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Neonatal Sepsis and Neutrophil Insufficiencies

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Abstract

Sepsis has continuously been a leading cause of neonatal morbidity and mortality despite current advances in chemotherapy and patient intensive care facilities. Neonates are at high risk for developing bacterial infections due to quantitative and qualitative insufficiencies of innate immunity, particularly granulocyte lineage development and response to infection. Although antibiotics remain the mainstay of treatment, adjuvant therapies enhancing immune function have shown promise in treating sepsis in neonates. This chapter reviews current strategies for the clinical management of neonatal sepsis and analyzes mechanisms underlying insufficiencies of neutrophil defense in neonates with emphasis on new directions for adjuvant therapy development.

Introduction to Neonatal Sepsis

Sepsis in children is a significant cause of morbidity and mortality. The incidence of severe pediatric sepsis in the United States exceeds 42,000 cases/year with a mortality rate of 10.3%. Total hospital costs for treating sepsis in children approaches \$1.7 billion a year [1]. Age is one of the greatest prognostic factors. Approximately 50% of severe pediatric sepsis occurs in infants less than one year of age, and half of those in infants with low or very-low birth weight (VLBW) [1, 2]. In the United States, resource use is highest amongst VLBW infants with an average hospital stay of 74 days and costs nearing \$75,000 per case [1]. Sepsis among infants is dominated by perinatal events with neonates particularly susceptible to severe bacterial infection [2]. In spite of enormous costs, the United States and other industrialized countries experience only 1% of the total number of neonatal deaths occurring worldwide. The other 99% occur in low and middle income countries, where approximately 25% of the 4 million neonatal deaths per year are related to neonatal sepsis [3].

Sepsis is a clinical syndrome characterized by the presence of both infection and a systemic inflammatory response. Clinical manifestations include hemodynamic instability, hypoxemia, and various signs of an acute inflammatory state [4]. Sepsis in the neonate less than 28 days old is particularly difficult to diagnose. One obstacle in diagnosing neonatal sepsis is accurately defining the disease state. There are multiple terms used to define sepsis-like syndromes in the adult including bacteremia, systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis, and septic shock. The American College of Chest Physicians and the Society of Critical Care Medicine use the following definitions. “Bacteremia” is defined as the presence of viable bacteria in the blood. “SIRS” is the systemic inflammatory response triggered by infectious or noninfectious conditions. “Sepsis” is defined by the presence of both infection and a systemic inflammatory response accompanied by more than

one of the following (1) temperature $>38^{\circ}\text{C}$ or $<36^{\circ}\text{C}$ (2) tachycardia >90 bpm (3) tachypnea (4) alteration in white blood cell count $>12,000/\text{cu mm}$ or $<4,000/\text{cu mm}$; or $>10\%$ immature neutrophils. Early signs of organ dysfunction may be the first signs of SIRS including hemodynamic instability, arterial hypoxemia, oliguria, coagulopathy, and altered liver function [4]. “Severe sepsis” refers to sepsis complicated by organ dysfunction and “septic shock” is acute circulatory failure characterized by persistent hypotension despite adequate fluid resuscitation [4, 5].

Due to distinct physiological and clinical manifestations in children, pediatric sepsis is defined differently than adult sepsis. The definition of pediatric sepsis, as agreed upon by the Pediatric Section of the Society of Critical Care Medicine, the American College of Critical Care Medicine, and the Section of Critical Care of the American Academy of Pediatrics, is a systemic inflammatory response in the presence of or as the result of *suspected or proven* infection. Systemic inflammatory response for children <1 year old includes bradycardia (mean heart rate $<10^{\text{th}}$ percentile for age) in the absence of external vagal stimulus, β -blocker drugs, or congenital heart disease, or otherwise persistent depression of heart rate over a 30 minute period [6]. Severe sepsis includes cardiovascular organ dysfunction or acute respiratory distress syndrome or two or more other organ dysfunctions (respiratory, renal, neurologic, hematologic, or hepatic) [6]. Diagnostic criteria for pediatric organ dysfunction have been published by Goldstein et al [6]. Neonates have higher vascular tone than adults, which allows shock to develop before hypotension is detected. Pediatric and neonatal septic shock is defined as sepsis with cardiovascular organ dysfunction despite fluid resuscitation. Cardiovascular dysfunction is defined differently in children than adults; hypotension (<2 SD below normal age), or need for vasoactive drugs to maintain blood pressure in normal range, or two of the following: unexplained metabolic acidosis (>5.0 mEq/L), increased arterial lactate (>2 times upper limit of normal), oliguria (<0.5 mL/kg/hr), prolonged capillary refill (>5 sec), and core to peripheral temperature gap $>3^{\circ}\text{C}$ [6]. Unique definitions for sepsis-like syndromes in children and neonatal patients further exacerbate the difficult clinical management of sepsis at the bedside.

The accurate diagnosis of sepsis is particularly challenging in the neonate as blood cultures and clinical presentation are less informative. Blood culture is often unreliable for diagnosis in neonates due to low sample volume, low bacterial density, culture contamination, or suppression of bacterial growth due to antibiotic administration [7]. This variability can lead to drastic underdiagnosis of culture-proven sepsis. In the United States, the leading bacterial causes of neonatal sepsis include *Staphylococcus* (22.7%), *Streptococcus* (12.1%), *Pseudomonas* (3.6%), *Haemophilus influenza* (1.6%), and *Meningococcus* (0.3%). Fungal infection is also common (10%) [2]. Common sites of infection include primary bacteremia (33.6%), respiratory (26.9%), soft tissue wounds (5.9%), central nervous system (5.3%), and genitourinary (4.1%) [2]. Besides attaining accurate blood cultures, another challenge for diagnosing neonatal sepsis is that clinical signs and symptoms can be more vague and nonspecific relative to older children and adults. Signs and symptoms include, but are not limited to, respiratory distress, irritability, lethargy, cyanosis, acidosis, poor feeding, vomiting, unexplained jaundice, petechiae, changes in body temperature or glycemic state, pulmonary hypertension, hypotonia, or seizure [8, 9]. The clinical status of neonates can worsen quickly and lead to rapid deterioration. These unique features dictate that the definition of neonatal sepsis remains vague and flexible to physician interpretation.

While the clinical presentation of sepsis in the pediatric population as a whole is different than that of adults, age-specific categorization of laboratory values is also necessary. Investigators have subdivided pediatric sepsis into six demographics including newborn (0-7 days), neonate (1 week – 1 month), infant (1 month – 1 year), toddler (2-5 years), school-age (6-12 years), and adolescent (13 to <18 years) [6]. These groups were determined by age-

specific risks for invasive infections, age-specific antibiotic treatment recommendations, and developmental physiologic cardiorespiratory characteristics. However, the physiologic and laboratory normative data to support such categorization and their validity in critically ill patients with sepsis have been challenged [10]. Many studies, including this review, define the neonatal period as the first 28 days of life and therefore do not distinguish newborns from neonates [2]. Other investigations have also divided neonatal infections into early or late onset categories. Early-onset infections occur during the first week of life and are most commonly associated with maternal to fetal disease transmission. Late-onset infections, however, are contracted from the environment and occur after the first week [7]. The transition from life *in utero* to birth is associated with significant immunological changes. *In utero*, the neonate is both threatened and protected by the maternal immune system. Once out of the womb, the neonate no longer faces the possibility of maternal immune rejection and begins to rapidly expand and develop its own immune defense mechanisms in order to protect the host against environmental challenges. Therefore, age-specific categorization of sepsis in the neonate is useful as it clarifies the capacity of the neonatal immune system to battle systemic infection at a specific stage of life. By indicating whether sepsis is occurring early or late in perinatal development, clinical laboratory values can be standardized for the appropriate stage of neonatal immune system development.

