Published in final edited form as:

Transl Res. 2017 December ; 190: 4–15. doi:10.1016/j.trsl.2017.08.003.

Neutrophil Chemotaxis and Transcriptomics in Term and Preterm Neonates

Steven L. Raymond, MD^a, Brittany J. Mathias, MD^a, Tyler J. Murphy^a, Jaimar C. Rincon, PhD^a, María Cecilia López^b, Ricardo Ungaro^a, Felix Ellett, PhD^c, Julianne Jorgensen^c, James L. Wynn, MD^d, Henry V. Baker, PhD^b, Lyle L. Moldawer, PhD^a, Daniel Irimia, MD, PhD^c, and Shawn D. Larson, MD^a

^aDepartment of Surgery, University of Florida College of Medicine; Gainesville, Florida

^bDepartment of Molecular Genetics and Microbiology, University of Florida College of Medicine; Gainesville, Florida

^cDepartment of Surgery, Massachusetts General Hospital, Harvard Medical School, Shriners Burns Hospital; Boston, Massachusetts

^dDepartment of Pediatrics, University of Florida College of Medicine; Gainesville, Florida

Abstract

Neutrophils play a crucial role in combating life-threatening bacterial infections in neonates. Previous studies investigating neonatal cell function have been limited because of restricted volume sampling. Here, using novel microfluidics approaches, we provide the first description of neutrophil chemotaxis and transcriptomics from whole blood of human term and preterm neonates, as well as young adults. *Ex vivo* percent cell migration, neutrophil velocity, and directionality to fMLP were measured from whole blood using time-lapse imaging of microfluidic chemotaxis. Genome-wide expression was also evaluated in CD66b⁺ cells using microfluidic capture devices. Neutrophils from preterm neonates migrated in fewer numbers compared to term neonates (preterm 12.3%, term 30.5%, $p=0.008$) and at a reduced velocity compared to young adults (preterm 10.1 $\mu\text{m}/\text{min}$, adult 12.7 $\mu\text{m}/\text{min}$, $p=0.003$). Despite fewer neutrophils migrating at slower velocities, neutrophil directionality from preterm neonates was comparable to adults and term neonates. 3,607 genes were differentially expressed among the three groups ($p<0.001$). Differences in gene expression between neutrophils from preterm and term neonates were consistent with reduced pathogen recognition and antimicrobial activity, but not neutrophil migration, by preterm neonates. In summary, preterm neonates have significant disturbances in neutrophil chemotaxis compared to term neonates and adults, and these differences in phenotype appear at the transcriptional level to target inflammatory pathways in general, rather than in neutrophil migration and chemotaxis.

Correspondence should be directed to: Shawn D. Larson, MD, FACS, Division of Pediatric Surgery, Department of Surgery, University of Florida College of Medicine, MSB N6-10, PO Box 100119, Gainesville, Florida 32610-0119, (352) 273-8761, (352) 273-8772 (fax), Shawn.Larson@surgery.ufl.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Despite advances in neonatal critical care medicine, mortality remains significant among neonates. The daily mortality rate during the neonatal period is almost 30-fold higher compared to the post-neonatal period with the absolute highest risk of mortality occurring among preterm neonates (gestational age less than 37 weeks) [1]. Infections including neonatal sepsis account for the greatest percentage of neonatal mortality worldwide, which exceeds one million neonatal deaths each year [1]. Bacteria are the predominant pathogens associated with neonatal sepsis with gram-negative bacteria accounting for 38 percent of neonatal septic shock cases and 63 percent of neonatal sepsis mortality [2]. To recognize and combat these life-threatening bacterial infections, neonates rely predominantly on their innate immune system [3]. As such, neutrophils play a crucial role in neonatal survival. Neutrophils act as early responders to pathogens and are the most predominant immune cells in human blood [4]. Despite their central role in the recognition and early response to infections, our understanding of human neonatal neutrophil function is limited. Recent advances have provided a unique opportunity to study low birth weight preterm population using whole blood assays in the first few days of life. In the past, such studies would have been impossible to perform because of the required large blood volumes, which have now been overcome with the use of novel microfluidic approaches [5]. These approaches circumvent the need for neutrophil purification, thus allowing the investigation of neonatal neutrophils from a single 400 microliter unprocessed whole blood sample. In addition, functional assays that utilize whole blood better reflect the intravascular milieu and thus should more closely approximate neutrophil capabilities *in vivo*. In this study, neutrophil chemotaxis and transcriptomics of both term and preterm neonates were characterized utilizing whole blood samples obtained in the first few days of life, as well as whole blood samples from healthy young adults.

Methods

Study Design

This prospective observational study was conducted between February and September 2016 at UF Health Shands Children's Hospital, a 202-bed tertiary medical center. The study was approved by the Institutional Review Board prior to initiation and carried out according to The Code of Ethics of the World Medical Association (Declaration of Helsinki). All neonates admitted to the Neonatal Nursery and Neonatal Intensive Care Unit were screened for inclusion. Neonates born greater than 24 weeks gestation age were eligible for participation. Neonates were excluded from the study if the sample collection did not coincide temporally with a required clinical blood draw. Healthy adults aged 21 – 45 years were also recruited for participation. Adult subjects were excluded from enrollment if any of the following was reported: severe 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 and from the parents of neonatal subjects prior to sample collection.

Sample Collection

Whole blood samples were collected from adults, term neonates, and preterm neonates. In adult subjects, a four milliliter blood sample was collected. In neonatal subjects, a single 700 microliter blood sample was collected once at the time of a clinically-required blood draw. Blood was collected for term neonates in the Neonatal Nursery prior to discharge between 24 and 36 hours of life. Blood was collected for preterm neonates in the Neonatal Intensive Care Unit on day four of life as not to interfere with the intense clinical management in the first 48 hours and to allow the family an appropriate time to absorb the trauma of a premature birth prior to discussing study enrollment.

