sets were hierarchically clustered, the expression pattern first distinguished adults from neonates (Fig. 2a). Adult and neonatal groups then separated based on the presence or absence of LPS stimulation. Leave-one-out cross-validation using a 3-nearest neighbor prediction model was able to correctly predict class label at a rate of 75% compared to the expected rate for chance alone of 17%. Based on 1000 random permutations this result was significant at $p < 0.001$. Thus, the gene expression differences between the groups have predictive validity and are informative as to the groups.

Analyses of neutrophil gene expression patterns from unstimulated groups and stimulated groups performed separately demonstrated distinct gene expression patterns of adults, term neonates, and preterm neonates. The expression of 398 genes differed significantly among the unstimulated samples ($p < 0.001$), and the expression of 1779 genes differed significantly among the stimulated samples ($p < 0.001$). Principal component analyses (PCA) demonstrated clear separation among unstimulated and stimulated samples from adults, term neonates, and preterm neonates. (Fig. 2b and 3c).

The number of genes that were differentially expressed in response to LPS stimulation increased with age (preterm: 760, term: 916, adult: 1129). A common cluster of 222 unique genes was identified whose expression changed in stimulated samples as compared to unstimulated samples regardless of age group. Shared upregulated genes included key components of innate immune response as shown in Table 2.

3.4. Functional predictions

Ingenuity® Pathway Analysis software was used to predict key canonical pathways represented by probe sets that were differentially expressed based on f-test at $p < 0.001$ and had greater than a 1.5 fold-change. In response to LPS stimulation, adults, term neonates, and preterm neonates had significant upregulation of genes representing innate inflammatory response pathways, including activation of interferon-regulator factor (IRF) by cytosolic pattern recognition receptors, inflammasome pathway, and role of retinoic acid-inducible gene I (RIG-I) -like receptors in antiviral innate immunity (Supplemental Table 2). In addition, preterm neonates uniquely had significant upregulation of phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signaling and triggering receptor expressed on myeloid cells 1 (TREM1) signaling, whereas adults and term neonates did not demonstrate significant changes in these pathways. Adults, term neonates, and preterm neonates had significant downregulation of sphingosine-1-phosphate signaling in response to LPS stimulation.

Biological function predictions in response to LPS stimulation demonstrated adults, term neonates, and preterm neonates had significant overexpression of genes involved in immune response of cells, cell-mediated response, and inflammatory response (Supplemental Table 3). Adults and term neonates also had a significant overexpression of genes involved in antimicrobial and antiviral response, whereas preterm neonates did not. Term and preterm neonates had a significant overexpression of genes involved in the recruitment of neutrophils. Preterm neonates exclusively had a significant overexpression of genes involved in the response of neutrophils. There were no significant differences in expression of genes involved in the cell movement, migration, or homing of neutrophils.

3.5. Cytokine production

In response to LPS stimulation, adults, term neonates, and preterm neonates had a significant increase in IL-1 β , IL-6, IL-8, MIP-1 α , and TNF- α plasma concentrations (Fig. 2d). IL-10 plasma concentrations were increased following LPS stimulation in term and preterm neonates, while adults demonstrated no significant change. IP-10 concentrations were significantly increased by LPS stimulation in adults and term neonates, while preterm neonates demonstrated no significant change. IL-12 concentrations were significantly increased by LPS stimulation in adults and preterm neonates, while term neonates had no significant change. IFN- γ concentrations were significantly increased by LPS stimulation in only preterm neonates. MCP-1 concentrations did not significantly change by LPS stimulation in any group, although baseline concentrations were significantly higher in term and preterm neonates.

4. Discussion

Previously, we demonstrated preterm human neonates had diminished chemotactic neutrophil migration and velocity [5]. Here, we report for the first time that preterm neonates had a decreased number of spontaneous migrating neutrophils compared to adults and term neonates. Term and preterm neonates also had reduced spontaneous migrating neutrophil velocity compared to adults. In addition, we demonstrate that TLR4 stimulation had no effect on the numbers of spontaneous migrating neutrophils in any age group. However, in the presence of a TLR4 agonist, spontaneous migrating neutrophil velocity was not significantly different among adults, term neonates, and preterm neonates, suggesting partial but incomplete mitigation.