Historical Overview of Neonatal Sepsis Research

Research into hematopoietic characteristics of the neonate was first documented in 1896 by Luther Emmett Holt in, “The Diseases of Infancy and Childhood.” In addition to differences in red blood cell numbers, he noted 3-4 fold higher granulocyte concentrations in newborns compared to adults [11]. Although granulocytes were greater in number, in 1910 Tunnicliff, found that opsonization and phagocytosis was limited in neonatal blood [12]. Due to technological limitations of the era, further investigation on functional deficiencies of neonatal granulocytes was restricted and research in the 1920s centered on quantitative neonatal white blood cell deficiencies [13]. During this time, neutropenia in the neonate was a known risk factor for bacterial infection. Lucas and Washburn in 1928 first linked neutropenia in children to the development of bacterial sepsis. Case reports by Dunham supported such a phenomenon when documenting that all of her neonatal patients who died from sepsis were neutropenic [13].

Today, neutropenia in the neonate is defined as an absolute neutrophil count $< 2.0 \times 10^9/L$ and is reported in approximately 8% of all patients admitted into the neonatal intensive care unit (NICU) [14, 15]. In the United States, this translates to 32,000 NICU admissions with neutropenia per year [15]. Low gestational age and low birthweight strongly correlate with the incidence of neutropenia in neonates due to age-dependent maturation of the immune system and/or nutrient deprivation [16-18]. However, of the postnatal causes of neutropenia, infection is responsible for fewer episodes than antibiotic administration, which is the most common cause of neutropenia in the second week of life. While the precise mechanism of antibiotic-mediated neutropenia in neonates is not known, Gessler et al suggest that adverse reactions to penicillins, including bone marrow depression and granulocytopenia, are higher in neonates due to the increased susceptibility of neonatal myeloid granulopoietic cells [16].

The functional deficits of neonatal neutrophils first suggested by Tunnicliff were better defined during the 1950s. Matoth in 1952 demonstrated that not only was phagocytosis impaired in neonatal granulocytes, but that the ability of neonatal granulocytes to intracellularly degrade phagocytosed foreign particles was deficient compared to adult cells. Using starch granules and other substrates, multiple investigators conducted a series of studies which confirmed this phenomenon [19]. During the 1970s, specific intracellular killing mechanisms of neonatal granulocytes were studied including the NADPH oxidase

system. Schlegel and Bellanti found this system was abnormal in the neonate as were other post-phagocytic, intracellular metabolic activities including hexose monophosphate shunting and glucose-6 phosphate dehydrogenase activity [19, 20]. During the 1980s, research by Anderson et al and Rider et al correlated the insufficient generation of hydroxyl radicals by neonatal neutrophils with reductions of lactoferrin and myeloperoxidase granule proteins [21-23]. Thus, early studies began to uncover qualitative deficiencies of neonatal neutrophils including deficient enzymatic and protein activity indispensable for later stages of the respiratory burst required for degrading phagocytosed pathogens.

From centuries of investigation, a multitude of evidence suggests that many aspects of granulopoiesis and the neutrophil response to infection are insufficient in neonates. The innate immune system is the primary defense against bacterial infection. As a major component of innate immunity, neutrophils are responsible for engulfing and destroying pathogenic bacteria during infection. Neonates have both quantitative and qualitative deficiencies in neutrophils, significantly diminishing their capacity to battle infection. Currently used adjuvant treatments for neonatal bacterial infections attempt to overcome these insufficiencies by enhancing neonatal neutrophil supply and function. In this chapter, we review current investigations identifying the link between neonatal neutrophil insufficiency and the development of neonatal sepsis. Subsequently, we discuss mechanisms of granulopoiesis, highlighting recent research on the granulopoietic response to infection. Specific quantitative and qualitative insufficiencies of neonatal neutrophils will be studied in order to analyze the efficacy of commonly used adjuvant strategies in overcoming such insufficiencies.

Granulopoiesis in Neonates

All mature blood cells are derived from pluripotent hematopoietic stem cells. In early development, hematopoietic stem cells emerge separately from the yolk sac, chorio-allantoic placenta, and aorta-gonad-mesonephros (AMG) region [24]. Following the initial erythropoietic stage, myeloid progenitor cells can be found in the yolk sac during the 3rd to 4th week of gestation [25]. From the yolk sac these progenitor cells sequentially seed the liver, thymus, spleen and eventually take up permanent residence in the bone marrow at the 11-12th week of gestation [26]. The production of mature granulocytes remains limited in the fetus until the end of the second trimester when the fetal bone marrow takes over hematopoietic production. Hematopoiesis is largely restricted to the bone marrow throughout adult life. By definition, hematopoietic stem cells can self renew and produce mature cells of all blood cell lineages by asymmetric division. During asymmetric division, long-term hematopoietic stem cells give rise to short term hematopoietic stem cells and multipotent progenitors. As new blood cell production is required, multipotent progenitor cells differentiate down either a lymphoid or myeloid lineage development pathway by producing common lymphoid progenitor cells (CLPs) or common myeloid progenitor cells (CMPs) respectively [27]. If lymphoid gene expression is not actively induced by cytokine or infectious stimuli, myeloid differentiation represents the default lineage commitment pathway [28]. Restricted to myeloid lineage development, CMPs can give rise to either megakaryocyte-erythrocyte progenitor cells (MEPs) or myelomonocytic progenitor cells (GMPs) [29]. After GMPs have committed to granulocyte lineage development, terminal neutrophil differentiation defines the sequential maturation of lineage restricted granulocytic progenitor cells into functional, mature neutrophils. Distinct stages of terminal neutrophil differentiation include the myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell, and mature neutrophil stages [30]. Each stage is characterized by a specific transcriptional program, cell cycle status, cytokine receptor expression, phagocytosis, granule and bactericidal protein content, reactive oxygen intermediate production, and ability to degrade phagocytosed matter. Mature neutrophils must also produce chemokines and cytokines that

regulate other immune effector cells and coordinate apoptotic programs so to resolve the inflammatory response and minimize excessive tissue damage [31]. Traditional strategies to identify the stage of terminal neutrophil differentiation rely on visual identification of specific granules or nuclear morphology. However, the complexity of neutrophil production and activity is largely regulated at the transcriptional level. New techniques such as flow cytometric cell sorting and molecular biological analysis have enabled researchers to study the transcriptional control of neutrophil lineage differentiation at various stages.

