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Mechanisms of Gut Barrier Failure in the Pathogenesis of Necrotizing Enterocolitis: Toll Like Receptors Throw The Switch

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Abstract

Necrotizing enterocolitis (NEC) is the leading cause of death from gastro-intestinal causes in premature infants, and its overall survival has not improved in the past three decades. While the precise cause of NEC remains incompletely understood, we and others have shown that a major predisposing factor in the development and propagation of NEC is a breakdown of the intestinal barrier which leads to bacterial translocation and systemic sepsis. In seeking to identify the causes involved, we and others have also determined that activation of the receptor for bacterial endotoxin, namely toll like receptor 4 (TLR4), is required for the development of intestinal barrier failure leading to NEC. We have also shown that the premature infant is endowed with strategies that can either limit or promote the extent of TLR4 signaling within the gut, which together determine the relative propensity with which NEC develops. In this review, we will highlight the evidence for TLR4 signaling in the pathogenesis of NEC through a survey of its effects on gut barrier failure. We will identify how TLR4 regulation within the gut can explain the unique susceptibility of the premature infant to the development of NEC, and will highlight how strategies to limit the degree of TLR4 signaling can serve as novel therapeutic approaches for this devastating disease.

Keywords

necrotizing enterocolitis; neonatal intestinal barrier; toll like receptor 4 regulation(TLR4); bacterial translocation

1. Introduction: a critical role for TLR4 in the pathogenesis of necrotizing enterocolitis

It is axiomatic that necrotizing enterocolitis (NEC) is both a devastatingly serious disease, and also one in which its very underpinnings remain mysterious. The disease develops quickly and without warning. Every clinician who takes care of neonates will attest to the

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typical scenario of a premature infant who is at first clinically stable, then subtly begins to manifest a series of symptoms that progress – often quite rapidly – from feeding intolerance, abdominal distention and bloody stools, to outright sepsis and hemodynamic instability. Every surgeon who operates on these fragile patients will have experienced that laparotomy reveals the presence of patchy necrosis of the intestine with a distribution and severity that may or may not match the clinical impression that was present preoperatively. A large number of studies have revealed that neither the type of surgery that is performed on patients with NEC, nor its timing, can significantly alter outcome in patients with this disease^{1–3}. These clinical impressions reveal the highly variable nature of patients with NEC, and also underscore the fact that if we are to improve the outcome of these young children, then a clearer understanding of the pathogenesis of NEC at the molecular level is required.

In seeking to understand the molecular events that lead to the development of NEC, we and others have discovered a critical role for the receptor for Gram negative bacterial lipopolysaccharide in its pathogenesis^{4–10}. The lipopolysaccharide receptor, called toll like receptor 4 (TLR4), was originally identified based upon its expression on leukocytes, and its function in host defense was discovered through its role in the activation of the pro-inflammatory transcription factor NFκB and the elimination of invading pathogens^{11, 12}. In a series of studies that were performed in 2004, we showed that TLR4 signaling can play important roles in the pathologic consequences observed in NEC (see below), suggesting but by no means proving that TLR4 is critical to NEC pathogenesis¹³. In further studies, Jilling and colleagues⁵ as well as our group⁴ showed that mice with inhibitory mutations in TLR4 were protected from the development of NEC. Further evidence that NEC development requires TLR4 activation is found in studies showing that the expression of TLR4 was significantly increased in the intestine of mice and humans with NEC as compared to controls without the disease⁴. While these findings did not identify the precise cellular compartment that was critical in the intestine, they provided important information regarding disease pathogenesis, by suggesting that a receptor for Gram negative bacteria – long known to be important in NEC – could be important in disease development. While these studies served to provide some insights into an important signaling process required for NEC development, they raised some intriguing and important questions. For instance, why would a receptor that is so crucial for host defense have a role in causing disease? And since the vast majority of NEC cases are seen in premature patients, could an understanding of TLR4 signaling in NEC pathogenesis provide any insights into the unique susceptibility of the premature infant for NEC development?

In the sections below, we will describe current research explaining how TLR4 signaling leads to the development of NEC, how an understanding of TLR4 can provide insights into the reasons why the premature infant is at risk for NEC, how the host curtails TLR4 signaling in order to restrain spontaneous NEC development, and how modulation of TLR4 signaling may be utilized therapeutically as a novel approach in the management of patients with NEC.