Patient Data Collection

Patient data including maternal age, Group B streptococcal (GBS) colonization, prolonged premature rupture of membranes (PROM), clinical chorioamnionitis, antepartum antibiotics use, antepartum corticosteroid therapy, antepartum magnesium administration, labor, method of delivery, neonatal gender, completed gestational age, birth weight, Apgar scores, neonatal antibiotic administration, neonatal corticosteroid therapy, indomethacin administration, use of invasive respiratory therapy, positive blood cultures, and mortality were collected from the electronic medical records of the birth hospitalization. Prolonged PROM was defined as any rupture of membranes that persisted for more than 24 hours and prior to the onset of labor. Presence of labor was defined clinically by occurrence of contractions. Apgar scores were accessed at one and five minutes following birth by a certified pediatric nurse or physician. Neonatal antibiotics, corticosteroids, and indomethacin were recorded if administered any time prior to blood sample collection. Use of invasive respiratory therapy was based on the status of the neonate at the time of blood sample collection. Blood cultures were considered if drawn any time prior to blood sample collection and considered positive if the culture grew a microorganism. Mortality was defined as death during the birth hospitalization.

Neutrophil Chemotaxis Assay

Microfluidic chemotaxis devices with a single whole blood loading chamber (WBLC), erythrocyte filters, and 16 identical migration tracks and focal chemoattractant chambers (FCCs) were utilized, as previously described and validated by Hoagn *et al.* and Jones *et al.* (Fig. 1) [5, 6]. In brief, the filters block the dispersion of erythrocytes and monocytes into the migration channel, whereas actively migrating neutrophils can migrate through without a decrease in speed. A bifurcation before each FCC facilitates quantification of directional neutrophil movement following the chemoattractant gradient. Microfluidic chemotaxis devices were primed with the chemokine N-formylmethionyl-leucyl-phenylalanine [1 μ M] (fMLP; Sigma-Aldrich, St. Louis, MO, USA). The device was then placed in a desiccator under vacuum for ten minutes to ensure complete filling of the FCCs. Following filling, the WBLC and the outer region of the device were washed thoroughly with phosphate buffered saline (PBS) to establish a gradient along the migration tracks with the greatest concentration in the focal chemoattractant chambers. The device was then filled with Hank's Balanced Salt Solution (HBSS) with albumin, and allowed to sit for a period of 10 minutes to generate a stable chemoattractant gradient. Finally, two microliters of whole blood stained

with Hoechst Stain Solution (Sigma-Aldrich, St. Louis, MO, USA) was pipetted into the WBLC. Time-lapse imaging was performed every 30 seconds for 10 hours on a Nikon Bio-Station IMq™ microscope inside of a biochamber heated to 37° C with 5% CO₂. Neutrophil percent migration was calculated using Nikon Imaging Software Elements™ High Content Analysis (Nikon Instruments Inc., Melville, NY, USA). Neutrophil instantaneous velocity and directionality were calculated using ImageJ software (NIH, Bethesda, MD, USA) [7].

Neutrophil Transcriptome Assay

Neutrophils were captured from 350 µl of whole blood using positive selection with anti-CD66b monoclonal antibody, clone 80H3 (BioRad, Hercules, CA, USA) coated microfluidic devices as previously described and validated by Kotz *et al.*, Warner *et al.* and Mathias *et al.* [8–10]. The microfluidic devices 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). 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). 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, USA).

Statistical Analysis

Patient data for samples in the neutrophil chemotaxis assay and transcriptome assay were assigned to term and preterm neonatal groups based on gestational age. Statistical analyses for subject data and functional analyses were performed using Prism GraphPad Software (La Jolla, CA, USA). Two-tailed Student's t-tests and Fischer's exact tests were used to compare continuous and categorical clinical variables, respectively. Mann-Whitney U test was utilized for non-parametric data. For each neutrophil chemotactic measurement (percent cell migration, neutrophil velocity, directionality), means and standard deviations were calculated and compared among adults, term neonates, and preterm neonates by use of a two-tailed Student's t test with Bonferroni correction.

Genome-wide expression was compared among adults, term neonates, and preterm neonates using both unsupervised and supervised analyses. In the unsupervised analysis, probe sets with a coefficient of variation greater than 50% were hierarchically clustered. Hierarchical clustering was generated using DNA-Chip Analyzer (dChip) software. In the supervised analysis, significant genes were identified using an F-test at $p < 0.001$. Leave-one-out cross-validation and Monte Carlo simulations and permutation analysis were used to determine whether the misclassification rate for each subject (term, preterm, or adult) was significantly better than predicted by chance, using BRB ArrayTools™ Version:4.5.1-Stable Release as described previously [11–13].

Once significant genes were identified, Gene Ontology™ analysis was conducted as implemented in BRB ArrayTools™. Fold changes of significant genes were calculated between groups. Functional pathway analysis was performed on significant genes using Ingenuity Pathway Analysis (IPA) software™ (Ingenuity Systems, Redwood City, CA, USA). IPA predicts significant canonical pathways, common upstream transcriptional regulators, and cellular functions based on the expression values from a group of genes and prior knowledge of expected effects of these gene products. Significance pathways were determined using a z-score greater than 2 or less than -2.

Results

Patient Data

Whole blood samples were collected from 15 young adults (21–45 years old), 20 term neonates (gestational age >37 weeks), and 20 preterm neonates (gestational age <37 weeks). Small sample volumes and technical issues prevented all measurements to be performed for every subject. The exact number of subjects for each experiment is given in the figure legends. Patient data regarding demographics, management, and outcomes are provided in Tables 1 and 2. Overall, there was a slight predominance of male neonates in the study cohort (55%). Sixty-three percent of neonates were born via cesarean section. Mean maternal age was 28 years with 41% of the mothers being positive for GBS. Method of delivery, gender, maternal age, and GBS colonization were similar between preterm and term neonatal groups. Mothers of preterm neonates had a greater incidence of prolonged PROM, clinical chorioamnionitis, and labor. Mothers of preterm neonates were also more likely to receive antepartum antibiotics, corticosteroids, and magnesium. Overall, term neonates had an average completed gestational age of 39 weeks, whereas preterm neonates had an average completed gestational age of 29 weeks. Preterm neonates had mean birth weight of 1,457 grams, which was significantly less than term neonates at 3,325 grams. Preterm neonates were also born with significantly lower Apgar scores at one and five minutes, compared to term neonates. Furthermore, preterm neonates were more likely to receive mechanical ventilation and to be administered antibiotics and indomethacin. There were no neonates with positive blood cultures within the first 96 hours of life. Finally, there were no neonatal deaths during the birth hospitalization.