Development of mature neutrophils from hematopoietic precursors is promoted by distinct granulopoietic transcriptional frameworks. CCAAT Enhancer Binding Protein (CEBP) family members, specifically CEBP- α and CEBP- ϵ , control key steps of the granulopoietic process. CEBPs homodimerize and heterodimerize with each other, as they exert their control of cell proliferation and cell cycle protein activity by binding the same CEBP consensus DNA site [32]. CEBP- α expression is activated during initial myeloid lineage commitment, where its expression is unique to granulocytes [33]. CEBP- α controls the transcription of genes encoding granulocyte growth factor receptors, secondary granule protein production, actively suppresses gene expression of other blood cell lineages, and negatively regulates hematopoietic stem cell renewal which promotes cell cycle exit and mature neutrophil production [34]. Hematopoietic deficiencies of CEBP- α knockout mice are specific to granulopoiesis. These mice lack mature neutrophils and demonstrate immature blast cell accumulation indicative of a block in differentiation [35]. This differentiation block specifically occurs at the CMP to GMP transition, as CEBP- α gene expression is not required for later stages of terminal neutrophil differentiation [36]. However, CEBP- ϵ is transcribed downstream of CEBP- α and is indispensable in the terminal stages of neutrophil differentiation. Because the expression of these two genes overlaps, CEBP- ϵ knockout animals similarly demonstrate myelodysplasia, impaired neutrophil production, and succumb to opportunistic infections [32]. During times of stress, a third CEBP family member, CEBP- β , becomes an integral regulator of granulocyte production. Although, CEBP- α and CEBP- ϵ , maintain steady state granulopoiesis, CEBP- β is upregulated in response to inflammatory stimuli and becomes the predominant transcription factor driving enhanced granulocyte production [37]. The balance between steady state CEBP- α and emergency state CEBP- β driven gene transcription can be altered in response to ligand-receptor binding of various cytokines and colony stimulating factors [38].

Colony stimulating factors are 18-90 kDa glycoproteins that stimulate the production of mature hematopoietic cells from bone marrow cultures. Colony stimulating factors that promote the production of mature neutrophils include granulocyte-colony stimulating factor (G-CSF), granulocyte/macrophage stimulating factor (GM-CSF), and interleukin-3 (IL-3) [39]. G-CSF is the principle cytokine controlling neutrophil development and function [40]. G-CSF receptors (G-CSFRs) are expressed early in development during the myeloblast stage and receptor density increases as myeloblasts differentiate into mature neutrophils [41]. G-CSFR-mediated cell signaling promotes proliferation, survival, and terminal differentiation of neutrophil precursors while also enhancing the release of both mature neutrophils and immature progenitors out of the bone marrow [40]. Circulating G-CSF concentrations remain low during steady state, but can increase more than 20 fold during infection [42]. Enhanced G-CSF production during infection can be initiated by inflammatory stimuli such as lipopolysaccharide (LPS), tumor necrosis factor- α (TNF- α), and interleukin-1 β through the translocation of nuclear factor-kB (NF- κ B), interleukin-17 receptor activation by T helper 17 cells, or through feedforward G-CSF production via CEBP- β [40, 42]. G-CSFR activates multiple downstream effector pathways including signal transducers and activators of transcription (STAT) proteins, the Ras/Mek/Erk pathway, Src related kinases p53/56lyn (LYN) and p59hck (HCK), the Akt pathway, Syk tyrosine kinases, and NF- κ B [40, 43, 44].

The extensive involvement of G-CSFRs in multiple signaling pathways suggests a well-coordinated signaling framework required for neutrophil production. Gene deletions in any one pathway downstream of G-CSFR activation can produce significantly impaired granulopoiesis.

Data on critical granulopoietic signaling pathways in neonates are sparse. Yan et al investigated LPS stimulated superoxide production by newborn neutrophils, focusing on Mitogen Activated Protein (MAP) and Src kinase signaling. Although toll-like receptor-4 (TLR-4) imparts ligand-specific recognition of LPS, it shares many downstream signaling molecules with G-CSFR that could affect neutrophil production and activity [45]. Yan et al found that subcellular localization of the MAP kinase family proteins extracellular-signaling regulated kinases 1/2 (ERK1/2) and p38 from newborn neutrophils differed in being more heavily distributed in membranes and granules than the cytoplasm, where they predominate in adult neutrophils. When stimulated with LPS, both newborn and adult ERK1/2 and p38 both localized exclusively to the cytoplasm [46]. Adult and newborn neutrophils exhibit no difference in the expression of the Src kinases p58fgr, HCK, and LYN. However, LYN displayed altered localization, elevated basal activity, and was unresponsive to LPS priming in newborn neutrophils. Insufficient LYN activity suggests one possible mechanism for the diminished responsiveness of neonatal neutrophils [46].

Recently insufficient Src kinase activity in neonatal neutrophils has also been demonstrated by Rashmi et al, as a mechanism for prolonged neonatal neutrophil survival. They found that the prolonged survival of neonatal neutrophils was due to less abundant sialic acid-binding immunoglobulin-like lectin-9 (Siglec-9) and Src homology domain-2 containing tyrosine phosphatase-1 (SHP-1), two pro-apoptotic proteins. Rashmi suggested that diminished SHP-1 function in neonatal neutrophils can explain the high basal phosphorylation of LYN previously reported by Yan et al [47]. Marodi et al investigated a third intracellular signaling pathway, the STAT family of proteins, using neonatal monocytes. Cord-blood derived mononuclear phagocytes, displayed attenuated STAT-1 phosphorylation in response to interferon- γ stimulation compared to adult cells [48, 49]. Altered STAT signaling, as well as differences in MAP and Src kinase activity, could play a large role in insufficient production and function seen in neonatal neutrophils. Current research focuses on impaired responsiveness of neonatal neutrophils to TLR ligands, neglecting alternative methods of cytokine stimulation of neonatal neutrophils. A better understanding of the mechanisms central to the differential response of neonatal neutrophils to the many cytokines of the inflammatory milieu is essential for moving the science forward.

Granulopoietic Response to Infection in Neonates

The normal lifespan of mature neutrophils is short, having a 7-10 hour half-life in the human circulation [50, 51]. The bone marrow compensates for short neutrophil half-lives by producing approximately 120 billion granulocytes per day [42]. In spite of this high steady state turnover rate, the bone marrow retains a large capacity to increase granulocyte production. During bacterial infection, bone marrow granulocyte production is markedly enhanced [42, 50]. In response to bacterial infection, bone marrow initially increases release of neutrophils from the marrow storage pool of neutrophils which houses 98-99% of all mature neutrophils during steady state conditions [52]. Subsequently, the granulopoietic activity in the marrow is activated, as reflected by an increased rate of precursor cell proliferation, decreased neutrophil storage time, and enhanced release of both immature and mature neutrophils into the circulation [53]. As a consequence, the concentration of immature neutrophils begins to rise in the peripheral blood that is presented as an increased immature-to-total (I/T) ratio. A functional bone marrow response can increase neutrophil

production from 1.6×10^6 kg/day during the steady state to 5.0×10^9 kg/day during infection [54].

