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Necrotizing Enterocolitis: Pathophysiology from a historical context

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Abstract

Necrotizing Enterocolitis (NEC) continues to afflict approximately 7% of preterm infants born weighing less than 1500 grams, though recent investigations have provided novel insights into the pathogenesis of this complex disease¹. The disease has been a major cause of morbidity and mortality in neonatal intensive care units worldwide for many years, and our current understanding reflects exceptional observations made decades ago. In this review, we will describe NEC from a historical context and summarize seminal findings that underscore the importance of enteral feeding, the gut microbiota, and intestinal inflammation in this complex pathophysiology.

HISTORICAL PERSPECTIVE

Although not described as classic NEC, a report from almost 200 years ago by Charles Billard described a case from the Hôpital Des Enfants Trouvés in Paris, France in which 'a foundling newborn developed a swollen abdomen with greenish then bloody diarrhea, developing a tense abdomen, cold extremities, bradycardia, and subsequent death.' The autopsy of this patient described an intensely red and swollen terminal ileum, with friable mucosa and the surface covered with blood. In fact, the mucosa was so soft that it 'turned to mash when scraped with the fingernail'. This description is consistent with clinical findings that are sometimes observed in our NEC patients now, and may be the first published account of NEC. (reference)

In 1944 Heinrich Willi reported on 62 cases of 'malignant enteritis' in newborns. These cases were of interest in that 2/3 of them had birthweights under 2500 grams, they seemed to be associated with overcrowding in the nursery, and they typically occurred in clusters. These observations may be the first describing what subsequently was reported as a 'NEC epidemic', though these events are distinctly uncommon in the current era. (reference)

There were 85 cases described in 1952 by Schmidt and Kaiser in two reports where newborn patients had abdominal symptoms, bloody stools, and pathological evidence of ulcerated and necrotic bowel that they termed 'enterocolitis ulcerosa necroticans', or the fore-runner of

necrotizing enterocolitis². These publications are often credited as the first descriptions of NEC, and framed the disease for many subsequent investigators to analyze and ascertain.

Around the same time, in 1951, a radiologist in Ann Arbor, Michigan, Arthur Steinen observed pneumatosis intestinalis in the radiograph from a newborn patient with bloody stools, and described ‘from the mesenteric root gas can dissect, extend to the mesenteric insertion of the intestine and from here either dissect along the subserosal layers or, following the blood vessels...enter the submucosa’. This finding has become the radiologic hallmark of NEC, and scientists have speculated on the contribution of this gas in the bowel wall to include hydrogen gas as a byproduct from bacterial fermentation on carbohydrate substrate. (reference) Though controversial, this may explain why NEC may occur in very preterm patients without evidence of pneumatosis intestinalis, particularly before enteral feedings have been well established.

In the 1950’s and 1960’s, as more and more preterm infants were able to survive due to advances in neonatal care for very low birth weight infants, there was a significant and consistent rise in cases of necrotizing enterocolitis. During that time, there were several colleagues at the Babies Hospital of Columbia University in New York that conducted extensive analyses from newborns with NEC and animal experiments in the laboratory setting. These investigators concluded that hypoxia was an important initiator of the development of intestinal injury in this population, but that enteral feedings, intestinal microbial flora, and inflammation all contributed to the final common pathway of disease. These studies demonstrated that Gram negative bacteria contributed significantly to the outcome of NEC, and that prematurity was an important risk factor, presumably due to impaired host defense. While the impact of mesenteric ischemia and hypoxia on NEC pathogenesis remains controversial, the remaining risk factors of enteral feeding, dysbiosis, and intestinal inflammation have gained additional support following innovative and reproducible observations over the course of time.

