Neutrophil Extracellular Trap Formation in Newborn Infants at Risk for Inflammatory Syndromes

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Background

Dysregulation of acute inflammation contributes to the pathogenesis of many neonatal and pediatric diseases including sepsis, acute respiratory distress syndrome (ARDS), neonatal chronic lung disease (nCLD), retinopathy of prematurity (ROP), and necrotizing enterocolitis (NEC).

nCLD causes significant morbidity and mortality in the United States. nCLD is a complication of preterm birth that results from prolonged mechanical ventilation required for chronic respiratory failure. It occurs in 30 - 50% of mechanically ventilated extremely low birthweight infants (ELBW) with respiratory distress. While surfactant therapy and pre- or postnatal steroids have decreased the severity of nCLD, this significant morbidity associated with preterm birth remains common in at – risk infants, with about 8,000 to 10,000 new cases occurring annually in the US. The mortality rate due to nCLD in ELBW infants remains high from 15 - 40%. Furthermore, nCLD remains the most common cause of long-term hospitalization in neonates and is also associated with developmental delay. Whether or not dysregulated NET formation contributes to the pathogenesis of nCLD remains unknown.

ROP is the most common cause of pediatric blindness. Dysregulated inflammation is also implicated in the pathogenesis of ROP leading to retinal detachment and blindness. To date, no one has determined whether dysregulated NET formation contributes to ROP.

NEC, predominantly a disease of premature infants, afflicts ≈ 5 - 10% of very low birth weight infants (< 1500 grams), develops typically in the 3rd to 4th week of life and often results in mortality. Infants born closer to term rarely develop NEC but those that do typically exhibit other risk factors such as ductal-dependent congenital heart disease or mesenteric ischemia. For these patients, NEC occurs typically in the first week of life and can prove fatal. Other sequelae of NEC include intestinal perforation, short gut syndrome, prolonged total parenteral nutrition with possible concomitant liver failure and a prolonged intensive care unit stay. While the etiology remains unknown, risk factors of prematurity, enteral feeds, infection and intestinal ischemia predict an increased risk for NEC[5].

The final common pathogenic pathway for NEC appears to depend on a dysregulated, pro-inflammatory response to bacterial translocation across an immature or damaged gastrointestinal epithelial barrier. Animal and human studies of NEC pathogenesis implicate inflammatory cytokines and phospholipids such as tumor necrosis factor alpha, Interleukin-8 and platelet activating factor (PAF). One animal model also demonstrated that aortic lipopolysaccharide (LPS) infusion caused a NEC-like syndrome in rats [6]. Each of these biologically active inflammatory mediators activates the polymorphonuclear leukocyte (PMN)[7], the primary effector cell of the acute inflammatory response to tissue injury or infection.

Data from animal models suggest a prominent role of the PMN in the pathogenesis of NEC. Musemeche et al. induced NEC-like disease in rats by intra-aortic injection of PAF[8]. They then used vinblastine, a chemotherapeutic agent, to induce neutropenia in rats four days prior to intra-aortic injection of PAF. The vinblastine-induced neutropenia protected against the clinical and pathologic manifestations of NEC-like disease.

The role of the human PMN in the acute inflammatory response is well documented. PMNs play a fundamental role in the innate immune response and are rapidly recruited to areas of injury or inflammation where they participate in bacterial phagocytosis and killing. Disorders associated with a deficiency or impairment of PMN function (neutropenia, chronic granulomatous disease (CGD), leukocyte adhesion deficiency) predispose to infections with gram-negative and gram-positive bacteria[9]. However, regulation of this potent component of the acute inflammatory response is imperative. Disorders such as ARDS, ischemia/reperfusion injury, and rheumatoid arthritis appear to result from the dysregulation of the PMNs' acute inflammatory response.