During infection, bone marrow granulocyte production is reciprocally enhanced over the production of other blood cell types [55]. Enhanced granulocyte production has classically been demonstrated by increased proliferation rates of hematopoietic stem cells and granulopoietic progenitors following a hierarchical pattern of differentiation. However, current opinions in stem cell biology recognize that stem cells may alternatively follow a dynamic continuum of development rather than unidirectional differentiation. This phenomenon is exemplified by induced pluripotent stem cells, in which mature cells are reprogrammed to a stem-cell like phenotype by altering the expression pattern of as few as a single gene. *In vivo*, however, reprogramming may occur if uncommitted progenitor cells migrate to different bone marrow niche space [56]. A stem cell niche is an interactive structural unit, organized to facilitate cell-fate decisions in a proper spatiotemporal manner. The niche shelters stem cells from stimuli which promote excessive production, differentiation, or apoptosis thus maintaining hematopoietic stem cells in a quiescent state [57]. During infection and times of stress, hematopoietic progenitor cells egress from the bone marrow and leave open niche space [58]. Open niche space can be repopulated by marrow derived or circulating progenitor cells [59]. The re-seeding of open niche spaces suggests an endogenous mechanism by which uncommitted progenitor cells can regain “stem cell like” phenotypes [60].

Work by our laboratory and others supports the stem cell continuum theory. Using mouse models, we have observed that myeloid lineage development during bacterial infection does not follow the classical hierarchical pattern of cellular differentiation. In mice, hematopoietic stem cells are enriched in the cell population bearing surface markers of lineage -, c-Kit+, Sca-1+ (lin-ckit+Sca-1+, also called LKS+) [61]. Sca-1 expression is lost as hematopoietic stem cells differentiate into more committed downstream progenitors [62]. In response to bacterial infection, phenotypic inversion of downstream lin-ckit+Sca-1- (LKS-) cells to more immature stem cell phenotypes allows a 10 fold expansion of the marrow LKS+ cell pool [63]. The expanded LKS+ cell population serves as a platform on which the genetic reprogramming of precursor cell lineage commitment takes place [63]. Sca-1 expression level is directly associated with myeloid lineage development [63, 64]. Enhancement of Sca-1 expression is associated with activation of several master myeloid transcription factor genes including CEBP- α , PU.1, and Irgm. This polarized transcription factor profile strongly promotes myeloid lineage commitment by primitive precursor cells. Thus reprogramming of hematopoietic precursor cells constitutes an additional mechanism to augment the granulopoietic response to bacterial infection [65]. Essers and colleagues have demonstrated that Sca-1 gene expression can be upregulated by IFN- α /STAT1 signaling [66]. However, our results have shown that Sca-1 is upregulated in response to multiple cytokines and therefore more than one pathway is likely to be involved. To this point, investigating the importance of Sca-1 signaling in hematopoietic responses to infection has largely been restricted to cell lines and adult animal models. However, in human and non-human primates, hematopoietic precursors express other Ly6 family molecules in which Sca-1 is a member, including Sca-2, CD59, and other GPI-anchored proteins. Lothian et al, have previously demonstrated that enhanced CD59 expression in adult neutrophils in response to infectious stimulation does not occur in neonatal neutrophils [67]. Further exploring the age-related difference in the function of these molecules will improve our understanding about the process of hematopoietic precursor cell reprogramming in neonates during bacterial infection.

Quantitative Insufficiency of Neonatal Neutrophils

The increased susceptibility of the neonate to widespread bacteremia can be associated with a quantitative deficit in neonatal neutrophils. Due to lack of adaptive immunity, neonates primarily rely on innate, first line defenses to protect them in the relatively immunodeficient state following birth. However, for many neonates the innate immune system does not mature fast enough to adequately defend against bacterial infection. Neutrophil kinetics in response to bacterial infection is vastly different in the neonate compared to the adult. Blood neutrophil counts of normal neonates exist in a dynamic flux during the first few days of life. Unlike relatively steady blood neutrophil concentrations of $4.4 \times 10^9/L$ in adults, in neonates blood neutrophil counts can fluctuate between $1.5 - 28.0 \times 10^9/L$ [68]. In an effort to standardize criteria for diagnosing neutropenia in neonates, the Manroe and Mouzinho systems are used clinically to evaluate term and very low birthweight neonates respectively [69, 70]. Both systems demonstrate that blood neutrophil concentrations in the neonate sharply increase during the first 12hrs of life and gradually decline towards a steady $2.0 - 6.0 \times 10^9/L$ range between 2-3 days [71]. However, these systems do not account for variations in altitude, gender, and maternal status that could each affect neonatal blood neutrophil concentrations [72].

Using a neonatal animal model of bacterial infection, Al-Mulla and Christensen have previously shown a delay in the initiation of neonatal innate immune responses during infection. Following bacterial inoculation, neutrophil kinetics follow characteristic patterns of delay, increase in I/T marrow neutrophil ratio, and export of neutrophils into the circulation. This is followed by a period of accelerated neutrophil progenitor cell production restoring the marrow neutrophil reserve. However, the timing of the neutrophil response to bacterial infection is different in neonates. Using both lethal and sublethal bacterial inoculations of neonatal animals, Al Mulla and Christensen have demonstrated that neutrophil responses to infection took 3-4 hours to initiate in the neonate compared to 30-90 minutes in the adult [13]. A prolonged delay response suggests deficiencies at two levels, the neutrophil storage pool and progenitor cell cycling in the bone marrow. The neutrophil storage pool is defined as the sum of all the post-mitotic segmented neutrophils, band neutrophils, and metamyelocytes within hematopoietic tissue. Both human and animal studies have demonstrated that the neutrophil storage pool in the neonate, especially in premature infants, is less than that of adults [73, 74]. Animal models have suggested that the neutrophil storage pool/g body weight of newborns is 25% that of adults [73]. However, this difference has not been found in humans [74, 75]. Animal studies have also demonstrated faster release of the neonatal neutrophil storage pool into the circulation during infection, leading to a faster depletion of neutrophil reserves [73]. Neonatal bone marrow is additionally deficient in its ability to increase the number of progenitor cells following infection. Progenitor cells in neonates are significantly fewer and are more actively proliferating during the steady state than those of adults. These features limit the capacity of neonatal progenitor cells to upregulate neutrophil production during times of stress [76]. This is in contrast to adults whose progenitor cells are found in higher numbers and remain relatively quiescent during the steady state. Using a rodent model, Carr et al have determined neonatal neutrophil production differences which include; total neutrophil count in neonates less than 25% that of adults, GM-CFU number less than 10% of GM-CFU/g body weight of the adult, and one third the number of neonatal neutrophils found in G_0 of the cell phase compared to adult neutrophils [54]. The high percentage of neutrophil progenitors in the cell cycle was supported by work of Christensen et al in their human neonate studies. Christensen's group found the GM-CFU activity of human neonates averaged 55% with a range of 40-75%, representing near maximum proliferative activity [77]. Greater steady state proliferation reduces the capacity of these progenitor cells to increase neutrophil production during infection. As it has been demonstrated, there are

quantitative deficits in mature neutrophils, neutrophil storage pools, and quiescent neutrophil progenitor cell populations, which contribute significantly to the insufficient capacity of the neonate to efficiently control bacterial infection.