ENTERAL FEEDINGS IN NEC PATHOGENESIS

In the early 1970’s, Dr. Barlow and colleagues in New York developed a newborn rat model of NEC that included formula feeding, intermittent asphyxia, and bacterial colonization³. Initial studies demonstrated that rat mother’s milk feedings completely protected against NEC compared to newborn pups who received formula feedings, and it was hypothesized that breast milk feedings provided mucosal immunity that promoted colonization with commensal microbes thus allowing for normal mucosal epithelial cell repair and regeneration. Formula feedings were suggested to promote gut dysbiosis with overgrowth of enteric bacteria, mucosal translocation, and the development of NEC. Subsequent studies showed that while fresh milk was protective, frozen milk was ineffective, and it was the leukocyte fraction that was important in preventing the development of disease⁴.

As cases of NEC were increasing, in the mid 1970’s Drs. Brown and Sweet at Mount Sinai Hospital in New York hypothesized that aggressive feeding approaches contributed to the development of NEC in premature infants, and embarked on a strict feeding regimen to reduce the risk in their population⁵. They devised a careful protocol in which feeding

volumes and formula strength were initially begun at low levels, and slowly advanced over a two week course to attain 'full' enteral volumes and calories. Based on this approach, they reported that they almost completely prevented NEC in their NICU population, and that a prospective, randomized and controlled trial was unethical to study their innovative protocol. Based on this supposition, they reported that only 1/932 low birth weight (less than 2500 grams) infant developed NEC with the strict feeding regimen compared to 14/1745 (0.8%, $p = 0.026$) of historic controls. This was a provocative report, and likely influenced the feeding strategies for many providers in NICU settings around the country.

Throughout the 1980's and 90's, several studies showed that early hypocaloric or trophic feedings (given at approximately 15–20 ml/kg/day) as compared to a prolonged period of nothing by mouth (NPO) benefitted preterm infants with less hyperbilirubinemia, less osteopenia of prematurity, and more rapid attainment of full enteral feedings without the risk of increased incidence of NEC^{6,7}. Therefore, most neonatologists began early trophic feedings, and the pendulum swung towards earlier and faster advancement of the feeding protocol. In 2003, Dr. Berseth and colleagues reported a prospective randomized trial that showed delayed advancement of hypocaloric feedings by 10 days reduced the risk of NEC significantly compared to patients that began earlier advancements (NEC incidence 10% for advancing volume group v. 1.4% for delayed advancement, $p = 0.03$)⁸. This result was provocative and some NICU's adopted this practice of delayed feeding advancement, though similar to Berseth's own data, these patients were at increased risk of central line infections associated with prolonged need for intravenous cannulation and hyperalimentation. It remained unclear whether the size of feeding volume increases on a day to day basis contributed to NEC, and some investigators conducted studies to evaluate this supposition. Based on a reasonably low number of patients randomized, a meta-analysis was recently published in the Cochrane Review that suggested the size of feeding volume increases did not influence the development of NEC⁹. It should be noted that the power of this conclusion was small, though the takeaway was that daily advances between 15 ml/feed up to 35 ml/feed did not differentially contribute to NEC pathogenesis. Overall, even today there exists significant variation in feeding protocols for extremely premature infants, though most regimens include conservative advancements for the smallest and sickest patients.

Human milk feedings for premature infants were advocated in the 1950's and 1960's, though most of the studies suggesting their benefit were retrospective and case-control in design. The most definitive report was published by Lucas and Cole in 1980, and this study demonstrated that human milk reduced the risk of NEC compared to formula feeding in each specific gestational age stratum¹⁰. Further, this is the only study that randomized patients prospectively to human milk v. formula. In this analysis, because of small numbers, there was not a statistically significant reduction in NEC in randomized patients, though the trend was strongly in favor of human milk protecting against NEC (NEC incidence: 1% human milk v. 5% formula feeding, $p > 0.05$). Nonetheless, many subsequent studies demonstrated beneficial effects of human milk on NEC, and despite the need for robust fortification to increase calories, protein, electrolytes, minerals, and vitamins for preterm infants, human milk should be, and is standard of care for the preterm infant. For patients that do not have maternal milk available, studies suggested that donor human milk may be advantageous

compared to formula feedings. All studies have not been definitive, though in a recent meta-analysis, donor milk reduced the risk of NEC compared to formula feedings. (reference)