Recently, neutrophils isolated from healthy adult donors were shown to undergo programmed cell death distinct from apoptosis and necrosis to form neutrophil extracellular traps (NETs). NETs are extensive lattices of extracellular DNA and chromatin decorated with anti-microbial proteins and degradative enzymes such as myeloperoxidase and neutrophil elastase (NE). NETs effect extracellular killing of bacteria and fungi. Our laboratory recently characterized impaired NET formation as a novel innate immune deficiency of human newborn infants and showed that PMNs isolated from the cord blood of newborn infants, both term and preterm, demonstrated impaired NET formation and extracellular bacterial killing as compared to PMNs isolated from healthy adults. However, the timing for developmental maturation of newborn infant PMN NET formation remains unknown. Furthermore, while failure of NET formation contributes to the predisposition for and severity of bacterial and fungal infections associated with disorders such as CGD, dysregulated and inappropriately robust NET formation may lead to immune-mediated host tissue damage such as autoimmune small vessel vasculitis.

Using data generated from the previous version of this IRB application (IRB_00039621 – Neutrophil Extracellular Trap Formation in Newborn Infants at Risk for Necrotizing Enterocolitis – Closed on 11/8/2018), we recently discovered a new regulatory mechanism governing NET formation in the first 2 weeks following birth for neonates, whether term or preterm. Using cord blood samples as well as samples of peripheral blood taken serially from neonates over the first 2 months of life, we recently identified a family of NET-Inhibitory Peptides (NIPs) which circulate in the fetus and neonate and potently inhibit PMNs from forming NETs. These NIPs inhibit NET formation despite stimulation with many NET-generating agonists like LPS, PMA, live gram+ or gram- bacteria, heme, or viruses. We further examined the use of these NIPs in the setting of in vivo sepsis. We found that pretreatment with the NIPs increased survival in the cecal ligation and puncture model of polymicrobial sepsis by 40-60%, with the survival advantage most likely due to decreased NET-mediated inflammatory tissue damage. With these published results (Yost CC, et al., JCI 2016), we are now funded by the NIH to evaluate the use of NIPs as therapeutic agents in preclinical models of neonatal sepsis. These studies will determine regulatory pathways governing NIP expression in the fetus and newborn, NIP mechanism of action, and efficacy in neonatal sepsis.

We hypothesize that NET formation contributes to the pathogenesis of inflammatory syndromes associated with sequelae of prematurity including sepsis, nCLD, ROP, and NEC by inappropriately releasing degradative proteins and tissue destructive enzymes into the inflammatory milieu of the premature infant. We further hypothesize that NIPs may provide protection against NET-associated inflammatory tissue damage.

Study Purposes and Objectives:

The following study is best described as a prospective, in vitro longitudinal cellular biology study of LPS/PAF-stimulated PMNs isolated from the cord and peripheral blood of premature infants at risk for inflammatory sequelae associated with prematurity (including neonatal Chronic Lung Disease (nCLD), early and late onset sepsis, necrotizing enterocolitis (NEC), and retinopathy of prematurity), and from term infants not considered at risk for such sequelae. In the specific case of NEC, we will also assay for NET formation in gastrointestinal tissue samples obtained at the time of surgery for severe NEC in enrolled prematurely born infants if that occurs. Such tissue samples will not be collected for other inflammatory syndromes of prematurity.

These studies are the first of their kind and aim to answer important questions such as the impact of dysregulated NET formation in the pathogenesis of these inflammatory sequelae of prematurity, the regulatory mechanisms

governing NET formation in newborn infants, the role of our newly discovered NET-Inhibitory Peptides (NIPs) as endogenous regulators of NET formation, and their potential use as novel therapeutic agents for dysregulated NET formation.

Inclusion Criteria:

Eligible Patients

1. Preterm infant patients delivered at UUMC and hospitalized in the NICU who are ≤ 1500 grams or < 30 weeks gestational age at birth

Cord blood sample obtained at delivery

Peripheral blood sample obtained during IV start under anesthesia at any elective surgeries during the first year of life

2. Term infants delivered at UUMC without complication, either via cesarean section or vaginal delivery.

Cord blood sample obtained at delivery

Heel-prick blood sample obtained at 24 hours of life

Exclusion criteria:

None

Study Procedures:

Recruitment: We will screen charts through a waiver of authorization for potential participants within the University of Utah covered entity. Eligible participants identified through the waiver of authorization will be identified once they are admitted to the hospital for clinical management, with the expectation of delivery during the current hospital admission.

Inclusion/exclusion criteria will be reviewed with the patient's chart and then approached once initial eligibility criteria have been verified.

Pregnant women admitted to labor and delivery and who meet the eligibility criteria for term deliveries without complication will be identified and approached about the study prior to delivery.