Qualitative Insufficiency of Neonatal Neutrophils

In addition to the insufficient capacity of the neonate to produce neutrophils, the neutrophils that neonates do produce have various functional deficits. In order for neutrophils to function properly they must recognize pathogenic bacteria, adhere to vascular endothelium, migrate to the site of infection along chemotactic gradients, engulf and intracellularly degrade the invading microorganism, and be removed from the circulation in order to prevent excess tissue injury. Neonatal neutrophils have deficiencies at virtually every step of normal neutrophil function.

Pathogen recognition

The initial recognition of invading bacteria is limited in the neonate by toll-like receptor (TLRs) deficiencies. TLRs bind to conserved molecular patterns on pathogens and initiate key events of the innate immune response. TLR4 is a transmembrane receptor for the gram negative bacterial product LPS. The expression of this receptor is markedly low in newborns and subsequently limits the extent to which bacterial-derived LPS can stimulate the monocyte production of inflammatory cytokines including TNF- α , IL-1, and IL-6 [78]. Neonates however are not deficient in the expression of other TLRs. TLR2 is expressed similarly in both neonates and adults, however, the downstream cascade stimulated by TLR2 binding is impaired in the neonate. Studies have also shown that the activation of intracellular signaling factors myeloid differentiation antigen 88 (MyD88) and p38 is insufficient following LPS binding to TLR4 on neonatal neutrophils. Decreased signaling activity of these proteins leads to a decreased production of TNF- α in response to LPS [79-81]. This blunted response has been partially attributed to a unique neonatal adenosine system. Neonatal blood has high concentrations of circulating adenosine and neonatal blood cells are highly sensitive to adenosine receptor binding. Heightened adenosine activity increases cytosolic cAMP concentrations which subsequently inhibit LPS induced TNF- α production [82]. cAMP dependent inhibition of TNF- α production is mediated by the activation of protein kinase A and is independent of the stimulatory effect of cAMP on interleukin-10 production [83]. Other cAMP elevating agents such as prostaglandin-E2 or cyclic nucleotide phosphodiesterase inhibitors can markedly suppress the TNF- α response [84, 85]. Decreasing pro-inflammatory cytokine production limits the ability of the neonatal immune system to be activated and reinforced when clearing an invasive bacterial infection.

Adherence

In addition to deficits in bacterial recognition, neonatal neutrophils also exert a limited capacity to adhere to vascular endothelium once pathogenic bacteria have been detected. Selectins mediate contact between neutrophils and the vascular endothelium as neutrophils are carried through the bloodstream [86]. This initial contact leads to the upregulation of other adhesion molecules and eventually helps localize circulating neutrophils at sites of infection. There are two principle selectins involved in this interaction, L-selectin expressed on the surface of neutrophils and E-selectin expressed on the surface of the vascular endothelium [21]. Analysis of cord blood from neonates less than one month old has demonstrated lower levels of L-selectin than seen in adults [87]. Less L-selectin expression suggests an insufficiency in the ability of neonatal neutrophils to adhere to the vascular endothelium and contribute to mounting an immune response [88]. While L-selectin mediates the initial loose adherence of neutrophils, further tight binding requires the participation of other proteins including LFA-1 and Mac-1 [89, 90]. The importance of

LFA-1 is seen in Leukocyte Adhesion Deficiency (LAD), an inherited childhood illness that leads to recurrent bacterial infections. Anderson et al, however, have demonstrated that Mac-1, also known as CD11b/CD18, is the more important of the two factors in neutrophil adhesion [91]. His group attributed a 50% reduction in transmigration to significantly reduced Mac-1 expression levels in neonates [92]. Mac-1 insufficiency in neonatal neutrophils includes reduced cell content [93, 94], reduced upregulation in response to stimulation, and reduced translocation of Mac-1 receptors to the cell surface [22]. Such deficiencies are closely associated with reduced adhesion and subsequent chemotactic capacity of neonatal neutrophils [22].

Chemotaxis

Following adhesion and transport through the endothelial layer, the next component of the neutrophilic response to infection includes migration toward the site of infection, a process known as chemotaxis. Dysfunctional chemotaxis is another deficiency of neonatal neutrophils. Einsfeld et al demonstrated this deficiency in their longitudinal study of neonatal neutrophil adherence and migration. They observed that neonatal chemotaxis was reduced 60% during the first week of postnatal life, but reached adult levels after the second week [95]. This chemotactic deficit was associated with decreased responsiveness to chemotactic factors including colchicine [96] and N-formyl-methionyl-leucyl-phenylalanine (fMLP) [97]. In studies using fMLP stimulation, neonatal neutrophils demonstrated a blunted rise in intracellular calcium that normally results from fMLP binding. Such a reduction in fMLP-induced intracellular calcium is believed to play a role in the attenuated chemotactic response of neutrophils in neonates [97]. Another cause of the blunted chemotactic response of neonates includes altered actin polymerization. The polymerization of monomeric G-actin to filamentous F-actin is a crucial component of neutrophil polarity and chemotaxis [98]. Cytoskeleton reorganization generates force for leukocyte deformation, adhesion, and migration [99]. While term neonates demonstrate similar F-actin concentrations as adults in an unstimulated state, when activated by chemotactic substances such as fMLP, neonates cannot increase F-actin to as great a degree as seen in adults [100, 101]. This limits the ability of the neonatal neutrophils to deform and penetrate the endothelial lining. Less deformability translates into less ability of neonatal neutrophils to migrate through the extracellular matrix of tissues toward the site of infection. Defective actin polymerization prevents neutrophils from performing their requisite motile functions and leads to recurrent, severe bacterial or fungal infections [99].

Phagocytosis

Phagocytosis requires the proper coordination and function of multiple cellular processes. The ability of neonatal neutrophils to phagocytose foreign particles is highly dependent on soluble factors within the serum. Matoth and Miller both found that in the presence of normal adult serum, cord blood derived neonatal neutrophils have similar abilities to phagocytose starch granules and baker's yeast respectively. However, neonatal neutrophils demonstrated deficient phagocytosis when adult serum was diluted [102]. It also appears that phagocytic ability in early life is dependent on the size of the infecting particle. Adult and neonatal neutrophils have similar abilities to phagocytose bacteria such as *Staphylococcus aureus* and *Escherichia coli*, while neonates are less able to phagocytose *Candida* [103]. Carr has attributed the poor phagocytosis of *Candida* to a deficit of neonatal neutrophils to engulf larger particles [21]. This relative deficiency in the phagocytosis of larger particles is resolved after the second week of life, correlating closely with the ultimate maturation of the chemotactic response [104].