Neutrophil Chemotaxis

Ex vivo whole blood chemotaxis was measured to the chemokine fMLP for term and preterm neonates, as well as young adults. A representative time-lapse video of the ten hour measurement is presented in the supplemental material section. The mean number of cells added to each device was not significantly different among groups (adult 1,036 cells, term 1,039 cells, preterm 1,343 cells; $p=0.2693$). One in eight neutrophils from preterm neonates successfully migrated to fMLP over ten hours, compared to approximately one in three neutrophils from term neonates (preterm 12.3%, term 30.5%; $p=0.008$) (Fig. 2A and B). Percent neutrophil migration in term neonates was greater, albeit not significantly from adults (term 30.5%, adult 22.2%; $p=0.36$). The velocity of neutrophils from preterm neonates that migrated to fMLP was significantly lower than that of adult neutrophils (preterm 10.1 $\mu\text{m}/\text{min}$, adult 12.7 $\mu\text{m}/\text{min}$; $p=0.003$) (Fig. 2C), while the velocity of

neutrophils from term neonates was intermediate (11.3 $\mu\text{m}/\text{min}$). Despite fewer neutrophils migrating at slower velocities, the capability of neutrophils from preterm neonates to follow the fMLP gradient (directionality index) was comparable to neutrophils from adults or term neonates (Fig. 2D).

Neutrophil Transcriptomics

To determine if the observed differences in neutrophil chemotaxis to fMLP were reflected by changes in the neutrophil transcriptome, genome-wide expression of CD66b⁺-captured cells was performed. An unsupervised analysis of gene expression from the three groups identified 2,077 genes that were differentially expressed among the three groups with a coefficient of variation greater than 50%. When the 2,077 genes were hierarchically clustered based upon the overall similarity of expression without any knowledge of the group assignment, the expression pattern could distinguish preterm neonates from term neonates and young adults (Fig. 3A). Following the first major division in the dendrogram, term neonates and young adults further separated into two distinct groups. The overall clustering was not perfect as the expression pattern of one term neonate clustered with the preterm neonates and the expression pattern of one preterm neonate clustered with the term neonates. Further review of the electronic medical records for the birth hospitalization of these two subjects did not identify any specific differences unique to these two neonates compared to the other neonates in their respective groups.

Using a supervised analysis, the expression of 3,607 genes significantly differed among the three groups at a confidence level of $p < 0.001$. Hierarchical clustering of these genes was similar to the previous unsupervised analysis with the gene expression patterns between young adults and term neonates being more similar to each other than to preterm neonates (Fig. 3B). Again, one term neonate incorrectly clustered with the preterm neonates in the supervised analysis.

Cross-validation methods were able to distinguish subjects by group with a mean percent of correct classification of 90% and 83% for 1-nearest neighbor classifier and 3-nearest neighbor classifier, respectively. The misclassification rate was significantly better than predicted by chance based on Monte Carlo simulations using 1,000 random permutations ($p < 0.001$).

Compared to adults, preterm neonates had 500 genes differentially expressed with greater than a two fold-difference ($p < 0.001$), while term neonates had 205 genes that were differentially expressed compared to adults ($p < 0.001$, > 2 fold-difference). One hundred twenty-five genes were differentially expressed in both preterm and term neonates compared to adults ($p < 0.001$, > 2 fold-difference). Compared to term neonates, 113 genes were differentially expressed in preterm neonates ($p < 0.001$, > 2 fold-difference). The genes with the greatest downregulation in preterm neonates compared to term neonates or young adults included key components of innate immune response to pathogens: peptidase inhibitor 3 (*PI3*, an antimicrobial peptide); pro-platelet basic protein (*PPBP*, a chemoattractant and neutrophil activator); C-type lectin domain family 2, member B (*CLEC2B*, role in pathogen recognition), guanylate binding protein 1 (*GBP1*, promotes antimicrobial activity);

ribonuclease RNase A family, k6 (*RNASE6*, a broad-spectrum antimicrobial protein) (Table 3).

Functional Pathway Analysis

To better understand the biologic differences, the 3,607 differentially expressed genes were examined using Ingenuity Pathway Analysis™ (IPA). Preterm neonates had significant downregulation in the canonical pathways for ‘interleukin (IL)-1 signaling’, ‘inducible nitric oxide synthase (iNOS) signaling’, and ‘inflammasome pathway’, compared to term neonates (z-score <−2) (Fig. 4). Preterm neonates also had significant inhibition of the common upstream transcriptional regulators for the cytokines *CXCL12*, *IL1A*, *IL1B*, *IL12A*, *IFNB1*, *IFNG*, *OSM*, *MIF*, *TNF*, and *TNFS10*, compared to term neonates (z-score <−2) (Table 4). Furthermore, preterm neonates had significant inhibition of upstream regulators for the primary inflammatory regulator, nuclear factor-κB (*NF-κB*) complex and toll-like receptor 2 (*TLR2*), compared to term neonates (z-score <−2). Genes involved in ‘cell movement of neutrophils’ and in ‘phagocytosis’ were surprisingly not significantly different between preterm and term neonates (z-scores 1.34 and 0.487, respectively). Interestingly, these genes were significantly increased in both preterm and term neonates compared to adults (z-score >2), again consistent with the reliance on innate immunity by neonates.

In comparison to young adults, both preterm and term neonates had inhibition of upstream transcriptional regulators for interferon beta 1 (*IFNB1*) and the classic T-cell transmembrane receptors *CD3* and *CD28* consistent with decreased role for adaptive immunity in neonates (z-score <−2) (Table 5). Both preterm and term neonates had significant activation of upstream regulators of multiple cytokines including *CXCL8*, *IL1B*, *IL2*, *IL4*, *IL5*, and *IL6*, compared to young adults consistent with their reliance on innate immunity (z-score >2).