Pregnant women admitted to labor and delivery in an emergent manner and who meet the eligibility criteria for preterm delivery at <30 weeks gestational age may be approached prior to delivery, when deemed appropriate by the patient's care team.

Consent: For consent of healthy term neonates, all mothers will be approached after admission to the Labor and Delivery Unit at UUMC. Informed consent for their neonate's participation in our study will be sought by Dr. Yost. If the delivery is precipitous and cord blood is collected, the families of potential healthy term neonate participants may also be approached after the fact but within 24 hours after delivery if appropriate. It is anticipated that this will be the exception and not the rule. Furthermore, any umbilical cord blood collected will be used and tested for any clinical indications before being considered for use in research for this project. Also,

research related activities connected with this study will not begin until after consent has been obtained from the family of the newborn.

For consent of preterm neonates born at less than 30 weeks gestation, all mothers admitted to the Labor and Delivery Unit at UUMC and meeting inclusion criteria will be approached for consent prior to delivery by Dr. Yost or his research team. In each case, the obstetrical clinical care team will determine whether initiating the consent process prenatally is appropriate. The family's physical and emotional state prior to delivery will be considered in this determination. If appropriate, Dr. Yost or his research team will consent for study participation prenatally and proceed with use of the umbilical cord blood at that moment. If not deemed appropriate, Dr. Yost's research team will collect the umbilical cord blood and approach the family for informed consent within 24 hours after delivery. Furthermore, any umbilical cord blood collected will be used and tested for any clinical indications before being considered for use in research for this project. Also, research related activities connected with this study will not begin until after consent has been obtained from the family of the newborn.

Phlebotomy:

Patient Group #1: Prematurely born infants in the UUMC and PCH NICUs

After delivery, cord blood will be drawn from the ligated umbilical cord and placenta. For premature deliveries of infants weighing less than 1500 grams, it is estimated that only 5 – 10 mL of cord blood will be obtained. The resuscitation and treatment of a premature infant often requires cord blood specimens for such clinical tests as arterial and venous cord blood gases and blood type and crossmatch in preparation for probable future blood transfusions. These specific clinical cord blood samples will be collected before study blood collection. The residual cord blood will be dripped into sterile 8 mL EDTA-anticoagulated vacutainers, labeled with a study generated patient identifier and transported by Dr. Yost or his laboratory technicians to his lab in the Eccles Institute of Genetics for immediate PMN isolation and sample stabilization. This rapid isolation and sample stabilization is necessary given the short life of PMNs *in vitro* and the potential for PMN activation after isolation.

The family will then be approached regarding informed consent as discussed below. If informed consent is obtained, preparations will be made to obtain 3 serial 0.5 ml samples of peripheral blood during the infant's NICU stay. Samples will be collected on the routine lab draw day that most closely correlates with the following days of life (DOL) #: 3, 7, and 14 if still in the NICU at either UUMC or PCH.

Longitudinal samples will be collected into a 0.5 ml EDTA-anticoagulated microtainers and processed in an identical manner to the cord blood already collected. These serial samples will only be collected during routine clinical laboratory draws occurring around the scheduled DOL. **No new lines, IVs or heel stick wounds will be started or made for the sole purpose of drawing the scheduled study sample.**

For the small subset of at-risk infants who develop NEC requiring surgery at PCH, whether born at UUMC or transported to PCH from a referring institution, we will also obtain fixed and paraffin-embedded gastrointestinal tissue samples at the time of surgery for analysis of NET formation. These samples will be obtained by the pediatric surgical team at the time of exploratory laparotomy and processed by the diagnostic pathology service at PCH. For those infants transported to PCH with surgical NEC from referring institutions, informed consent will be requested upon admission to PCH NICU. If informed consent is obtained, the samples would be processed and transferred by Dr. Yost to his laboratory for further analysis.

Patient Group #2: Healthy term infants born at UUMC

After delivery, cord blood will be drawn from the ligated umbilical cord and placenta. For term deliveries at UUMC, it is estimated that approximately 15 - 25 mL of cord blood can be routinely obtained without compromising needs for possible clinical studies such as infant blood-typing. Samples will be dripped into sterile 8

mL EDTA-anticoagulated vacutainers, labeled with a study generated patient identifier and transported by Dr. Yost or his laboratory technicians to his lab in the Eccles Institute of Human Genetics for immediate PMN isolation and sample stabilization. Again, this rapid isolation and sample stabilization is necessary given the short life of PMNs *in vitro* and the potential for PMN activation after isolation.