Intracellular killing

Engulfed particles are normally digested by neutrophils using reactive oxygen species and various enzymes. Chemiluminescence linked to the oxidative metabolism in the neutrophil is closely associated with cell killing [105]. In response to opsonized zymogen and GBS, chemiluminescence production in the term neonate is similar to the adult. However, this response is blunted during times of infection and immunological stress [106, 107]. The Ambruso group has studied NADPH oxidase system kinetics in the neonate. The NADPH oxidase system is composed of multiple proteins found both on the plasma membrane surface as well as the cytosol. Oxidative activity is based upon the translocation of cell cytosolic phox proteins and their combination with specific granules to upregulate cytochrome b558 [108]. Ambruso et al have found that while cytosolic phox proteins are reduced in the neonate, plasma membrane cytochrome b558 levels are nearly double. Balanced changes result in oxidative function of neonatal neutrophils similar to that seen in adults. Under prolonged periods of infection, however specific deficiencies in cytosolic components and granules might restrict neutrophil response [109].

Clearance

Apoptosis, also referred to as programmed cell death, represents the major mechanism for neutrophil removal and inflammatory control and is another area of functional insufficiency of neonatal neutrophils. Compared to factors such as GM-CSF, IFN- γ and LPS which stimulate neutrophil activation, mediators such as IL-10 and the Fas ligand are anti-inflammatory and promote apoptosis. When compared to adults, cord blood neutrophils from healthy neonates do not respond as efficiently to Fas-mediated apoptosis as adults [110, 111]. Pretreatment of cord blood neutrophils with anti-Fas antibodies induced significantly less apoptosis (24% v. 63%) than seen in adult neutrophils [110]. The limited responsiveness to apoptotic stimuli is related to decreased surface receptor expression of the Fas receptor, decreased pro-apoptotic intracellular signaling molecule caspase-3, and decreased activity of the pro-apoptotic proteins Siglec-9 and SHP-1 [47, 111, 112]. Adult and neonatal cells were thought to be equally responsive to anti-apoptotic factors including GM-CSF, IFN- γ , and LPS [111]. However, the mRNA expression and production of these factors, particularly GM-CSF, has been found to be less in neonatal cord blood [47, 113].

NETosis

Neutrophil extracellular traps (NETs) are extracellular fibers composed of granule and nuclear constituents that provide high concentrations of microbicidal products to disarm and kill bacteria extracellularly [114, 115]. NETs are products of the reactive oxygen-dependent cell death pathway termed "NETosis," which is distinct from apoptosis and necrosis. Upon stimulation by interleukins, LPS, bacteria, fungi, or activated platelets, NETosis is initiated by high concentrations of reactive oxygen species which cause the condensation of nuclear chromatin, dissolution of the nuclear envelope, phagosome breakdown and granular enzyme mixing, and the ultimate expulsion of intracellular content to acquire an extracellular volume several folds larger than the cell itself [114]. More than 25% of all adult neutrophils will initiate NETosis [114]. As discussed above, human neonates have various phagocytic and intracellular killing deficiencies. Recently, Yost et al have determined that both term and premature neonates have deficient neutrophil NET formation and NET-mediated extracellular killing [116]. This defect is shared by neutrophils isolated from chronic granulomatous disease (CGD) patients. CGD patients are severely immunodeficient and suffer from recurrent and opportunistic infections [115]. Therefore, a deficit in extracellular killing by NETosis is an additional mechanism by which neonatal neutrophils lack the ability to contain infection and thus predispose neonates to widespread sepsis.

Adjuvant Therapies for Treating Neonatal Sepsis

Multiple pharmacologic strategies for treating neonatal sepsis have emerged. During the 1990s, intrapartum antibiotic therapy using penicillin and ampicillin became the principal prevention strategy while ampicillin was the first line empirical treatment of suspected sepsis [117]. Current recommendations for empiric therapy include combination therapy of intravenous aminoglycoside and an expanded spectrum penicillin antibiotic [118]. However, implementing the appropriate pharmacological regimen is particularly difficult in the relatively immunodeficient neonate in which bacteremia can quickly be lethal [119]. During the last three decades, physicians and researchers have been investigating therapeutic approaches to strengthen neonatal immunity while they concurrently treat patients with antibiotics. A number of adjuvant strategies to improve neonatal immune function during sepsis have been explored.

Colony stimulating factors

Colony stimulating factors play a vital role in the development of a hematopoietic stem cell into mature blood cells. In the process of granulocyte development, colony stimulating factors regulate processes such as hematopoietic stem cell lineage commitment and precursor cell proliferation and differentiation [120]. They also modulate granulocyte function including phagocytosis and cell mediated killing. Two extensively studied colony stimulating factors for the treatment of neonatal sepsis are G-CSF and GM-CSF. Both G-CSF and GM-CSF regulate the production and release of neutrophils from the bone marrow [121]. Investigators have applied their use in two strategies to combat neonatal sepsis: prophylactically stimulating neutrophil production and enhancing bactericidal function of existing granulocytes [122].

In 1991, Roberts et al were the first to demonstrate increased neutrophil production as a result of G-CSF administration to a neutropenic neonate [123]. Recombinant G-CSF (rG-CSF) was administered to compensate for known deficits in the production of colony stimulating factors by stimulated neonatal mononuclear cells, including G-CSF and IL-3 [124, 125]. While rG-CSF can significantly enhance circulating neutrophil number in adults, its ability to enhance absolute neutrophil number in neonates has been questioned [126, 127]. The limited studies attempting to demonstrate additional benefits of rG-CSF adjuvant use with antibiotic therapy in treating neonates with infection are plagued by low statistical power and unclear inclusion criteria [126]. Recently a multicenter trial by Kuhn et al, including 200 infants, studied the benefit of prophylactic rG-CSF treatment in preterm neonates with neutropenia in preventing nosocomial infections during 4 weeks of treatment. Although rG-CSF increased the number of neutrophils in these patients during the initial two weeks of treatment, this difference did not persist to the conclusion of the study. Kuhn et al concluded that prophylactic rG-CSF did not increase infection free survival four weeks after initiating treatment [128]. rG-CSF prophylaxis however may prove beneficial in bolstering neutrophil number and preventing infection in some subsets of neonates including those that are small for gestational age and those with early established postnatal neutropenia [129-131].

Similar to G-CSF, GM-CSF is upregulated in response to inflammatory stimuli. The effects of GM-CSF are more broad spectrum than G-CSF as it is produced by many different cell types and promotes the growth of precursors for multiple myeloid and megakaryocytic lineages [132]. When administered prophylactically recombinant GM-CSF (rGM-CSF) can significantly increase neutrophil counts in at risk neonates [129, 133]. rGM-CSF can also improve neonatal neutrophil function. *In vitro* incubation of neonatal neutrophils with GM-CSF increases their functional activity as seen by enhanced CD11b expression and reactive oxygen species generation [121]. However, in spite of proliferative and functional benefits

of GM-CSF on the neonatal neutrophil population, rGM-CSF administration does not significantly increase sepsis free survival and there is insufficient evidence to support its use both as a prophylactic and treatment strategy [126].