Finally, cellular function analysis revealed preterm neonates specifically had under-expression of genes involved in ‘differentiation of blood cells and leukocytes’, compared to term neonates (z-score <−2) (Supplemental Table 1). Both preterm and term neonates had significant under-expression of genes involved in cell death, necrosis, and apoptosis, and significant overexpression of genes involved in cell viability, cellular growth, and cell survival, compared to adults (z-score <2).

Discussion

Sepsis remains a prominent cause of neonatal mortality especially among preterm neonates. Previous studies have demonstrated that neonatal neutrophils specifically have qualitative impairments of adherence, chemotaxis, phagocytosis, neutrophil respiratory burst activity, and release of extracellular traps, compared to adults [14–21]. However, these findings have been largely based on experiments that utilized cord blood from term neonates and required detailed neutrophil isolation. Here, we focused on neutrophil chemotaxis and transcriptomics of both preterm and term neonates utilizing whole blood samples obtained from the neonates themselves in the first few days of life. The chemotactic measurements obtained here cannot be achieved using any of the traditional cell migration technologies, including the commonly used transwell assay [22]. Transwell assays provide only end-point results for populations of cells and lack single cell resolution. Additionally, transwell assays

require several million neutrophils isolated from at least five milliliters of whole blood, which is not possible to collect in extremely low birth weight neonates where total blood volumes may be less than 100 milliliters [23].

We demonstrate that preterm neonates have inherent defects in neutrophil migration in response to a microbial product, whereas neutrophil migration in term neonates was not significantly different from adults. Subsequently, we asked whether changes in neutrophil transcriptome could explain the functional changes associated with preterm neonates. A survey of the published literature found no studies looking at genome-wide expression of neutrophils from preterm and term neonates. Here, we demonstrate that preterm neonates have significant differences in gene expression compared to term neonates and young adults. Analysis of the most downregulated genes suggests that at the transcriptional level, preterm neonates have a generalized, diminished ability to mount a successful innate immune response compared to term neonates or young adults. Subsequent functional genomic analysis revealed preterm neonates also had deficiencies in pathogen recognition and pathogen destruction, compared to term neonates, as demonstrated by inhibition of the IL-1 signaling, iNOS signaling, and inflammasome pathway [24–26]. Preterm neonates also had inhibition of upstream regulators for multiple cytokines, NF- κ B, and TLR2, compared to term neonates. NF- κ B is a central regulator of innate immunity and inflammation, and plays a role in neutrophil development and survival [27]. TLR2 is important for the recognition of gram-positive and gram-negative bacteria [28]. As demonstrated by these findings, preterm neonates have significant deficiencies in the neutrophil transcriptome for the innate arm of the host protective immune response, when compared to term neonates.

In comparison to healthy young adults, both preterm and term neonates had inhibition of key promoters for T-cell activation [29]. Although the expression of these T-cell genes is not significant in neutrophils, the observation that their upstream transcriptional regulators are suppressed in neonates suggests that they are likely to have defects in the regulation of gene expression in adaptive immunity. This supports previous studies which have demonstrated neonates fail to produce a robust adaptive immune response to infection [30]. Likewise, it is well known that neutrophils communicate bidirectionally with the adaptive immune response through neutrophil development and the expression of regulatory cytosolic and cell surface molecules. Compared to healthy young adults, preterm and term neonates had significant activation in transcriptional regulators for various cytokines including IL-6, which has been shown to have anti-inflammatory properties and inhibit neutrophil accumulation at sites of inflammation [31]. Neonates also had significant downregulation of genes involved in cellular death and upregulation of genes involved in cellular growth, compared to adults. These findings are not unexpected since neonates are in a period of rapid growth and development, and are consistent with our previous work comparing the transcriptome of naïve neonatal mice and healthy young adult mice [32].

There are number of limitations to the study that require further discussion. First, the relatively small number of study participants are underpowered to detect all significant differences between young adults, term neonates, and preterm neonates. However, the study had at least 80% power to find a difference in the percentage of neutrophils migrating if one exists and 5% chance that the differences would not be observed if the number of patients

enrolled were increased. Second, the neonatal population was heterogeneous with preterm neonates being born at lower birth weights and Apgar scores compared to term neonates. However, this is expected given the differences in gestational age. Preterm neonates were more likely to be exposed to prolonged premature rupture of membranes, clinically suspected chorioamnionitis, and labor, compared to term neonates. Prior studies have suggested microbial exposure and labor may have influential roles in neutrophil function. Specifically, some investigators believe that neonatal neutrophils reside in a “pre-primed” state and become reactive following labor [33]. Despite preterm neonates being exposed to these potentially activating factors, preterm neutrophils still demonstrated deficiencies in chemotaxis and transcriptomics.

In the study cohort, preterm neonates were also more likely to be administered antibiotics following birth given their prematurity. The use of broad-spectrum antibiotics in preterm neonates is known to be associated with microbiome modification and decreased microbial diversity which may have global effects on neonatal health [34]. Changes in the microbiome may specifically influence neutrophil chemotaxis. For example, previous murine studies have demonstrated that the microbiota drives neutrophil ageing and that aged neutrophils have reduced capacity for migration to site of inflammation [35, 36]. We recommend future studies investigating the human neonatal intestinal microbiome and its impact on neutrophil function.

Since electronic medical records were only reviewed for neonatal subjects, maternal characteristics, such as presence of labor and antenatal medication administration, are subject to reporting bias in the neonates’ record. Additionally, this study was not designed to directly access the impact of medications on neonatal neutrophil migration; however, previous cord blood studies investigating isolated neutrophil migration to zymosan-activated serum demonstrated defective chemotaxis with betamethasone and magnesium sulfate exposure [37, 38]. The presence and duration of these effects *in vivo* remains unknown. Herein, samples from term neonates were collected at the same time as blood for the state’s newborn screening program and occurred 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, primarily out of respect for the family dealing with the trauma of a premature birth, as well as the intense clinical management commonly required in the first 48 hours. This delay may additionally allow antenatal medications to be cleared from the neonatal circulation, as well as minimize the influence of the physiological stresses from labor and delivery on the observed neutrophil chemotaxis and transcriptomics.