Neutrophil transcriptomic and cytokine profiles were compared among adults, term neonates, and preterm neonates with and without TLR4 stimulation. Transcriptomic profiles were significantly different with and without stimulation. Moreover, the number of genes that differed between adults, term neonates, and preterm neonates was greater with stimulation than without stimulation. This finding suggests that although there are differences in neutrophil transcriptome at baseline as demonstrated here and previously [5], differences in transcriptomics are amplified in a TLR4-stimulated state. This is in agreement with our previous results comparing neutrophil transcriptomics of umbilical cord blood and healthy adults at baseline and after 24 hours of TLR4 stimulation [7]. We also observed a positive correlation between the number of genes that changed in response to stimulation

and age. This correlation with age is consistent with findings from our multi-cohort, pooled analysis of 636 adults, children, infants, and neonates with and without clinical sepsis [14].

Functional predictions from the neutrophil transcriptome suggest that in response to TLR4 stimulation, adult and neonatal neutrophils have a significant upregulation of innate inflammatory response pathways. In addition, preterm neonatal neutrophils had significant upregulation of PI3K/AKT signaling. Several murine studies have demonstrated that PI3K plays an important role in neutrophil migration [15–17]. Both adult and neonatal neutrophils had downregulation of sphingosine-1-phosphate signaling which has previously been shown to inhibit TLR-activated CXCL8 secretion [18]. In response to TLR4 stimulation, preterm neonates had no significant change in expression of genes involved in antimicrobial and antiviral response, whereas there was significant overexpression in term neonates and adults. This finding is in agreement with previous studies that report a diminished transcriptomic immune response in neonatal sepsis [14]. Importantly, this is the first time the impact of *ex vivo* TLR4 stimulation on neutrophil transcriptomics has been reported from whole blood of term and preterm neonates. Following TLR4 stimulation, preterm neonates had a significant overexpression of genes involved in the recruitment and response of neutrophils which was not observed in adults. Somewhat surprisingly, there were no overall differences in the expression of genes involved in the cell movement, migration, or homing of neutrophils, suggesting that the differences are more likely due to variations in the host inflammatory response than in the machinery involved in cell movement.

Whole blood from neonates and adults stimulated with a TLR4 agonist exhibited significant increase in the concentrations of multiple pro-inflammatory cytokines and chemokines including IL-1 β , IL-6, IL-8, MIP-1 α , and TNF- α . In particular, IL-8 has a key role in the migration, recruitment, and activation of neutrophils [19]. Preterm neonates alone had a significant increase in IFN γ , but an attenuated IP-10 response suggesting that interferon-signaling pathways may be implicated [20]. Cuenca et al. previously showed that TIR-domain-containing adapter protein-inducing interferon- β (TRIF) dependent pathways are differentially regulated in neonatal mice [21]. Term and preterm neonates had a significant increase in the concentrations of the anti-inflammatory cytokine IL-10, while there was no significant change in adults. Conversely, adults had a significant IL-12 response to TLR4 stimulation. Similarly, we previously reported a significant increase in IL-10 levels in umbilical cord blood following 24 hours of *ex vivo* TLR4 stimulation, which was not observed in peripheral blood obtained from healthy adults [7]. In neonatal mice, IL-10 levels were significantly increased 4 hours following *in vivo* administration of a TLR4 agonist [6]. Taken together, these findings suggest that adults have a more biased Th1 response than neonates who have a more Th2 inflammatory response.

There are a number of potential limitations that require further discussion. First, the neonatal populations are inherently heterogeneous. As expected, preterm neonates were born at lower birth weights and Apgar scores compared to term neonates and were more likely to receive mechanical ventilation, antibiotics, and indomethacin following birth given their prematurity. These clinical characteristics mirror the vast differences of these two neonatal populations. Since electronic medical records were only reviewed for neonatal subjects, maternal characteristics such as antenatal medication administration are subject to reporting

bias in the neonates' records. This bias is expected to be minimum and affect all neonates similarly. Additionally, based on the neonates' records, the presence and duration of labor could not be accurately determined. The impact of this on our reported measurements is unknown, however is presumed to be less impactful in this study compared to umbilical cord blood studies. Samples from term neonates were collected between 24 and 36 hours of life because of the current practice of rapid discharge of healthy term newborns. In contrast, samples from preterm neonates were delayed until day four of life. The difference in sample timing was deliberate to reduce the impact of potentially immunomodulating medications that were administered to the mother prior to delivery and to the preterm neonate after birth. In addition, insufficient blood sample volumes prevented measurement of cell counts and differentials in the *ex vivo* whole blood studies. Therefore, we are unable to determine whether the differences in cytokine production reflect differences in cell number and type, or whether they can be explained by differences in cell functionality. Importantly, both microfluidic devices used are not dependent on the starting number of neutrophils. Despite these potential study limitations, the findings that we have presented accurately reflect the clinical scenario and are in themselves novel and contribute to the literature providing previously unpublished knowledge and understanding of human neonatal immune response particularly in preterm neonates which are lacking due to limited sampling volumes and opportunities to study human preterm neonates.