The family will then be approached regarding informed consent as discussed below. If informed consent is obtained, preparations will be made to obtain one additional 1 ml sample of peripheral blood during the infant's heel stick wounding for the 24-hour metabolic screening test performed on all infants before DOL #3 and before hospital discharge. The sample will be collected into 2 0.5 ml EDTA-anticoagulated microtainers and processed in an identical manner to the cord blood already collected. This additional sample will only be collected during the routine 24 – 48-hour metabolic screening laboratory draw. **No new lines, IVs or heel stick wounds will be started or made for the sole purpose of drawing the scheduled study sample.**

Experimental Procedures:

After parental consent is obtained, PMNs will be isolated via positive immunoselection against CD15 antigen expression as previously described. PMNs will be stimulated with PAF or LPS and analyzed for NET formation via live cell imaging, quantitative extracellular DNA content and quantitative neutrophil elastase activity. For NEC tissue samples, NET formation will be qualitatively assessed via immunohistochemistry. NET-Inhibitory Peptide concentrations in blood plasma will be assessed via quantitative Western blotting and/or HPLC/MS^2 assays.

Live Cell Imaging:

Isolated PMNs will be allowed to adhere to lysinated glass coverslips and stimulated with PAF [10 nM] or LPS [100 ng/ml] for 1 – 4-hour time points. Cell impermeable and cell permeable intercalating DNA dyes will be used to differentially stain NET-associated, extracellular DNA and intracellular nuclear DNA. Qualitative assessment of NET formation will be captured and recorded via confocal microscopy.

Quantitative extracellular DNA Content Assay:

Supernatants following PAF/LPS stimulation and confocal imaging will be treated with DNase (66 U/ml for 15 minutes) to break up NETs and gently removed and analyzed for total DNA content. Supernatant DNA concentrations will be quantified by incubating the samples with Sytox Orange (1mM) and comparing the values against a concentration curve constructed with varying concentrations of a known DNA standard using fluorimetry with filter settings of 530(excitation)/ 645(emission) (BioTek Synergy HT).

Quantitative Myeloperoxidase (MPO)-DNA ELISA:

NET-associated MPO activity will be determined at selected time points as described. Briefly, the incubation medium will be gently removed and replaced with medium containing DNase (66 U/ml) for 15 min. A capture antibody against MPO will be employed as part of a MPO-DNA ELISA to provide quantitative levels of NET formation. A detection antibody against human DNA will serve as the detection antibody.

Quantitative NIP Concentration in Human Plasma:

Plasma samples will be obtained from whole blood samples via centrifugation. The aliquots of the plasma will then be run on Tris-Tricine precast gels to resolve proteins and peptides with a molecular weight between 1 and 30 kD. A standard curve with increasing concentration of synthesized NIPs will be run on the same gel and concentration will be determined by comparing the fluorescent intensity of the plasma band to those of the

standard curve. Alternatively, we are developing a HPLC/MS2 assay which will use a synthesized "heavy" variant of NIPs to quantify NIP concentration in plasma compared to a known amount of spiked "heavy" NIP.

Qualitative assessment of NET formation in NEC tissue specimens via immunohistochemistry:

NEC tissue specimens processed by pediatric pathology at PCMC and embedded in paraffin will be transported to Dr. Yost's laboratory and analyzed for NET formation via immunofluorescence. Surgical samples will be sectioned at 5 – 10 μm thickness. NETs will be visualized by examining the colocalization of antibodies against DNA, histones, and neutrophil elastase.

Data Analysis:

NET formation as assessed qualitatively via live cell imaging and immunohistochemistry and quantitatively via extracellular DNA quantitation and neutrophil elastase activity assays.

We anticipate enrolling 40 prematurely born infants in patient group # 1, 20 healthy term newborn infants in patient group # 2 during this study.

Statistical comparisons for the quantitative assays of extracellular DNA and NE activity will be made via one-way ANOVA with post hoc Pairwise Multiple Comparisons made via the Holm-Sidak method.