The small volume of whole blood collected for neonatal subjects prevented additional assessments of neutrophil function, such as phagocytosis, oxidative burst, and bacterial killing. Likewise, the time-lapse imaging microscope and microfluidic chemotaxis device design permit only one chemoattractant to be studied during a given chemotaxis assay, thus fMLP was the only chemoattractant used here. Regarding the neutrophil transcriptome assay, anti-CD66b monoclonal antibody coated microfluidic device were utilized; however, it is important to note that CD66b is expressed on other granulocyte subsets including eosinophils [39]. Nevertheless, neutrophils comprise the majority of the CD66b positive

population. A previous validation study comparing the microfluidic technique used herein to neutrophil isolation by dextran-Ficoll demonstrated the biological and analytical artifacts introduced by microfluidics processing are small and possibly less than the variability among subjects or due to dextran-Ficoll isolation [9]. Furthermore, microfluidic approaches may result in the introduction of less artifactual *ex vivo* stimulation, as it requires significantly fewer sample manipulations and less exposure to nonphysiologic solutions and *g* forces. RNA hybridization-based microarrays have general limitations as well. Specifically, their ability to make predictions of the overall genome is restricted to gene-level, although the GeneChip™ Human Transcriptome Array 2.0 contains probe sets for future exon-level and alternative splicing determinations [40]. Although predicted canonical pathways and regulatory networks can provide a representation of molecular interactions within cells, the actual regulatory pathways are dynamic and change with environmental stresses [41]. Despite the study limitations, the authors feel that the results are applicable and represent the first time microfluidic techniques were used to analyze neutrophil chemotaxis and transcriptomics from the whole blood of preterm and term neonates.

In conclusion, we demonstrated that preterm neonates have significant differences in neutrophils, compared to term neonates and young adults. These differences include reduced percent neutrophil migration and velocity in response to a microbial product, as well as transcriptomic evidence of impaired pathogen recognition, cytokine production, and anti-microbial activity. These findings suggest that the defects in neutrophil migration may be just one component of a more generalized transcriptional down regulation of promoters and genes involved in inflammation and innate immunity. Future studies should focus on investigating the impact of specific maternal and neonatal factors, as well the development of targeted interventions aimed at enhancing neutrophil function in preterm neonates with the ultimate goal of developing therapies to prevent and treat neonatal sepsis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

All authors have read the journal's policy on disclosure of potential conflicts of interest and have none to declare. All authors have read the journal's authorship agreement, and the manuscript has been reviewed by and approved by all named authors. No editorial support was used for preparation of the manuscript.

This study was supported by grants from the National Institute of General Medical Sciences (NIGMS; K08 GM106143, R01 GM097531, and P50 GM111152). SLR was supported by a clinical research training fellowship award from the Surgical Infection Society Foundation. BJM was supported by a NIGMS post-doctoral training grant (T32 GM-008721).

LLM, DI, JLW, and SDL developed the hypothesis and designed the experiments. FE, JJ, and DI provided the microfluidic chemotaxis devices and technical support. SLR, BJM, TJM, JCR, MCL, and RU performed the experiments and analyzed the results. SLR, JLW, HVB, LLM, DI, and SDL reviewed the results and prepared the manuscript.

The authors thank all study participants as well as the research and clinical staff at UF Health Shands Children's Hospital who made this study possible.

Abbreviations

CLEC2B	C-type lectin domain family 2, member B
dChip	DNA-Chip Analyzer
FCC	Focal chemoattractant chamber
fMLP	N-formylmethionyl-leucyl-phenylalanine
GBS	Group B streptococcal
HBSS	Hank's Balanced Salt Solution
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IPA	Ingenuity Pathway analysis
NF-κB	Nuclear factor- κ B
PBS	Phosphate buffered saline
PI3	Peptidase inhibitor 3
PPBP	Pro-platelet basic protein
PROM	Premature rupture of membranes
RNASE6	Ribonuclease RNase A family, k6
SAM	Significance Analysis of Microarrays
TCR	T-cell receptor
TLR2	Toll-like receptor 2
WBLC	Whole blood loading chamber

References

1. Lawn JE, Cousens S, Zupan J. 4 million neonatal deaths: when? Where? Why? *Lancet* (London, England). 2005; 365:891–900.
2. Kermorvant-Duchemin E, Laborie S, Rabilloud M, Lapillonne A, Claris O. Outcome and prognostic factors in neonates with septic shock. *Pediatric critical care medicine : a journal of the Society of Critical Care Medicine and the World Federation of Pediatric Intensive and Critical Care Societies*. 2008; 9:186–91.
3. Cuenca AG, Wynn JL, Moldawer LL, Levy O. Role of innate immunity in neonatal infection. *American journal of perinatology*. 2013; 30:105–12. [PubMed: 23297181]
4. Wynn JL, Wong HR. Pathophysiology and treatment of septic shock in neonates. *Clinics in perinatology*. 2010; 37:439–79. [PubMed: 20569817]
5. Jones CN, Hoang AN, Martel JM, Dimisko L, Mikkola A, Inoue Y, et al. Microfluidic assay for precise measurements of mouse, rat, and human neutrophil chemotaxis in whole-blood droplets. *Journal of leukocyte biology*. 2016; 100:241–7. [PubMed: 26819316]