In conclusion, we demonstrate that a novel microfluidic technology can be utilized in the neonatal population to successfully measure spontaneous neutrophil migration. For the first time, preterm neonates have been shown to have a significant reduction in spontaneous neutrophil migration counts and velocities compared to adults, which along with a decreased ability to follow a chemoattractant gradient to fMLP, may help explain their increased susceptibility to infection. With TLR4 stimulation, spontaneous neutrophil migration counts remained decreased among preterm neonates, but velocity was not significantly different among stimulated groups. Finally, we demonstrated unique, age-specific transcriptomic and cytokine profiles in response to TLR4 stimulation that suggest neonates have an attenuated interferon-dependent and biased Th2 response. Further studies are required to evaluate the efficacy of TLR4 agonist administration to enhance neonatal immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Spontaneous neutrophil migration assay

a. Microfluidic device. Neutrophils migrate from the center whole blood loading chamber (WBLC), past the erythrocyte filters, and into the migration channels that are sized specifically for neutrophils but not other cell populations. Once in the migration channels, neutrophils are able to spontaneously migrate. Posts located at the end of the migration channels allow neutrophils to change direction and continue spontaneous movement. Individual neutrophils are tracked and neutrophil migration count and velocity are obtained with time-lapse imaging.

b. Unstimulated neutrophil migration. Average spontaneous neutrophil migration profiles over 10 hours for unstimulated adults (n=10), unstimulated term neonates (n=10), and unstimulated preterm neonates (n=10). Unstimulated preterm neonates had a reduction in number of spontaneous migrating neutrophils compared to unstimulated term neonates (p=0.0034) and adults (p=0.0280). **c. Unstimulated neutrophil velocity.** At baseline, both term and preterm neonates had a reduction in spontaneous

migrating neutrophil velocity compared to adults (unstimulated preterm 13.57, unstimulated term 15.33, unstimulated adult 21.48 $\mu\text{m}/\text{min}$; $p=0.0007$). ** indicates values that are significantly different between unstimulated term neonates and adults, ($p<0.01$). *** indicates values that are significantly different between unstimulated preterm neonates and adults, ($p<0.001$). Lines represent means with standard deviation. **d. Stimulated neutrophil migration.** Average spontaneous neutrophil migration profiles over 10 hours for stimulated adults ($n=10$), stimulated term neonates ($n=10$), and stimulated preterm neonates ($n=10$). Stimulated preterm neonates had a reduction in number of spontaneous migrating neutrophils compared to stimulated term neonates ($p=0.0332$) and adults ($p=0.0151$). **e. Stimulated neutrophil velocity.** There was no significant difference in neutrophil velocity among stimulated groups (stimulated preterm 17.47, stimulated term 17.06, stimulated adult 19.18 $\mu\text{m}/\text{min}$; $p=0.3607$). Lines represent means with standard deviation.

Figure 2. Neutrophil transcriptomics and cytokine production in response to TLR4 stimulation

a. Supervised analysis hierarchical clustering. Using a supervised analysis, the expression of 3977 genes significantly differed among the six groups ($p < 0.001$). The significant genes were then hierarchically clustered based on the Pearson correlations. The gene expression patterns between unstimulated and stimulated neonates were more similar to each other than to unstimulated and stimulated adults. A: Adults, N: Neonates, U: Unstimulated, S: Stimulated. **b. Principal component analysis of unstimulated samples.** The expression of 398 genes significantly differed among the three unstimulated groups ($p < 0.001$). Expression patterns from unstimulated adult samples (red) are seen on the left side of the image, while expression patterns from unstimulated term samples (orange) and unstimulated preterm samples (green) are seen on the upper and lower right of the image, respectively. **c. Principal component analysis of stimulated samples.** The expression of 1779 genes significantly differed among the three stimulated groups ($p < 0.001$). Expression patterns from stimulated adult samples (dark blue) are seen tightly clustered on the left side of the image, while expression patterns from stimulated preterm samples (purple) and stimulated term samples (light blue) are seen on the upper and lower right of the image, respectively. **d. Cytokine production.** Median concentrations and interquartile ranges of cytokines from unstimulated and stimulated adults ($n=12$), term neonates ($n=13$), and preterm neonates ($n=14$). In response to TLR4 stimulation, adults and neonates significantly increased IL-1 β , IL-6, IL-8, MIP-1 α , and TNF- α concentrations. IL-10 concentrations were increased by TLR4 stimulation in neonates, while adults demonstrated no significant change. IP-10 concentrations were significantly increased by TLR4 stimulation in adults and term neonates, while preterm neonates had no significant change. IL-12 concentrations were