Maternal Immunization

While immunoglobulin has no direct influence on neutrophil production or functional maturation, its indirect enhancement of phagocytosis can be significant. Transplacental transfer of immunoglobulin to the developing fetus occurs after 32 weeks gestation and provides passive protection from infection prior to the complete maturation of the neonatal immune system [134]. The neonate's immune system relies upon these maternal immunoglobulins until it reaches functional maturity several weeks after birth [135]. The degree to which protection is conveyed to the neonate is based upon the prior antigen exposure and vaccination of the mother. It is believed that such protection is pathogen specific [136] and corresponds with the duration of protection for the neonate [135]. Due to the benefit that maternal immunoglobulins provide for the neonate early in life, vaccination of pregnant women first began in the United States in 1957. Since then, thousands of women have been vaccinated against such pathogens as polio, hepatitis B, and pneumococcus [135]. It wasn't until 1988 that Baker et al studied the first capsular polysaccharide vaccine for pregnant women against GBS, the leading cause of neonatal sepsis at the time. Pregnant mothers were administered a polycapsular vaccine against the GBS Type III strain at 31 weeks gestation that stimulated the production of significant titers of Type III specific antibodies in the newborn. Resulting antibodies were able to activate complement and opsonization when studied *in vitro* [137]. Multiple clinical trials and political lobbying by the Baker group have sought to instill GBS conjugate vaccination to be as commonplace as Haemophilus influenza type B vaccination. However, they recognize that a significant obstacle remains in the liability issues facing immunizing pregnant women and the necessity for pharmaceutical partnership [138].

Immunoglobulin therapy

Some investigators have attributed high rates of sepsis in neonates to low levels of IgG immunoglobulin and have hypothesized that intravenous immunoglobulin therapy in the neonate is an acceptable approach for treatment. Similar to most immunoglobulins, the transplacental transport of IgG from the mother to fetus begins around 32 weeks gestation and increases until term. Premature infants born prior to 32 weeks gestation have profound IgG deficiencies. The major function of IgG in host defense is to opsonize bacteria and neutralize viruses. Levels of postnatal IgG are often low due to insufficient production by the immature neonatal immune system and catabolism of maternal IgG [139, 140]. Opsonic activity is also type-specific; therefore humoral immunity transferred to the neonate will be insufficient if the mother does not have immunity to the specific pathogen [140].

Shigeoka et al in 1978 correlated increased survival in GBS infected infants with acquiring serotype-specific opsonins following whole blood transfusions. Nine of nine infants transfused with blood containing antibodies against the infecting strain of GBS survived, while only three of six survived when transfused with blood lacking antibodies to that strain [141]. Sidiropoulos et al in 1981 demonstrated that administering immunoglobulin concurrent with antibiotics was more effective in treating neonatal sepsis than administering antibiotics alone. They co-administered a polyvalent immunoglobulin preparation, Sandoglobulin, with antibiotics to treat 35 newborns with bacterial infection. The mortality rate of preterm infants receiving antibiotics supplemented with immunoglobulin was 10% (2/20) compared to those receiving antibiotics alone 26% (4/15), and no term infants died. Follow up of the experimental groups at one and five years demonstrated no adverse sequelae from immunoglobulin therapy [142]. Haque et al found similar improvements in mortality when

instead of IgG immunoglobulin, they co-administered IgM immunoglobulin with antibiotics into 30 septic neonates [143]. This study was similar in size to that of Sidiropoulos and found similar improvement in survival. A larger follow up study in 130 septic neonates comparing intravenous IgM to IgG adjuvant therapy with concurrent antibiotic therapy demonstrated no significant difference in survival between the two formulations while both improved mortality rates compared to non-immunoglobulin treated groups [144]. Due to positive findings in recent clinical trials, the Surviving Sepsis Campaign, the international collaboration of experts who in 2004 released the first outline for proper clinical management of sepsis, suggested that only polyvalent intravenous immunoglobulin therapy was a promising adjuvant therapy for the treatment of sepsis and septic shock in the neonate [145].

The prophylactic benefit of immunoglobulin therapy was assessed by Baker's group in 1989 with a double blinded study of 588 low birth weight neonates. Although there was no difference in mortality rate, those neonates transfused with immunoglobulin demonstrated a reduction in the incidence of infection (relative risk of 0.7), fewer days of total hospitalization (62 v. 68d), and fewer days of hospitalization for septic neonates (80 v. 101d) [146]. Christensen sought to explain how the benefits seen with Baker's prophylactic immunoglobulin therapy were associated with improvements in innate immunity of the neonate. Using a randomized group of 20 premature neonates with early onset sepsis, 10 patients were given intravenous immunoglobulin (IVIG) while the other 10 were given albumin controls. While there were no differences in mortality rate between the two groups with all experimental participants surviving, there were significant differences in innate immunity seen in those participants receiving IVIG. The transfusion of 750 mg/kg caused an increased concentration of complement, increased opsonic activity for bacteria, more rapid resolution of neutropenia, and more rapid elevation in the I/T ratio [147]. The rapid elevation of I/T ratio was similarly reported by Cairo, and attributed to a rapid release of neutrophils into the circulation from the neutrophil storage pool of the bone marrow [124]. Jenson and Pollock's 1997 meta-analysis of 110 septic neonates treated with IVIG concluded that the use of IVIG in the treatment of neonatal sepsis can prolong the life of septic neonates by nearly six fold. They found unequivocal and substantial benefits of its use [148].

A recent Cochrane Meta-Analysis by Ohlsson reviewed the benefit of prophylactic administration of IVIG for preventing nosocomial infection in preterm neonates. Ohlsson concluded that prophylactic IVIG produced a 3% reduction in sepsis and a 4% reduction in other incidences of severe infection but did not lead to any difference in mortality rate. This finding significantly limits the scope of prophylactic improvements in neonatal immune defense first described by Baker and coincides with the Jenson and Pollack meta-analysis in which they found no overall benefit from prophylactic immunoglobulin therapy [148]. The administration of immunoglobulin to neonates also comes with risks. Side effects include blockage of the reticuloendothelial system, development of *Cytomegalovirus* or *Candida* infection, transmission of non-A non-B hepatitis, immune complex formation, and development of intracranial hemorrhage [149]. However, it has been suggested that administering doses of 750 mg/kg can help to avoid problems related to fluid overload in the neonate [150]. At the present time, it is suggested that concurrent IVIG adjuvant therapy and not prophylaxis may provide one method to improve the function of innate neonatal immunity during sepsis [119].