6. Hoang AN, Jones CN, Dimisko L, Hamza B, Martel J, Kojic N, et al. Measuring neutrophil speed and directionality during chemotaxis, directly from a droplet of whole blood. *Technology*. 2013; 1:49. [PubMed: 24809064]
7. Arena ET, Rueden CT, Hiner MC, Wang S, Yuan M, Eliceiri KW. Quantitating the cell: turning images into numbers with ImageJ. *Wiley interdisciplinary reviews Developmental biology*. 2017:6.
8. Kotz KT, Xiao W, Miller-Graziano C, Qian WJ, Russom A, Warner EA, et al. Clinical microfluidics for neutrophil genomics and proteomics. *Nature medicine*. 2010; 16:1042–7.
9. Warner EA, Kotz KT, Ungaro RF, Abouhamze AS, Lopez MC, Cuenca AG, et al. Microfluidics-based capture of human neutrophils for expression analysis in blood and bronchoalveolar lavage. *Laboratory investigation; a journal of technical methods and pathology*. 2011; 91:1787–95. [PubMed: 21931299]
10. Mathias B, Mira JC, Rehffuss JP, Rincon JC, Ungaro R, Nacionales DC, et al. LPS Stimulation of Cord Blood Reveals a Newborn-Specific Neutrophil Transcriptomic Response and Cytokine Production. *Shock (Augusta, Ga)*. 2017; 47:606–14.
11. Wynn JL, Guthrie SO, Wong HR, Lahni P, Ungaro R, Lopez MC, et al. Postnatal Age Is a Critical Determinant of the Neonatal Host Response to Sepsis. *Mol Med*. 2015; 21:496–504. [PubMed: 26052715]
12. Simon R. Microarray-based expression profiling and informatics. *Curr Opin Biotechnol*. 2008; 19:26–9. [PubMed: 18053704]
13. Simon R. Analysis of DNA microarray expression data. *Best Pract Res Clin Haematol*. 2009; 22:271–82. [PubMed: 19698933]
14. Al-Hadithy H, Addison IE, Goldstone AH, Cawley JC, Shaw JC. Defective neutrophil function in low-birth-weight, premature infants. *Journal of clinical pathology*. 1981; 34:366–70. [PubMed: 7240423]
15. Eisenfeld L, Krause PJ, Herson V, Savidakis J, Bannon P, Maderazo E, et al. Longitudinal study of neutrophil adherence and motility. *The Journal of pediatrics*. 1990; 117:926–9. [PubMed: 2246695]
16. Krause PJ, Herson VC, Boutin-Lebowitz J, Eisenfeld L, Block C, LoBello T, et al. Polymorphonuclear leukocyte adherence and chemotaxis in stressed and healthy neonates. *Pediatric research*. 1986; 20:296–300. [PubMed: 3703618]
17. Anderson DC, Hughes BJ, Smith CW. Abnormal mobility of neonatal polymorphonuclear leukocytes. Relationship to impaired redistribution of surface adhesion sites by chemotactic factor or colchicine. *The Journal of clinical investigation*. 1981; 68:863–74. [PubMed: 7287906]
18. Drossou V, Kanakoudi F, Tzimouli V, Sarafidis K, Taparkou A, Bougiouklis D, et al. Impact of prematurity, stress and sepsis on the neutrophil respiratory burst activity of neonates. *Biology of the neonate*. 1997; 72:201–9. [PubMed: 9339291]
19. Filias A, Theodorou GL, Mouzopoulou S, Varvarigou AA, Mantagos S, Karakantza M. Phagocytic ability of neutrophils and monocytes in neonates. *BMC pediatrics*. 2011; 11:29. [PubMed: 21492472]
20. Merry C, Puri P, Reen DJ. Defective neutrophil actin polymerisation and chemotaxis in stressed newborns. *Journal of pediatric surgery*. 1996; 31:481–5. [PubMed: 8801296]
21. Yost CC, Cody MJ, Harris ES, Thornton NL, McInturff AM, Martinez ML, et al. Impaired neutrophil extracellular trap (NET) formation: a novel innate immune deficiency of human neonates. *Blood*. 2009; 113:6419–27. [PubMed: 19221037]
22. Boyden S. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. *The Journal of experimental medicine*. 1962; 115:453–66. [PubMed: 13872176]
23. Sisson TR, Whalen LE, Telek A. The blood volume of infants. II. The premature infant during the first year of life. *The Journal of pediatrics*. 1959; 55:430–46. [PubMed: 14447226]
24. Cybulsky MI, Colditz IG, Movat HZ. The role of interleukin-1 in neutrophil leukocyte emigration induced by endotoxin. *The American journal of pathology*. 1986; 124:367–72. [PubMed: 3532812]
25. von Moltke J, Ayres JS, Kofoed EM, Chavarria-Smith J, Vance RE. Recognition of bacteria by inflammasomes. *Annual review of immunology*. 2013; 31:73–106.
26. Nussler AK, Billiar TR. Inflammation, immunoregulation, and inducible nitric oxide synthase. *Journal of leukocyte biology*. 1993; 54:171–8. [PubMed: 7689630]

27. Hayden MS, Ghosh S. NF-kappaB in immunobiology. *Cell research*. 2011; 21:223–44. [PubMed: 21243012]
28. Raymond SL, Holden DC, Mira JC, Stortz JA, Loftus TJ, Mohr AM, et al. Microbial recognition and danger signals in sepsis and trauma. *Biochimica et biophysica acta*. 2017
29. Smith-Garvin JE, Koretzky GA, Jordan MS. T cell activation. *Annual review of immunology*. 2009; 27:591–619.
30. Raymond SL, Stortz JA, Mira JC, Larson SD, Wynn JL, Moldawer LL. Immunological Defects in Neonatal Sepsis and Potential Therapeutic Approaches. *Frontiers in Pediatrics*. 2017;5. [PubMed: 28168186]
31. Xing Z, Gauldie J, Cox G, Baumann H, Jordana M, Lei XF, et al. IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *The Journal of clinical investigation*. 1998; 101:311–20. [PubMed: 9435302]
32. Gentile LF, Nacionales DC, Lopez MC, Vanzant E, Cuenca A, Cuenca AG, et al. Protective immunity and defects in the neonatal and elderly immune response to sepsis. *Journal of immunology (Baltimore, Md : 1950)*. 2014; 192:3156–65.
33. Lawrence SM, Corriden R, Nizet V. Age-Appropriate Functions and Dysfunctions of the Neonatal Neutrophil. *Front Pediatr*. 2017; 5:23. [PubMed: 28293548]
34. Raymond SL, Rincon JC, Wynn JL, Moldawer LL, Larson SD. Impact of Early-Life Exposures to Infections, Antibiotics, and Vaccines on Perinatal and Long-term Health and Disease. *Frontiers in immunology*. 2017;8. [PubMed: 28144241]
35. Zhang D, Chen G, Manwani D, Mortha A, Xu C, Faith JJ, et al. Neutrophil ageing is regulated by the microbiome. *Nature*. 2015; 525:528–32. [PubMed: 26374999]
36. Rankin SM. The bone marrow: a site of neutrophil clearance. *Journal of leukocyte biology*. 2010; 88:241–51. [PubMed: 20483920]
37. Fuenfer MM, Herson VC, Raye JR, Woronick CL, Eisenfeld L, Ingardia CJ, et al. The effect of betamethasone on neonatal neutrophil chemotaxis. *Pediatric research*. 1987; 22:150–3. [PubMed: 3658539]
38. Mehta R, Petrova A. Intrapartum magnesium sulfate exposure attenuates neutrophil function in preterm neonates. *Biology of the neonate*. 2006; 89:99–103. [PubMed: 16479090]
39. Yoon J, Terada A, Kita H. CD66b regulates adhesion and activation of human eosinophils. *Journal of immunology (Baltimore, Md : 1950)*. 2007; 179:8454–62.
40. Xu W, Seok J, Mindrinos MN, Schweitzer AC, Jiang H, Wilhelmy J, et al. Human transcriptome array for high-throughput clinical studies. *Proceedings of the National Academy of Sciences of the United States of America*. 2011; 108:3707–12. [PubMed: 21317363]
41. Califano A. Rewiring makes the difference. *Molecular systems biology*. 2011; 7:463. [PubMed: 21245848]