2. TLR4 activation leads to NEC through the induction of gut barrier failure

Under normal conditions, microbial products are shielded from the immune system of the host through a complex and tightly regulated series of cellular and molecular processes that together serve to limit bacterial translocation. A major determinant of the ability of the host to prevent bacterial translocation across the gut is an intact crypt-villus axis, in which healthy intestinal epithelial cells originate from progenitor cells within the intestinal crypts, and then migrate along the villi where they undergo apoptosis and are then shed into the lumen, 3–5 days later^{14, 15}. While this process is of great importance to the homeostasis of the small intestine – which is the site that is most severely and most frequently affected in

patients with NEC – the cycle of proliferation, migration and apoptosis is of importance in the maintenance of homeostasis throughout the entire gastrointestinal tract. There is ample evidence that prior to the development of systemic sepsis, or perhaps as a direct consequence of it, there is significantly impaired breakdown of the intestinal barrier that leads to translocation of bacteria and the development of a systemic inflammatory response^{16, 17}. Given evidence that TLR4 signaling is required in the pathogenesis of NEC – a disease that is characterized by gut barrier failure – these findings suggest the possibility that TLR4 regulation could play some role in maintaining the integrity of the intestinal barrier, and that exaggerated TLR4 signaling – as we observed in mouse and human NEC – could lead to disease development by disrupting the intestinal barrier.

In support of the notion that exaggerated TLR4 signaling could lead to a breakdown of the intestinal barrier, we have shown that TLR4 is expressed in the intestinal epithelium, as well as in cultured enterocytes, and that TLR4 activation leads to a significant loss of intestinal epithelial integrity due to a profound increase in enterocyte apoptosis, which leads to mucosal disruption^{4, 18, 19}. Under normal conditions, mucosal disruption induces a rapid and largely effective healing response that occurs in two distinct phases. In the first phase, termed *intestinal restitution*, healthy enterocytes migrate into the site of mucosal disruption from sites adjacent to the injured area. In the second phase, termed *enterocyte proliferation*, new enterocytes are generated from stem cells located within the intestinal crypts. Importantly, TLR4 activation within enterocytes was found to negatively regulate both phases of these healing responses. Specifically, TLR4 activation was shown to prevent the normal migration of enterocytes into a site of mucosal disruption¹³. The inhibition of enterocyte migration by TLR4 activation was found to be mediated through a marked increase in the degree of force with which the enterocyte adhered to the underlying surface, which effectively eliminated further cell movement¹³. In turn, the increased adhesiveness occurred through both an increase in the expression of matrix binding molecules on the surface of the enterocyte – called integrins²⁰ – as well as through an increase in the expression and function of small molecules that mediate cell-matrix binding, namely focal adhesion kinase⁴. By interfering with the exaggerated release of integrins, and by blocking the increase in focal adhesion kinase, the inhibition in enterocyte migration could be reversed, and mucosal healing could occur normally.

In addition to an inhibition in enterocyte migration, the increase in TLR4 signaling also led to a marked reduction in enterocyte proliferation at the base of the intestinal crypts, resulting in a net loss of available enterocytes for healing to occur^{18, 21}. In seeking to understand how TLR4 activation could lead to an inhibition of enterocyte proliferation, we further demonstrated that TLR4 plays a critical role in the normal regulatory signaling pathway that determines the degree of cell division within the small intestine, namely the Wnt- β -catenin signaling pathway, and that TLR4 activation inhibits β -catenin signaling through upregulation of the inhibitory kinase GSK3 β . Moreover, by restoring intact β -catenin/Wnt signaling in the premature intestine, the negative effects of TLR4 on enterocyte proliferation were reversed, and the severity of NEC was significantly reduced in the face of TLR4 activation. Taken together, these findings provide insights into how TLR4 activation leads to barrier failure and the development of NEC, and lead us towards a unifying understanding of the development of mucosal inflammation in this disease, as will be provided below.