For many years, fortification of human milk has been provided by concentrated bovine-based products that include high protein content, increased fat and carbohydrate, and a large array of electrolytes, minerals, and vitamins. Recently, human milk-based fortification has been developed, and a study from 2010 suggested that NEC was reduced using human milk-based fortifier compared to bovine-based fortifiers¹¹. Though this study was not powered to evaluate NEC as the primary outcome, the data are of interest, and while a large, definitive, appropriately powered study would be worthwhile, it remains unclear as to the mechanism responsible for this possible impact. Previous studies have shown that human milk reduces the risk of NEC in a dose-dependent manner, and suggest that many bioactive factors in human milk (that are not present or lower in preterm formula), such as leukocytes, polyunsaturated fatty acids, oligosaccharides, immunoglobulins such as IgA, lactoferrin, commensal microbes, and many others could provide antimicrobial and anti-inflammatory protection for the immune-compromised host. Alternatively, it has been suggested that cow's milk protein from the bovine-based fortification could initiate an inflammatory response and intestinal necrosis as part of the NEC cascade. This mechanism has been suggested in human observations in which peripheral blood monocytes from NEC patients respond with more inflammatory mediator production following bovine lactoglobulin compared to preterm monocytes that have normal intestinal health (reference). Nonetheless, it is known that human milk protein content includes approximately 10% of cow's milk proteins from women who are not on a dairy free diet, so the possible impact of human milk fortification and cow's milk protein exposure at this stage remains conjectural.

In summary, enteral feedings may contribute to the initiation of NEC, and while the understanding of these risk factors changed over the last 30–40 years, specific strategies and feeding protocols are not uniformly agreed upon by all clinicians.

EFFECT OF MICROBIOME

It was suggested over 50 years ago that bacteria contributed significantly to the development of NEC. Mizrahi and colleagues in NYC cultured blood and peritoneal fluid from patients with disease and demonstrated a predilection for Gram negative bacteria, particularly *E. Coli* as offending agents associated with NEC¹². Additional studies in the early 1970's showed that 'epidemics' of NEC occurred in some NICU's, and in one of the first reports, Stein et al showed that Salmonella organisms were involved in 11 consecutive cases¹³. Nonetheless, many cases of NEC were not associated with bacterial organisms in the blood, and further investigation suggested that intestinal bacteria might initiate NEC by activating the inflammatory response. It was suggested in the 1970's that the intestinal microbiome in newborns housed in the NICU environment was different than in healthy full-term infants, yet clear description of microbial differences were first described between preterm infants and full-term infants by Gewolb and colleagues in 1999¹⁴. These investigators cultured stool from 29 preterm infants born weighing less than 1000 grams, and showed a significant lack of diversity in the organisms as compared to full-term infants. Further, the study showed that breast milk exposed infants had more diversity, and those treated with early antibiotics had

much less. Finally, results showed that only 1/29 patients had evidence of commensal bacteria compared to the majority of full-term infants who had significant quantities of these organisms in routine stool cultures. This breakthrough report set the stage for additional and intense investigation into the role of the microbiome in preterm infants and NEC, and has led to further understanding of NEC pathophysiology.

As the technology improved and analyses of microbial communities advanced to sophisticated DNA methodology, many additional studies evaluated differences in the microbiome from preterm infants and in those with NEC. The first convincing report demonstrating differences between preterm infants with NEC and preterm infants without NEC showed that NEC patients had less microbial diversity and a predominance of one of many different Gram negative enteric pathogens¹⁵. In addition, further analysis showed that preterm patients without NEC had significantly more commensal Gram positive bacteria compared to the NEC patients. While many additional reports have confirmed these differences, some did not identify significant changes in patients who went on to develop NEC. In the largest prospective trial to date, Warner and colleagues evaluated 166 patients with 46 developing NEC, and they found that NEC patients had increasing amounts of *Proteobacteria* species prior to the onset of disease with decreasing numbers of *Negativicutes* and *Bacilli*¹⁶. These changes were prominent only in those patients less than 28 weeks gestation at birth, and there was significantly less microbial diversity in those patients who went on to develop NEC. Overall, using a modified meta-analysis approach, all studies together seemed to corroborate these important findings, and it is generally accepted that changes in the composition of intestinal microbes contribute to the development of NEC¹⁷.