significantly increased by TLR4 stimulation in adults and preterm neonates, while term neonates had no significant change. IFN γ concentrations were significantly increased by TLR4 stimulation in only preterm neonates. MCP-1 concentrations did not significantly change by TLR4 stimulation in any group. * indicates values that are significantly different between unstimulated and stimulated groups, ($p < 0.05$).

Table 1

Patient Demographics, Clinical Variables, and Outcomes

Maternal and pregnancy characteristics	Term (n=22)	Preterm (n=21)	p value
Maternal age, median (range)	29 (16–36)	30 (18–39)	0.7132
GBS Colonization, n (%)			
Positive	7 (32)	8 (38)	0.7546
Negative	14 (64)	10 (48)	0.3640
Unknown	1 (5)	3 (14)	0.3449
Prolonged PROM, n (%)	0 (0)	7 (33)	0.0036
Clinical Chorioamnionitis, n (%)	0 (0)	0 (0)	1.0000
Antepartum antibiotics, n (%)	6 (27)	14 (67)	0.0148
Antepartum corticosteroids, n (%)	0 (0)	13 (62)	<0.0001
Antepartum magnesium, n (%)	1 (5)	15 (71)	<0.0001
Cesarean section, n (%)	7 (32)	16 (76)	0.0058
Neonatal characteristics	Term (n=22)	Preterm (n=21)	p value
Male, n (%)	11 (50)	11 (52)	1.0000
Gestational age, median (range)	39.8 (37.3–41.7)	32.3 (25.0–35.7)	<0.0001
Birth weight in grams, median (range)	3610 (2633–4342)	1878 (470–3446)	<0.0001
Apgar score at 1 min, median (range)	8 (2–9)	5 (1–9)	0.0004
Apgar score at 5 min, median (range)	9 (8–9)	7 (3–9)	<0.0001
Mechanical ventilation, n (%)	0 (0)	5 (24)	0.0211
Neonatal antimicrobial treatment, n (%)	0 (0)	15 (71)	<0.0001
Neonatal corticosteroids, n (%)	0 (0)	3 (14)	0.1078
Indomethacin, n (%)	0 (0)	6 (29)	0.0089

Table 2

Top 20 shared genes whose expression increased in response to LPS stimulation and associated fold changes^a

Symbol	Name	Adult	Term	Preterm
IL23A	interleukin 23 alpha subunit	74.4	29.3	16.9
IRG1	immunoresponsive 1 homolog	20.5	26.6	18.5
CXCL10	chemokine (C-X-C motif) ligand 10	46	10	6.2
IL6	interleukin 6	25.2	19.6	14.4
CCL20	chemokine (C-C motif) ligand 20	22.1	16.6	12.8
IL1A	interleukin 1 alpha	12.2	13	15.5
SLC39A8	solute carrier family 39 member 8	20.4	9.8	6.6
ITGB8	integrin subunit beta 8	13.4	11.3	6.2
AK4	adenylate kinase 4	7.1	7.2	8.1
SLAMF7	SLAM family member 7	10.5	6.3	4.6
FLT1	fms related tyrosine kinase 1	6.6	6.2	7.3
CCL7	chemokine (C-C motif) ligand 7	8.6	4.2	5.5
CD69	CD69 molecule	9.4	3.2	3.8
PI3	peptidase inhibitor 3	2.6	3.9	9.7
TNFAIP6	TNF alpha induced protein 6	6	5	5.1
MX1	MX dynamin like GTPase 1	8.4	3.7	3.4
GCH1	GTP cyclohydrolase 1	5.9	5.3	4.2
RIN2	Ras and Rab interactor 2	4.3	4.7	6.2
CLIC4	chloride intracellular channel 4	7.3	4.6	3.2
NCR3LG1	natural killer cell cytotoxicity receptor 3 ligand 1	5.7	4.8	3.6

^aTop genes were sorted by greatest mean fold change across all groups