3. What is the cellular site of TLR4 signaling in the gut?

The findings described above indicate that TLR4 signaling plays a key role in the pathogenesis of NEC. As stated earlier, TLR4 was identified as an immune receptor on hematopoietic cells, where it serves an important role in host defense by recognizing and mounting a response to Gram negative bacterial endotoxin. TLR4 was shown to also be

expressed on intestinal epithelial cells^{22, 23}, where its activation not only leads to an increase in NFkB by these epithelial cells, but also to an increase in apoptosis as well as a reduction in migration and proliferation, as described above. These findings raise the important question of the cellular site at which TLR4 can signal in the pathogenesis of NEC – whether on the intestinal epithelium directly, or indirectly via effects on hematopoietic cells of the immune system. To resolve these possibilities, and to determine directly whether TLR4 signaling on the intestinal epithelium versus leukocytes is critical in NEC pathogenesis, we deleted TLR4 specifically from the intestinal epithelium, and subjected these mice to a well validated model of NEC, that involved four days of formula gavage every 3 hours, as well as twice daily hypoxia treatment. Importantly, newborn mice lacking TLR4 on the intestinal epithelium were significantly protected from the development of NEC as compared to the wild-type littermates that expressed TLR4 on the intestinal epithelium, and in fact, were as protected as the mice in which TLR4 was deleted from all of the cells in the host. Moreover, as compared with wild-type mice, newborn mice lacking TLR4 on the intestinal epithelium showed normal proliferation and reduced enterocyte apoptosis when subjected to experimental NEC²⁴. These data demonstrate that TLR4 signaling on the intestinal epithelium – as opposed to other cells – is critically important in NEC pathogenesis, and further, that TLR4 signaling on the intestinal epithelium is important in the regulation of intestinal injury and repair in disease pathogenesis. We will next consider the clinical implications of TLR4 signaling on the intestinal epithelium, by evaluating first whether these findings can explain perhaps why the premature infant is particularly susceptible to NEC development.

4. Can an understanding of TLR4 signaling within the intestinal epithelium explain the unique susceptibility of the premature infant to the development of NEC?

It has been long understood that the development of NEC strongly favors the premature infant, although the precise explanation for the particular prevalence of NEC in this population remains incompletely explained. Previous authors have provided evidence that the premature infant is at risk for NEC through the following characteristics: (1) a general impairment of the immune system; (2) decreased secretory IgA with diminished phagocytosis, which could lead to impaired microbial clearance and bacterial overgrowth leading to NEC; (3) impaired epithelial tight junctions and reduced mucin, which lead to increased bacterial permeability of the intestinal barrier, and could result in an enhanced septic response; and (4) non-defined factors within formula leading to mucosal injury in the immature intestine and the development of NEC. While these factors are each likely to play some role in NEC development, each factor alone is insufficient to explain the major inflammatory response that is observed within the intestinal mucosa in infants with NEC, and none of these factors can provide a complete explanation as to why the premature infant is at particular risk for disease development. In order to further address this gap in our knowledge, and given our finding that TLR4 signaling within the intestinal epithelium, as opposed to within other cells, is required for NEC development, we investigated whether the pattern of TLR4 signaling or expression could play a role in determining the particular susceptibility of the premature infant to NEC. Recently, we have identified that the premature infant gut is characterized by significantly increased expression of TLR4 as compared with the full term infant, in both mice and humans²⁵. We also demonstrated that the expression of TLR4 within the gut normally drops precipitously at the time of full term birth, while factors that are associated with NEC development – including hypoxia and the exposure to high concentrations of LPS – significantly increase TLR4 expression within the intestinal epithelium²⁵. In a series of additional studies, we demonstrated that TLR4 is not only expressed at high levels within the intestine of the premature (i.e. fetal) as compared

with the full term neonate, but it is also functionally active²⁵. In support of this concept, we performed experiments in which we delivered the TLR4 agonist LPS directly into the intestinal lumen of the developing mouse using ultrasound guided microinjection, followed by analysis of TLR4 signaling in the intestinal mucosa using quantitative RT-PCR. Based upon these findings, we now propose that in the setting of prematurity, TLR4 levels are high (for reasons that will be explained below), but normally decrease shortly after birth to levels that allow for the normal adaptation of the intestinal mucosa to bacteria. However, in the setting of prematurity, levels of TLR4 remain high, and are maintained at high levels after birth due to the normal ontogeny of gene expression. When the premature intestine with accompanying elevated expression levels of TLR4 becomes colonized in the neonatal intensive care unit, exaggerated TLR4 signaling occurs, which leads to increased enterocyte apoptosis and delayed mucosal repair, which together favors bacterial translocation and the development of systemic sepsis and NEC (see Figure 1). These findings provide a conceptual framework upon which to study NEC development, and may also provide additional insights into the increased susceptibility of the premature infant to NEC as compared to full term infants. It is noteworthy that in full term infants, TLR4 expression in the gut is either markedly reduced, or confined to the distal intestine, which perhaps non-coincidentally correlates with the pattern of NEC observed in full term infants.