Based on the supposition that the microbiome contributes to NEC and that preterm infants have a modified microbiome with less commensal species, more Gram negative enterics, and less overall diversity, many studies have focused on the potential impact of probiotic supplementation as a preventive strategy for the disease. In the 1990's, two animal studies demonstrated that probiotic supplementation reduced the risk of NEC in newborn quail and rodent models, and subsequent clinical trials were therefore performed in high risk premature infants^{18,19}. There have now been a large number of trials, though most are underpowered and utilize various combinations of organisms. However, the recent meta-analyses demonstrate significant reduction in the incidence of NEC²⁰. With increasing numbers of studies, the effect size for the impact of probiotics is lower, though there remains obvious excitement in the neonatal community about the potential efficacy of this simple approach to prevent NEC. Further quality control, safety, and efficacy studies are ongoing in the US before routine standard of care is initiated using this approach.

INFLAMMATION AND NEC

For many years, the pathogenesis of NEC was thought to be multifactorial and occurred in response to several key risk factors of prematurity, enteral feeding, intestinal ischemia, and bacterial effects²¹. In addition, it was suggested in the 1960's and 1970's that these risk factors stimulated an inflammatory response that either localized in the intestine, or could become more systemic in more severe cases of NEC. In early reports, it was suggested that

bacterial products such as endotoxin (also known as lipopolysaccharide or LPS) were associated with NEC and that these cell wall ligands initiated the inflammatory response leading to intestinal necrosis. At the time, a limited number of inflammatory mediators were well described and understood, though studies suggested the potential role of prostaglandin analogues such as thromboxanes and leukotrienes.

In 1979, platelet activating factor (PAF) was discovered, and this endogenous phospholipid mediator was shown to initiate potent inflammatory responses in a variety of tissues including the intestine²². In 1983, Gonzalez-Crussi and Hsueh demonstrated that PAF infusions into adult rats resulted in severe intestinal necrosis with limited pathology in the other organs²³. Subsequently, these investigators showed that PAF receptor antagonists prevented hypoxia and LPS-induced intestinal injury, and that PAF content was elevated in intestinal tissue recovered from animals with intestinal inflammation. Caplan and others reproduced these studies in a neonatal rat model of NEC, and showed that PAF receptor antagonists and the PAF-degrading enzyme, PAF-acetylhydrolase decreased the incidence of disease^{24,25}. Further, they showed that preterm infants with NEC had higher PAF levels and lower PAF-acetylhydrolase activity compared to gestational age-matched controls²⁶. These results together identified an important central role of PAF in intestinal inflammation and NEC in the newborn environment.

BACTERIAL-ENTEROCYTE SIGNALING IN THE PATHOGENESIS NEC?

The role for bacteria in the pathogenesis of NEC is highlighted by several clinical and experimental observations. First, NEC typically develops at several weeks of age, corresponding to the time at which bacterial colonization develops^{27–29}. Second, the administration of antibiotics is used as a treatment for NEC especially in its early stages, consistent with the notion that bacterial signaling may play a role in driving its development towards more advanced stages³⁰. Third, patients with NEC may demonstrate growth of enteric organisms in the blood, as well as elevated levels of LPS (a cell wall component of Gram negative bacteria), suggestive that a breakdown of the intestinal barrier and egress of bacteria into the circulation occurs during the course of the disease^{31,32}. Given that the disease process in NEC is initiated in the intestines – in contradistinction to other septic conditions in infants in which remote infection may lead to secondary intestinal pathology – we and others have proposed that one of the earliest events that lead to the development of NEC involve abnormal signaling in the premature intestine in response to colonizing microbes. We will now consider how abnormal bacterial-intestinal signaling plays a central role in the pathogenesis of NEC, and how strategies to disrupt abnormal bacterial-intestinal signaling can potentially prevent or treat this disease.