Background

Neutrophils play a crucial role in combating life-threatening bacterial infections in neonates. Despite their importance, our understanding of neonatal neutrophil function is limited. Recent advances in microfluidics have provided a unique opportunity to study this vulnerable population using small volume whole blood samples.

Translational Significance

This study provides the first description of neutrophil chemotaxis and transcriptomics in both preterm and term neonates. These findings will aid in the development of reliable tests using micro-quantities of blood to diagnose defects in immune function, to evaluate potential therapies for infectious complications, and to establish criteria for which babies would benefit from these treatments.

Figure 1. Neutrophil chemotaxis assay

Neutrophils migrate out of the center whole blood loading chamber (WBLC) through the filters along the migration tracks towards the focal chemoattractant chambers (FCC), which are primed with the chemoattractant fMLP. Neutrophil percent cell migration and velocity are obtained with time-lapse imaging. A bifurcation prior to the FCC allows for the quantification of neutrophil directionality index.

Figure 2. Characterization of neutrophil chemotaxis of adults, term neonates, and preterm neonates

A. Neutrophil chemotaxis. Average neutrophil migration profiles in response to fMLP over 10 hours for adults (n=8), term neonates (n=11), and preterm neonates (n=12). **B. Percent cell migration.** Preterm neutrophils migrated in fewer numbers than term neutrophils (preterm 12.3%, term 30.5%; $p=0.008$). **C. Neutrophil velocity.** Adult (12.7 $\mu\text{m}/\text{min}$) and term (11.3 $\mu\text{m}/\text{min}$) neutrophils migrated with comparable velocities, whereas preterm neutrophil velocity was significantly lower than adult neutrophils (preterm 10.1 $\mu\text{m}/\text{min}$, adult 12.7 $\mu\text{m}/\text{min}$; $p=0.003$). **D. Neutrophil directionality index.** Adult (0.84), term (0.74), and preterm (0.81) neutrophils migrated with comparable directionality index.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3. Neutrophil transcriptome of adults, term neonates, and preterm neonates

A. Unsupervised analysis hierarchical clustering (2,077 genes). When 2,077 genes with a coefficient of variation >50% were hierarchically clustered, differences in the patterns of gene expression were revealed among young adults, term neonates, and preterm neonates. The dendrogram above the hierarchical clustering summarizes the Pearson correlations. The length of each branch is equivalent to (1-R), so that small branches indicate high correlation coefficients in expression among branches at that level. The first branch indicates the clusters that are more closely related, and in this case, is between adults and term neonates, indicating that their gene expressions are more closely to each other than they are to the expression of preterm neonates. **B. Supervised analysis hierarchical clustering (3,607 genes).** Using a supervised analysis, the expression of 3,607 genes significantly differed among the three groups ($p < 0.001$). The significant genes were then hierarchically clustered and the dendograms show the clustering based on the Pearson correlations. Based on this supervised analysis, the gene expression patterns between young adults and term neonates were again more similar to each other than to preterm neonates.

Figure 4. Predicted differences in canonical pathways among preterm and term neonates

Preterm neonates had significant upregulation in the canonical pathways for 'eukaryotic initiation factor 2 (EIF2) signaling', 'cyclins and cell cycle regulation', and 'nuclear factor erythroid 2-related factor 2 (NRF2) mediated oxidative stress response', compared to term neonates (z-score >2). Preterm neonates had significant downregulation in the canonical pathways for 'interleukin (IL)-1 signaling', 'inducible nitric oxide synthase (iNOS) signaling', and 'inflammasome pathway', compared to term neonates (z-score <-2).

Table 1

Maternal, pregnancy, and neonatal characteristics for subjects in the neutrophil chemotaxis analysis.

	Term (n=18)	Preterm (n=16)	p value
Maternal and pregnancy characteristics			
Maternal age (years)	26 (18–36)	30 (21–39)	0.0347 *
GBS Colonization (%)	35% [‡]	45% [‡]	0.7011
Prolonged PROM (%)	6%	38%	0.0348 *
Clinical Chorioamnionitis (%)	0%	31%	0.0157 *
Antepartum antibiotics (%)	39%	88%	0.0052 *
Antepartum corticosteroids (%)	0%	69%	< 0.0001 *
Antepartum magnesium (%)	11%	63%	0.0033 *
Presence of labor (%)	44%	81%	0.0386 *
Cesarean section (%)	56%	63%	0.7385
Neonatal characteristics			
Male (%)	39%	63%	0.3028
Gestational age (completed week)	39 (37–40)	30 (24–36)	< 0.0001 *
Birth weight (grams)	3326 (2556–5028)	1612 (505–4576)	< 0.0001 *
Apgar score at 1 min	8 (7–9)	5 (0–9)	< 0.0001 *
Apgar score at 5 min	9 (9)	7 (0–9)	< 0.0001 *
Mechanical ventilation (%)	0%	25%	0.0392 *
Neonatal antibiotics (%)	0%	81%	< 0.0001 *
Neonatal corticosteroids (%)	0%	6%	0.4706
Indomethacin (%)	0%	25%	0.0392 *
Positive blood cultures (%)	0%	0%	1.0000
Mortality (%)	0%	0%	1.0000

[‡]Percentages are based on subjects with known status for GBS colonization. Subjects with unknown statuses were not included in this calculation.