These studies serve not only to shed light on the particular susceptibility of the premature infant to the development of NEC, but also raise two additional questions. First, why is TLR4 expression elevated during prematurity in the first place, and second, what factors if any curtail TLR4 signaling in the host to limit the propensity for spontaneous NEC development? These questions will be addressed in the subsequent sections.

5. TLR4 expression in the developing gut regulates normal intestinal epithelial differentiation

One of the central questions raised from the work described above, in which TLR4 signaling and expression are elevated in the premature host^{4, 6, 26}, involves why TLR4 would be elevated in the premature bowel in the first place? As a corollary, why would the host express a receptor on the intestinal surface that has deleterious effects? In order to reconcile this apparent conflict, we note that TLR4 was first identified as the receptor Toll in the fruit fly, where it was initially found to play a critical role in development of the fly^{27, 28}, and subsequently shown to mediate innate immunity²⁹. These early findings therefore raised the exciting possibility that perhaps the elevated expression of TLR4 within the intestinal mucosa of the premature mouse and human could reflect a role for TLR4 in normal gut development. To address this possibility directly, we evaluated in detail the pattern of intestinal development in mice that lacked TLR4 in the intestinal epithelium, and compared this developmental pattern to mice in which TLR4 was present in the intestinal epithelium. These studies revealed that in mice in which TLR4 was lacking, there was a striking effect on the differentiation of the intestinal epithelium, characterized by a marked increase in goblet cells, as compared to mice in which TLR4 was present²⁴. Moreover, the lack of TLR4 in the premature host – in which goblet cells were increased – conferred an advantage to the premature intestine, as the increase in goblet cells and the subsequent increased release of protective mucin – was found to exert a dampening effect on NEC development²⁴. In fact, when premature mice were administered an agent that increased goblet cells in the bowel, they were protected from the development of NEC²⁴. These results may explain the reasons for which TLR4 expression is elevated during development, particularly at a time when no expected role in host defense is anticipated. Moreover, these findings also indicate how the infant, when born at a premature state in which TLR4 expression levels are high through their parallel role in regulating normal gut differentiation, could lead to NEC through persistent and exaggerated TLR4 signaling in the post-natal period.

6. How the host curtails TLR4 signaling in the gut in order to restrain spontaneous NEC development

i. The role of TLR9 activation by microbial DNA in restraining TLR4 signaling in the gut