In seeking to understand the role of bacterial signaling in the pathogenesis of NEC, we also seek to address several key questions in the field, as follows:

- What is it about the premature intestinal epithelium that predisposes the host to the development of NEC in the first place?
- Why does the intestinal mucosa actually die – as opposed to simply becoming extremely inflamed – in the pathogenesis of NEC?

- What explains the fact that breast milk is so often protective against the development of NEC?
- What explains the reasons for which one individual patient may develop NEC, while another infant, born at the same gestational age and housed in the same NICU, does not develop NEC?

By addressing each of these questions in turn, and placing the potential responses within the historical context described above, we seek to not only gain a greater understanding of the pathogenesis of NEC, but also to develop novel and specific therapies for these patients.

BACTERIAL SIGNALING IN THE PREMATURE HOST LEADS TO NEC.

Several investigators have focused on elucidating the mechanisms by which bacterial signaling occurs in the premature host, and how abnormal bacterial signaling can lead to the development of NEC. One of the most exciting discoveries in this regard was the identification of the role of the Toll-like receptors as pathogen recognition molecules that allow the host to respond to potential pathogens – including bacteria – without ever having seen them before. The first family member identified – toll like receptor 4 - (TLR4) recognizes LPS, and induces a pro-inflammatory response involving the activation of the cytokine inducing transcription factor NFkB³³. Caplan's group showed in 2006 for the first time that mice deficient in TLR4 were protected from NEC development³⁴, providing the first evidence for TLR4 in the pathogenesis of NEC, and more importantly, providing proof that bacterial-enterocyte signaling plays a role in this disease. In additional independent studies published in 2007, Hackam and colleagues showed that expression of TLR4 was higher in the intestine of mice and humans with NEC as compared with mice without NEC, and showed that a different strain of TLR4 deficient mice were protected from the development of this disease^{35,36}. Furthermore, in a series of additional studies, Hackam and colleagues showed that TLR4 signaling in the intestinal epithelium as opposed to other cells is required for the development of NEC in mice, as mice lacking TLR4 on the intestinal epithelium were protected from NEC³⁷.

In seeking to understand the mechanisms by which TLR4 activation leads to NEC, Hackam and colleagues further showed that TLR4 signaling on the intestinal epithelium led to increased cell death via an induction of endoplasmic reticulum and enterocyte apoptosis, as well as a reduction in mucosal repair via inhibition of enterocyte migration and enterocyte proliferation^{38,39}. These deleterious effects of TLR4 on the intestinal mucosa result in mucosal breakdown and bacterial translocation, and the septic picture that characterizes NEC. It is noteworthy that the clinical factors most often seen in association with NEC, including hypoxia, remote infection, and prematurity, are all associated with induced TLR4 expression in the intestine, suggesting a biologic explanation for these clinical observations. The principal findings that the LPS recognition molecule TLR4 is elevated in experimental and human NEC, and that it plays a key role in the pathogenesis of this disease, has been confirmed by a variety of investigators^{40,41,42,43,44,45}.

In seeking to gain a greater understanding of the role of TLR4 in the premature intestine, Hackam and others have performed *in situ* hybridization studies using mRNA probes for

TLR4, and note that TLR4 is expressed specifically on the intestinal progenitor cells (stem cells) within the crypts^{46,47}. It was further determined that TLR4 is expressed at higher levels in the premature as compared with the full term host, which reflects in part the role of TLR4 in the regulation of normal stem cell function, as TLR4 was found to be an important regulator of Notch signaling in the stem cells of the gut^{35–37,46,48}. As a result, the premature host, in whom the intestine is normally still developing and expresses very high levels of TLR4, is at particularly higher risk for NEC development when exposed to bacteria within the harsh, bacteria rich extra-uterine environment of the neonatal intensive care unit. These findings provide insights into why the premature infant is at risk for NEC development, and why bacterial intestinal signaling is so much higher in the premature as compared with the full term population. These findings were explored in greater detail in a recent review from the Hackam group⁴⁹.

