Pediatric RESEARCH

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COMMENT

# Pediatric immunothrombosis—Understudied… but what potential!

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Brinkmann and Zychlinsky reinvigorated the fields of neutrophil biology and innate immunity with their discovery of Neutrophil Extracellular Traps (NETs) in 2004.[1] This initial excitement led to key discoveries regarding the regulatory pathways governing "NETosis" as a regulated cell death process distinct from apoptosis and necrosis, and led to evidence regarding NETs protective role as an essential component of the acute inflammatory response.[2] However, continued study uncovered a "dark side" of NET formation where dysregulated NETosis and NET components such as histones and granule proteases contribute to inflammatory tissue damage in many inflammatory conditions covering the spectrum of disease—sepsis, acute lung injury, atherosclerosis, autoimmune disorders, thrombosis, and cancer.[3,4] These in vitro and in vivo studies conducted mostly in mature vertebrate animal models or adult humans highlight the importance of NET-mediated inflammatory tissue damage in mature subjects. This phenomenon of immunothrombosis, defined as the interaction of NETs with activated platelets in the inflammatory milieu, remains understudied in the pediatric population and unknown to many pediatric practitioners.

In this issue of *Pediatric Research*, Franchi et al.[5] share their effort to correct this lack of awareness. They undertook a comprehensive review of this intersection of NETosis with thrombosis in pediatric syndromes of dysregulated inflammation—pediatric immunothrombosis. Using prospectively designed search criteria, the authors combed the National Library of Medicine (MEDLINE/PubMed) from the year 2000 through May of 2018. They identified 272 papers using pertinent, searchable keywords. They excluded duplicate studies and non-English papers. Finally, the authors qualitatively assessed each article and determined that 24 papers met the criteria for inclusion in their review.

The potential role of immunothrombosis in health and disease remains underappreciated in pediatric patients. The review by Franchi et al. highlights the lack of quality studies of immunothrombosis in syndromes of inflammation occurring during childhood or directly after birth. Yet, their review serves a more important purpose—highlighting the need for high quality, discovery based studies aimed at defining the role of immunothrombosis and NETosis in the pathogenesis of inflammatory syndromes of childhood. This purpose includes discovery, evaluation, and development of potential therapeutic agents specifically targeting immunothrombosis.

For the studies reviewed by Franchi et al., investigators employed many different methods to study and modulate NETosis and its downstream effects in both in vitro and in vivo systems. Many of these studies yielded conflicting results prompting the question—Does immunothrombosis protect or harm in the acute inflammatory response? While the answer to this question is clearly yes, a more nuanced approach reveals the importance of clinical context in evaluating the role of immunothrombosis in inflammatory syndromes of childhood. Sharing our experience examining the perinatal milieu for human neonates, born either at term or prematurely, may prove illustrative in evaluating other pediatric clinical contexts where the complicated role of immunothrombosis must be considered.

## NEONATES LEAD THE WAY

Neonates account for more cases of severe infection than all other pediatric age groups combined. For term and very low birth weight (VLBW) neonates, experts estimate the risk for sepsis at 1–3 cases/1000 live births and 10–17 cases/1000 live births, respectively. VLBW neonates also demonstrate high rates of inflammatory sequelae of premature birth—necrotizing enterocolitis (NEC), neonatal chronic lung disease, and retinopathy of prematurity to name a few. Thus, neonates are at the highest risk for severe infection but also at high risk for pro-inflammatory syndromes creating tissue damage in the gastrointestinal tract, lungs, and eyes. With the initial report from Brinkmann, et al.,[1] we began investigating a role for failed NET formation in neonates as an explanation for their increased risk of infection. We and other laboratories soon confirmed delayed or absent NET formation in stimulated PMNs in vitro following isolation from the umbilical cord blood of term or preterm neonates.[6] We also identified total and extracellular, NET-specific bacterial killing deficits in these "neonatal" PMNs. These reports added failed NET formation to the long list of immune deficiencies associated with neonates and infants, and would seem a case in point for Franchi et al.'s suggestion of augmentation of immunothrombosis as a potential therapeutic intervention.

But the story became much more interesting as we studied NET formation by prematurely born neonates over developmental time.[7] We followed 11 preterm neonates born at gestational ages of less than 30 weeks and assessed LPS-stimulated NET formation by their PMNs serially over the first 2 months of life. In each case, umbilical cord blood PMNs failed to form NETs but PMNs isolated from their peripheral blood showed increasing in vitro NET formation over the first two weeks of life. By 14 days after delivery, NET formation in response to LPS by PMNs isolated from preterm neonates equaled that of control PMNs isolated from healthy adults. Further experiments soon confirmed that the umbilical cord blood plasma but not autologous plasma isolated from the

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same neonates on day of life 60 inhibited LPS-stimulated NET formation. This suggested that umbilical cord blood plasma contained an endogenous inhibitor of NET formation. Using proteomic techniques, we then identified a 29 amino acid carboxy-terminus fragment of alpha-1-antitrypsin, a serine protease inhibitor, with NET inhibitory properties, which we named neonatal NET-Inhibitory Factor (nNIF). We synthesized this peptide and a scrambled peptide control for use in additional studies. We subsequently found that nNIF inhibits NET formation by PMNs in vitro following induction by a variety of clinically relevant inflammatory agonists including bacteria, fungi, dengue virus, heme (a damage-associated molecular pattern molecule), and pharmacologic agonists. Yet, nNIF does not inhibit other key neutrophil functions such as chemotaxis, phagocytosis, reactive oxygen species generation, intracellular phagolysosome-mediated microbial killing, or neutrophil-platelet interactions. Armed with these data, we tested the hypothesis that inhibition of NET formation might improve outcomes in preclinical models of infection by decreasing NET-associated inflammatory tissue damage. In a model of pathogenic *E. coli* peritoneal infection, we documented NET inhibition in vivo following nNIF intraperitoneal injection but not with the scrambled peptide control. Using the *E. coli* model and two other models of peritoneal inflammation, LPS-induced peritonitis and the cecal ligation and puncture model of polymicrobial sepsis, we found significantly improved survival (40-60%) and decreased clinical illness severity scores for mice pretreated with nNIF compared to the scrambled peptide control. These findings would seem to suggest that targeted inhibition of immunothrombosis within the right clinical context might provide protection in syndromes of dysregulated inflammation such as sepsis. Thus, nNIF and other compounds capable of specifically inhibiting NET formation may represent targets for therapeutic drug development. We have since gone on to document NET formation in gastrointestinal samples from prematurely born infants with surgical NEC.[8] Still, the use of agents to induce or inhibit immunothrombosis as clinically indicated therapy in neonates and children remains a contemplated but distant goal.

In summary, our experience dovetails nicely with the on-point messages delivered by Franchi et al. Immunothrombosis does play a role in the health and disease of pediatric patients. Increased clinical and research interest into this under recognized and novel immunophenotype will lead to improvements in clinical care and development of potential therapeutic strategies to augment or limit immunothrombosis in a context-dependent, personalized manner. Together with Franchi et al., our experience suggests that research conducted in newborns and children can yield novel and potentially transformative translational findings that may drive improvements in clinical care delivered to patients of all ages.

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## ADDITIONAL INFORMATION

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