As mentioned above, the development of NEC is dependent on the activation of TLR4 within the intestinal epithelium. Moreover, TLR4 expression is significantly increased in the premature as compared with the full-term intestine, which may explain in part the unique susceptibility of the premature infant to NEC development. However, since all premature infants are expected to express high levels of TLR4 and to be exposed to colonizing microbial flora, yet only a fraction of these individuals will ever develop NEC, it stands to reason that there would be a negative feedback system that curtails TLR4 activation in the premature gut. Further, in the absence of such a system of negative feedback, one would expect to observe the development of spontaneous NEC, and the fact spontaneous NEC is rarely seen argues in favor of a negative feedback loop. In order to explore this possibility directly, we turned to evidence that argues that in addition to expressing high concentrations of lipopolysaccharide, the bacteria within the intestinal lumen are rich in DNA. Of note, the DNA of bacteria differs from the DNA of mammals in that the former is enriched with CpG-DNA motifs and is also largely de-methylated³⁰. Bacterial DNA is recognized by a unique signaling receptor called toll like receptor 9 (TLR9), which is a close homologue of TLR4, yet unlike TLR4 does not recognize LPS³¹. In a variety of studies, TLR9 activation with CpG-DNA was shown to limit the extent of experimental colonic inflammation³². We extended these observations by showing that in the setting of NEC, TLR9 expression was reduced, which was particularly dramatic as TLR4 expression was increased in a reciprocal manner^{25, 33}. Moreover, activation of TLR9 with CpG-DNA was found to inhibit TLR4-mediated signaling in enterocytes, in a mechanism that was dependent upon the inhibitory signaling molecule IRAK-m²⁵. The administration of CpG-DNA was also found to cause a re-distribution of the inhibitory kinase IRAK-M to a subcellular region within the Golgi apparatus, the location at which TLR4 is known to respond to LPS in enterocytes³⁴. Importantly, activation of TLR9 in vivo with the administration of CpG-DNA to newborn mice significantly reduced the incidence of experimental NEC, through reduced enterocyte apoptosis and bacterial translocation²⁵, and indicates that the prematurely born infant may be at risk for the development of NEC in part due to the persistently elevated functional TLR4 in the absence of counter-regulatory TLR9. These findings together provide evidence that the activation of TLR9 within the intestinal mucosa can serve to dampen TLR4 signaling and limit the predisposition to develop NEC, and suggest that the administration of TLR9 agonists including CpG-DNA can serve as a novel therapeutic approach for patients with this disease. We note that in these studies, the administration of CpG-DNA did not reduce the inflammatory response to LPS in isolated macrophages, a finding consistent with previous studies that identify CpG-DNA as an “activator” rather than an “inhibitor” of myeloid cells³⁵. Given that the net effect of the administration of CpG-DNA was to reduce the overall development of systemic inflammation in experimental NEC, these findings raise the exciting possibility that administration of CpG-DNA may exert a relative intestinal-specific effect without causing broad myeloid cell immunosuppression which would be important in the premature infant who may also have additional defects in host immunity.

The finding that the administration of the TLR9 agonist CpG-DNA significantly decreases the severity of NEC could provide insights into recent advances in the expanding field of probiotics as it relates to protection from NEC. In this respect, several recent randomized controlled trials have demonstrated that administration of probiotic bacteria can decrease the incidence or severity of NEC^{36–38}, and similar results have been observed in animal studies³⁹, suggesting a potential benefit of probiotics for NEC⁴⁰. Importantly, Raz and colleagues have shown that TLR9 signaling mediates the anti-inflammatory effects of

probiotics in an experimental model of experimental colitis in mice⁴¹. In view of the finding that TLR9 activation with bacterial DNA can limit TLR4 signaling, it is tempting to speculate that the protective effects observed in human NEC that were attained through the administration of probiotics may have been achieved in part through the activation of TLR9 in the human intestine by CpG-DNA contained within the probiotics themselves, and that this protective effect may have been achieved through a limitation in TLR4 signaling. Further clinical and laboratory studies will be required to evaluate this possibility further, and to determine the relevant safety parameters.

ii. The role of nod like receptor 2 activation in restraining TLR4 signaling in the gut

In additional studies, we evaluated whether other bacterial components that are present within the intestinal lumen could also limit the degree of TLR4-mediated intestinal barrier dysfunction that leads to the development of NEC. In this regard, we note that bacteria within the intestine express ligands that are recognized not only by receptor of the toll like receptor family, but also by receptors of the NOD receptor family, which stands for nucleotide-binding oligomerization domain-containing proteins⁴². In particular, NOD2 is the receptor for the ligand muramyl-dipeptide (MDP) that is present on many Gram positive bacteria, and whose activation leads to the recruitment of a platform of proteins called the inflammasome, resulting in the activation of caspases-1 and the release of IL-1 β and IL-18⁴³. While it is primarily used to recognize bacterial peptides by immune cells, NOD2 has also been shown by our group and others to be expressed in intestinal epithelial cells⁴⁴, and mutations in NOD2 mutations are known to be associated with familial Crohn's disease^{45, 46}. In a series of studies, we extended these findings and demonstrated that NOD2 activation in the newborn intestine inhibits TLR4 signaling within enterocytes⁴⁴. NOD2 activation was found to inhibit TLR4 in enterocytes but not macrophages, and was found to reverse the deleterious effects of TLR4 activation on intestinal mucosa injury and repair⁴⁴. Further, the failure of appropriate NOD2 signaling within the enterocyte was found to be a major factor that leads to the development of NEC, through increased TLR4-mediated enterocyte apoptosis. In seeking to define the mechanisms involved, we used protein expression array technology and identified an unexpected mechanistic role for the oncogenic protein SMAC-diablo in mediating this effect. These studies not only revealed a novel mechanism by which the interplay between two innate immune receptor within the enterocyte – namely NOD2 and TLR4 – drive the extent of intestinal injury in the pathogenesis of NEC, but also raise the possibility that a therapeutic approach based upon the ability of MDP-mediated signaling to occur could be used clinically in patients with NEC. Moreover, the fact that NOD2 levels were decreased in NEC patients as compared with controls suggests the possibility that the ability to screen for the expression of NOD2 may be used to identify premature infants who are at risk for NEC development, in a similar manner as is being performed in patients at risk for IBD.