Recent studies have shed light on the cells that migrate to the inflamed intestinal mucosa of the premature infant, leading to the development of NEC. Several labs have shown that the development of NEC is characterized by an influx of pro-inflammatory lymphocytes, while Hackam and colleagues have determined that NEC is characterized by a TLR4-mediated influx of pro-inflammatory Th17 lymphocytes and a loss of anti-inflammatory T regulatory lymphocytes in the newborn mucosa. The influx of Th17 lymphocytes was *sufficient* for NEC development, as the adoptive transfer of these inflammatory cells into naïve mice resulted in the development of spontaneous NEC-like injury in the recipient intestinal mucosa⁵⁰. Importantly, strategies that either block the influx of Th17 cells, or which promoted the induction of anti-inflammatory Treg cells such as the administration of all trans retinoic acid, resulted in significant NEC protection in experimental studies⁵⁰. Taken together, these findings explain in part the effector mechanisms by which TLR4 responses to bacterial signaling cause the profound inflammation observed in the premature intestine in the pathogenesis of NEC, and provide a rationale for further study of bacterial signaling in order to prevent this disease.

MECHANISMS OF INTESTINAL DEATH IN THE PATHOGENESIS OF NEC.

While the above findings explain the mechanisms by which bacterial intestinal signaling in the premature host may lead to inflammation as seen in NEC, additional findings explain the mechanisms that underlie the tissue death that is characteristic of this disease. Specifically, as noted above, TLR4-mediated bacterial signaling leads to increased mucosal injury and reduced mucosal repair, resulting in mucosal defects through which enteric organisms can readily translocate into the circulation in the setting of prematurity^{39,51}. Upon entering the circulation, bacteria then interact with TLR4 on the lining of the mesenteric blood vessels, which leads to a dramatic inhibition of expression of the major vasodilator eNOS and reduction of nitric oxide (NO), which leads to profound vasoconstriction⁵². The resultant dramatic decrease in intestinal perfusion explains the tissue necrosis that is the hallmark of severe NEC. Accordingly, strategies which augment nitric oxide signaling, such as provision of the nitric oxide precursor sodium nitrate, or treatment with sildenafil, restore mesenteric perfusion in experimental models and attenuate NEC severity⁵². These findings also provide insights into the protective mechanisms of breast milk, which was found to be especially enriched in nitric oxide precursor molecules as well as human milk oligosaccharides, both of

which augment intestinal perfusion, and prevent the deleterious effects of exaggerated bacterial-intestinal signaling in the premature host in the pathogenesis of NEC⁵².

CURTAILING BACTERIAL SIGNALING IN THE PREMATURE INTESTINE TO PREVENT NEC: AMNIOTIC FLUID, BREAST MILK AND PROBIOTICS.

The above findings provide evidence that exaggerated bacterial signaling plays a role in the development of NEC in premature infants, and suggest the possibility that strategies may exist which curtail the degree of TLR4 signaling, and limit the propensity for NEC development. In this regard, several groups have shown that amniotic fluid administration can limit NEC^{38,53–62}, which Hackam et al showed to be due to effects on inhibition of the degree of TLR4 signaling³⁸. It is noteworthy that as the intestinal mucosa of the developing fetus is bathed in swallowed amniotic fluid and TLR4 activation is restricted, the fetus is protected from unexpected activation of TLR4 by ascending bacteria in utero, which could otherwise lead to intestinal injury and premature birth. The protective effects of amniotic fluid were found to be attributable to the high concentrations of epidermal growth factor (EGF) which limit TLR4 signaling via activation of inhibitory p38 MAPK pathways³⁸.

In additional studies, breast milk was determined to serve as a powerful inhibitor of TLR4 signaling, again through the rich concentration of EGF that is secreted by the mammary gland into the breast milk particularly in the first weeks of life⁶³. Removal of EGF from breast milk or administration of exogenous mouse breast milk to mice lacking the EGF receptor significantly attenuated the protective benefits of breast milk, confirming that EGF is the key protective factor in breast milk responsible for TLR4 inhibition⁶³, and which may explain by extension the profound protection that breast milk offers against NEC development in premature infants.