Granulocyte transfusion

Some scientists have forgone attempts to treat neonatal sepsis by stimulating the proliferation and function of existing neonatal neutrophil precursors and have instead administered adult neutrophils to at risk neonates. The first documented granulocyte

transfusion in neonates was performed by Laurenti et al. In their trial, they transfused 20 neonates suffering from systemic *Klebsiella* infections with granulocytes acquired from adults. Results found significant reductions in mortality, 10% in transfused groups and 72% in non-transfused groups. Laurenti's work however was a retrospective study that was not controlled or randomized and included different granulocyte administration schedules [151]. This study functioned as a proof of principle that granulocyte transfusions could provide benefit for septic neonates. Christensen expanded on Laurenti's findings in his prospective study of granulocyte administration to neutropenic, septic neonates. Granulocyte transfusion produced 100% survival in seven septic neonates, while only one of nine septic neonates untransfused survived. While their protocol originally designated granulocyte transfusion to occur every 12 hours, they found a single transfusion reestablished granulocyte numbers in the neonate within normal range. This was a surprising finding for the group because it suggested that the transfusion somehow stimulated endogenous production of granulocytes [152]. Following similar protocols, this trial was repeated by Cairo in 1992, when they compared granulocyte transfusion to IVIG transfusion for the treatment of sepsis in neutropenic neonates. This trial including 35 infants found 100% survival (21/21) in the granulocyte treated group and 64% survival (9/14, $p < 0.03$) in the IV immunoglobulin treated group [124]. However, the 2003 Cochrane review by Mohan and Brocklehurst questioned the efficacy of granulocyte transfusions. They determined that the relative risk of eligible trials was not significantly different in all-cause mortality when compared to placebo. They did however agree that the increased protection of granulocyte transfusion over IV immunoglobulin use determined in the 1992 Cairo trial was of borderline statistical significance. The limited statistical significance was attributed to a small number of trials with relatively few study subjects. They suggested that since neonatal progenitor cells in the bone marrow can be directly stimulated by colony stimulating factors, the use of G-CSF and GM-CSF may provide a more promising therapy. These therapies are more accessible, less costly, and pose less risk to the neonate than what is currently attainable with granulocyte transfusions [153]. However, to date, the only treatments proven to reduce mortality in neonatal infection are IVIG therapy and granulocyte transfusion [150].

Pentoxifylline

Pentoxifylline is a xanthine derived phosphodiesterase inhibitor which suppresses pro-inflammatory cytokines including TNF- α and IL-1. In doing so, it decreases neutrophil oxidative burst, adherence, and lysozyme degranulation in response to various cytokines [154]. For adults, pentoxifylline is FDA approved for the treatment of chronic peripheral arterial occlusive disease because it increases microcirculatory blood flow by improving red and white blood cell deformability and motility, lowering blood viscosity, decreasing platelet aggregation, decreasing fibrinogen levels, and decreasing prostaglandin synthesis [155].

The use of pentoxifylline as an adjuvant treatment for sepsis was first investigated by Zeni et al in a small group of sixteen adults with septic shock. They demonstrated that pentoxifylline could be administered safely and decreased *in vivo* production of TNF- α [156]. In the same year, Laughterbach demonstrated the same phenomenon in 29 premature newborn infants with confirmed sepsis. He showed that the decreased production of TNF- α also resulted in improved clinical outcomes in the groups receiving adjuvant pentoxifylline therapy. One in four septic infants in the placebo group survived versus all of the five infants in the pentoxifylline group. There were also lower rates of metabolic acidosis, necrotizing enteritis, and renal insufficiency with pentoxifylline therapy [157]. In 1999 his group then expanded this investigation to include 100 septic preterm neonates in a prospective, randomized, and double blinded study. A similar reduction in symptoms of sepsis was seen as well as a reduction in mortality with 6 in 38 in the placebo group and only 1 in 40 in the

pentoxifylline group. In addition to decreasing TNF- α production, pentoxifylline selectively decreases IL-6 production while having no effect on IL-1 [158].

The ability of pentoxifylline to promote blood cell deformability can significantly enhance neonatal neutrophil chemotactic function. Using a murine model, Krause et al. demonstrated that pentoxifylline administration increased neutrophil chemotaxis, accumulation of leukocytes at the infection site, and added protection against *Staphylococcus aureus* infection [159]. The augmented function was dose dependent, lower doses enhanced neutrophil function and higher doses were inhibitory in both neonatal and adult neutrophils. This group also identified similar benefits for neutrophils acquired from human cord blood. In response to pentoxifylline addition in culture, the F-actin content of neonatal neutrophils was reduced, increasing their membrane deformability and allowing greater motility [160]. These findings have been replicated by Ruef et al using blood samples from term neonates [161].

While pentoxifylline modulates the function of neonatal neutrophils, the support of its use as an adjuvant therapy in treating neonatal sepsis focuses on its ability to modulate the cytokine response to infection. The Cochrane Database Review of the use of adjuvant pentoxifylline in the treatment of neonatal sepsis focuses predominantly on Lauterbach's work. In this review, Haque suggests that pentoxifylline administration can significantly reduce all cause mortality of neonates with sepsis, however, has only provided a borderline statistical improvement in cases of confirmed gram negative sepsis [162]. Benefits are likely as a result of the administration schedule. Pentoxifylline is co-administered with antibiotic therapy and therefore attenuates the massive inflammatory response that occurs with the initial large-scale bacterial lysis produced by the antibiotics. Pentoxifylline administration continues over 6 days, further limiting the extent by which an inflammatory response is mounted [163]. It should be noted however, that both of Lauterbach's studies include preterm infants and studies of pentoxifylline use have yet to be reviewed in term neonates. Recent investigation using pentoxifylline in rodents also focuses on preterm neonates [164]. Lauterbach's work demonstrates statistical significant reduction in mortality and trends toward earlier correction of metabolic and hemodynamic derangements in preterm neonates with confirmed late onset sepsis [162]. By controlling the cytokine response to infection and improving neutrophil motility and localization to the infection focus, pentoxifylline dynamically regulates multiple facets of the immune response during infection. It appears that by regulating multiple targets simultaneously, pentoxifylline possibly allows for a better regulated and functioning response in the neonate.

Perspectives

In spite of significant improvement of technology and clinical practice, sepsis remains a leading cause of neonatal morbidity and mortality. Neonatal sepsis is difficult to diagnose due to the limitations of clinical tests, vague symptoms, and potential for rapid disease progression. In the neonate, multiple quantitative and qualitative neutrophil insufficiencies increase the risk of systemic bacterial infection. Antibiotics remain the mainstay of treatment for sepsis in neonates. However, as more has been learned regarding the pathophysiology of neonatal sepsis and the insufficiencies of neonatal host defense, it is increasingly clear that adjuvant treatments can bolster neonatal immune function. Common adjuvant treatments primarily target the modulation of the innate immune response. Although each strategy has demonstrated certain potential benefits, controversy remains regarding their general clinical application and potential side effects. Recent studies have revealed that hematopoietic stem cell reprogramming plays an essential role in initializing the granulopoietic response to bacterial infections [63, 65]. Characterizing differences in hematopoietic stem cell reprogramming during granulocyte lineage commitment between

neonates and adults will potentially advance our knowledge about the mechanisms underlying the insufficient granulopoietic response in neonates. This line of investigation may also establish a foundation for developing new adjuvant strategies for treating sepsis in neonates.

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