* p value < 0.05.

Abbreviation: GBS = Group B streptococcal; PROM = premature rupture of membranes.

Table 2

Maternal, pregnancy, and neonatal characteristics for subjects in the neutrophil transcriptome analysis.

	Term (n=10)	Preterm (n=10)	p value
Maternal and pregnancy characteristics			
Maternal age (years)	26 (18–35)	28 (21–33)	0.4172
GBS Colonization (%)	67% [‡]	14% [‡]	0.0601
Prolonged PROM (%)	0%	33% [‡]	0.0867
Clinical Chorioamnionitis (%)	0%	22% [‡]	0.2105
Antepartum antibiotics (%)	60%	80%	0.6285
Antepartum corticosteroids (%)	0%	70%	0.0031 [*]
Antepartum magnesium (%)	0%	60%	0.0108 [*]
Presence of labor (%)	30%	80%	0.0698
Cesarean section (%)	80%	60%	0.6285
Neonatal characteristics			
Male (%)	40%	50%	1.0000
Gestational age (completed week)	38.7 (37–40)	27.7 (24–36)	< 0.0001 [*]
Birth weight (grams)	3325 (2622–5028)	1348 (505–4576)	0.0003 [*]
Apgar score at 1 min	7 (1–9)	5 (1–9)	0.0755
Apgar score at 5 min	9 (5–9)	6 (1–9)	0.0085 [*]
Mechanical ventilation (%)	0%	60%	0.0108 [*]
Neonatal antibiotics (%)	0%	90%	0.0001 [*]
Neonatal corticosteroids (%)	0%	0%	1.0000
Indomethacin (%)	0%	60%	0.0108 [*]
Positive blood cultures (%)	0%	0%	1.0000
Mortality (%)	0%	0%	1.0000

[‡]Percentages are based on subjects with known status for GBS colonization, prolonged PROM, and clinical chorioamnionitis.

^{*}p value < 0.05.

Abbreviation: GBS = Group B streptococcal; PROM = premature rupture of membranes.

Table 3

Top five downregulated and upregulated genes between groups.

Preterm/Term Fold-Change	Gene	Preterm/Term Fold-Change	Gene
-7.7	PI3	6.7	CTSG
-5.6	PPBP	5.9	MYB
-5	CASP5	5.6	HIST1H4E
-3.4	CLEC2B	5.2	STMN1
-2.9	SOX6	5.1	MPO
Preterm/Adult Fold-Change	Gene	Preterm/Adult Fold-Change	Gene
-11.2	HCP5	12.8	STMN1
-9	PI3	12.5	OLFM4
-8.1	GBP1	11.2	CTSG
-7.8	P2RY10	10.2	MPO
-5.3	RNASE6	10	HP
Term/Adult Fold-Change	Gene	Term/Adult Fold-Change	Gene
-12	RNASE6	16.1	HBA2
-5.7	GBP1	10	PPBP
-5.5	P2RY10	7.7	MCEMP1
-5.2	MIRLET7G	7.7	TNFAIP3
-4.5	IFI44	6.7	CD177

Table 4

Predicted differences in upstream transcriptional regulators among preterm and term neonates.

Upstream Regulator	Molecule Type	Preterm/Term z-score
Igm	complex	2.644
CSF2	cytokine	2.233
MTORC1	complex	2.219
TREM2	transmembrane receptor	2.186
IL12A	cytokine	-2.007
IL1A	cytokine	-2.205
TLR2	transmembrane receptor	-2.224
CD44	enzyme	-2.296
PDGF BB	complex	-2.298
TNFSF10	cytokine	-2.303
NFkB	complex	-2.379
IFNB1	cytokine	-2.453
OSM	cytokine	-2.503
MIF	cytokine	-2.594
TCR	complex	-2.634
IL1B	cytokine	-2.767
CXCL12	cytokine	-2.891
TNF	cytokine	-2.976
CD28	transmembrane receptor	-3.706
CD3	complex	-4.009
IFNG	cytokine	-4.61

Table 5

Predicted differences in upstream transcriptional regulators among neonates and adults.

Upstream Regulator	Molecule Type	Preterm/Adult z-score	Term/Adult z-score
CSF2	cytokine	5.001	5.567
CSF1	cytokine	4.448	4.27
CD38	enzyme	4.409	3.931
IL5	cytokine	4.212	5.101
IL4	cytokine	4.119	2.847
PDGF BB	complex	4.071	4.542
EPO	cytokine	4.029	4.063
TNFSF11	cytokine	3.806	3.968
SPP1	cytokine	3.569	3.316
IGF1R	transmembrane receptor	3.414	4.723
CD40LG	cytokine	3.281	2.362
IL6	cytokine	3.165	3.657
Pdgf	complex	3.095	2.589
LIF	cytokine	3.08	3.528
CSF3	cytokine	2.99	3.115
IL1B	cytokine	2.87	5.1
IL2	cytokine	2.804	3.01
RHOA	enzyme	2.77	3.338
ICAM1	transmembrane receptor	2.577	2.759
EDN1	cytokine	2.568	3.086
C5	cytokine	2.484	2.853
CSF2RB	transmembrane receptor	2.433	2.433
CCL5	cytokine	2.421	2.713
PI3K	complex	2.391	3.227
LDL	complex	2.346	2.847
CCL2	cytokine	2.211	2.574
CXCL8	cytokine	2.209	2.761
Cg	complex	2.087	2.852
IFNB1	cytokine	-2.262	-2.711
CD28	transmembrane receptor	-3.118	-2.56
CD3	complex	-4.452	-3.6