Additional studies have shown that probiotic bacterial DNA can significantly inhibit TLR4 signaling in the developing intestine of mice and piglets, providing a potential explanation for the protective benefits of probiotics observed against NEC in infants³⁶. It is noteworthy that the DNA of probiotic bacteria – which differs structurally from mammalian DNA – was sufficient to inhibit TLR4, suggesting that live bacteria may not be required to achieve the benefit of probiotics⁶⁴. In exploring the mechanisms involved, probiotic bacterial DNA was found to activate TLR9, a homologous receptor to TLR4 which recognizes bacterial DNA, and inhibits TLR4 through activation of the inhibitory molecule IRAK-m^{36,64,65}.

Taken together, these findings provide evidence that while bacterial signaling may lead to NEC, several natural biological mechanisms exist to limit the degree of TLR4 signaling and thus attenuate bacterial recognition, leading to NEC protection. Such findings raise the possibility that pharmacologic approaches to limit TLR4 activation by endogenous bacteria may provide novel approaches to the prevention or treatment of NEC as explored further below.

PHARMACOLOGIC INHIBITION OF TLR4 FOR THE PREVENTION AND TREATMENT OF NEC

Having shown a critical role for bacterial signaling in the pathogenesis of NEC through activation of the LPS receptor TLR4, we and others have sought to identify whether pharmacologic inhibition of TLR4 may represent a novel approach for the prevention or treatment of NEC. To do so, the Hackam laboratory performed an *in silico* screen of potential binding partners to the LPS docking site within TLR4, based upon the recently published crystal structure of this molecule^{66,67}. This screen yielded many potential hits, of which approximately 80 were then verified using *in vivo* models of mouse endotoxemia. This secondary screen then confirmed a lead compound, which was provisionally entitled C34, which is a 2-acetamidopyranoside (MW 389) with the formula C₁₇H₂₇NO₉ that fits into the internal pocket of TLR4. C34 was found to inhibit TLR4 *in vivo*, and in human ileum *ex vivo* that had been resected from infants with NEC, suggesting the possibility of effectiveness in humans. Interestingly, structurally related molecules to C34 was shown to be present in breast milk, thereby suggesting an additional mechanism by which breast milk inhibits TLR4 activation and thus prevents NEC^{66,67}. Supplementation of infant formula with C34 prevented experimental NEC in animal models, raising the possibility that a clinical developmental strategy could be developed based upon TLR4 inhibition using compounds like C34.(reference)

POTENTIAL MULTI-AGENT APPROACHES FOR NEC PREVENTION AND TREATMENT

Based upon the above understanding of the role of bacterial signaling in the pathogenesis of NEC, a variety of potential molecular approaches could be proposed for the prevention or treatment of NEC, using a strategy of TLR4 inhibition. Such approaches may include inhibition of the upstream TLR4 signaling pathways using C34 or other novel and potentially more potent analogues, or the administration of probiotic bacteria or probiotic DNA, which serve to limit TLR4 activation via effects on TLR9. The administration of agents capable of bypassing the deleterious effects of exaggerated TLR4 signaling, which may include nitric oxide precursors to enhance intestinal perfusion, or growth factors that either promote intestinal healing or act to further limit downstream TLR4 signaling responses, could be utilized in combination as a supplement to human milk feeding. Such an approach would essentially represent “the surfactant of the gut” by developing a strategy capable of promoting an adaptive response to bacterial colonization of the premature intestine, and thus limit the deleterious effects of exaggerated bacterial signaling that can lead to the development of NEC.

SUMMARY

Necrotizing enterocolitis has caused significant morbidity and mortality in neonatal units for many years, and the historical context provides an interesting variety of scientific insights. As the pathogenesis of NEC has become better understood, our recent findings on the role of bacterial signaling in the pathogenesis of NEC suggest possible answers to the questions

posed above regarding the epidemiology of this disease. Specifically, it appears that the premature intestinal epithelium is at particular risk for the development of NEC in part due to the elevated expression of TLR4 within the premature as compared with the full term intestine, which is a consequence of the recently identified role for TLR4 in normal gut development, and its expression on the intestinal stem cells within the premature intestine. When the premature gut is colonized by bacteria in the postnatal period (particularly Gram-negative Proteobacteria), activation of TLR4 leads to mucosal injury, reduced mucosal repair, and bacterial translocation which then causes vasoconstriction, leading to the death of parts of the intestine, which characterizes NEC. Breast milk – a powerful protective agent for NEC – is endowed with molecules that inhibit TLR4 signaling, including growth factors such as EGF, but also novel oligosaccharides similar to our recently identified TLR4 inhibitor, C34. These factors lead us to speculate that differences in the susceptibility to the development of NEC between individual patients may lie in part in genetic alterations in TLR4 signaling pathways, or in those pathways which serve to normally restrain TLR4 signaling from leading to disease.

FUTURE CONSIDERATIONS

Based in part on early successes in the understanding of the role of bacterial signaling in NEC pathogenesis, there is reason to be optimistic that the field of NEC research is poised to enter a period of significant advancement towards the ultimate goal of developing a specific cure for this disease. To accomplish this goal, we will need to understand with greater accuracy which patients are most susceptible to the development of NEC; this will likely require the identification of specific genetic and molecular biomarkers. Such a strategy may allow physicians to tailor specific anti-NEC therapies to individual patients, such as providing anti-TLR4 agents for those with exaggerated TLR4 levels above a certain threshold. It is likely that additional emphasis on NEC prevention – perhaps through a greater understanding of how and when to feed premature infants, in combination with probiotic mediated manipulation of the microbial environment – may offer additional advances in the care of these fragile patients. Through careful and focused research, we hope to finally offer a specific cure to patients and families whose premature infants develop this devastating disease.

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Figure 1. The development of necrotizing enterocolitis requires TLR4 activation on the intestinal epithelium.

i-iv: representative H&E micrographs from sections of the terminal ileum in mice without (**i**) or with (**ii-iv**) NEC that are either wild-type (**i-ii**), globally deficient in TLR4 (**iii**), or lacking TLR4 on the intestinal epithelium (**iv**). Shown also is the expression by RT-PCR of iNOS and IL-6, and NEC severity in the indicated groups. Reproduced with permission from *Gastroenterology* 2012 Sep;143(3).

Figure 2. The development of necrotizing enterocolitis is associated with induction of Th17 cells and reduction in Tregs in the newborn intestine of mice and humans.

A-H: Representative confocal micrographs from sections of the terminal ileum in mice (**A-D**) and human infant (**E-H**) with or without NEC, immunostained for CD3 or CD4 as indicated. Characterization of the immune cell infiltrate in the lamina propria in mouse (**I**) and human (**J**) is shown. Scale bar = 10mm. **K-M:** qRT-PCR expression of the lamina propria CD4+ T cells in mice with and without NEC for the indicated lymphocyte transcription factors in murine CD4+ T cells. **N-R:** Flow cytometric analysis of Foxp3+ (**N-O**) or RORgt+ (**P-Q**) cells in the lamina propria of mice with and without NEC; quantification is shown in **R**. Reproduced from Journal of Clinical Investigation, 2016 Feb; 126(2):495–508.

Figure 3. Breast milk attenuates TLR4 signaling in vivo via EGFR.

A: Pseudocolor images demonstrating whole animal NF- κ B luciferase activity treated with either saline (**i**), LPS (5 mg/kg, 6 hr, **ii**), gavage pretreatment with breast milk (50 μ L/gram body weight, **iii**) and/or cetuximab (Cetux), an EGFR inhibitor (100 μ g/day for 3 days, **iv**) 1 h prior to LPS administration, quantification of images in Total Flux (photons/sec $\times 10^4$, **v**). **B:** IL-6 (**i**) and TLR4 mRNA expression (**ii**) for above groups. * $p < 0.05$ versus saline (white bar), ** $p < 0.05$ versus LPS, *** $p < 0.05$ versus LPS + breast milk (BM). Data are mean $\pm$ SD. Representative of at least three separate experiments with at least three mice per group. Reproduced with permission from Mucosal Immunol. 2015 Sep;8(5):1166–79.