iii. Intracellular protection against TLR4 signaling by Hsp70

Several investigators have identified that mammalian cells contains a variety of stress response proteins, collectively termed heat shock proteins (Hsp's) based upon the fact that their expression is potently induced by heat, which serve to mitigate against external stressors and thus to promote cell survival. Importantly, we identified that in both mice and humans with NEC, the expression of the most widely expressed heat shock protein, namely Hsp70, is significantly reduced in the intestine when compared to healthy controls¹⁹. In additional studies we showed that the activation of TLR4 within enterocytes served to increase the expression of Hsp70, indicating that Hsp70 could serve as a mechanism of auto-inhibition of TLR4 signaling in the gut. Indeed, the upregulation of Hsp70 was able to reduce TLR4 signaling, as Hsp70 caused the ubiquitination and subsequent proteosomal degradation of TLR4¹⁹. These findings pointed to the fact that a loss of Hsp70 protection

from TLR4 signaling could lead to NEC, and in support of this possibility, mice that over-expressed Hsp70 within the intestine were protected from NEC development while mice that lacked Hsp70 showed significant increased NEC severity. Most importantly perhaps, pharmacologic upregulation of Hsp70 using the drug Celastrol limited the extent of TLR4-induced inflammation, reversed TLR4-induced enterocyte apoptosis, and both prevented and treated experimental NEC. Thus, these findings show how a novel TLR4 regulatory pathway exists within the newborn gut that may be pharmacologically activated to limit NEC severity.

7. TLR4 signaling exerts different roles in inflammatory bowel disease and NEC

The current work highlights the importance of TLR4 activation within the intestinal mucosa in the pathogenesis of NEC. However, it should be mentioned that the role of TLR4 in the pathogenesis of NEC may not apply to other disease of intestinal inflammation including ulcerative colitis and Crohn's disease, in which TLR4 is known to play a protective role, at least in experimental models^{47, 48}. Several reasons may account for the apparent discrepancy between the roles for TLR4 in NEC versus colitis. In NEC, it is apparent that TLR4 activation leads to intestinal injury in a well-defined and physiologically relevant context, namely the newborn small intestine, as TLR4 activation with LPS leads to increased enterocyte apoptosis in the terminal ileum of newborn mice but not adult mice, and in the small intestine but not the newborn colon⁴⁴. Further, reports that demonstrate a protective role for TLR4 in models of colitis have typically been based upon the use of global TLR4 knockout mice, in which TLR4 signaling is disrupted in enterocytes as well as T-cells and myeloid cells. We have recently shown that TLR4 signaling within the enterocyte itself is important for the induction of intestinal injury leading to NEC, as described above. It is therefore reasonable to conclude that the protective effects attributed to TLR4 signaling in the gut by previous authors may reflect in part the mitigating effects of TLR4 signaling on other cells. In support of this possibility, Fukata and colleagues have recently shown in an elegant study using chimeric mice that TLR4 signaling in colonic epithelial cells worsened intestinal inflammation⁴⁹. These findings argue that the effects of TLR4 in the development of intestinal inflammation are strongly influenced by a variety of factors, including the effector cells involved, developmental factors and involved region of the intestine. The precise effects therefore of Hsp70 or other TLR4 regulatory pathways at these varying stages of development and within these different cell types remains to be explored in further detail, but are likely to provide important clues to the underlying causes of these diseases.

8. Amniotic fluid regulates TLR4 signaling within the gut of the developing fetus and can attenuate the severity of experimental NEC

In studies described above, we have reported that the expression of TLR4 within the intestine of the premature infant is significantly greater than that of the full term infant, and moreover, that the expression rises during development and then falls shortly after birth. During development, the developing fetus continuously swallows amniotic fluid, and as a result, the fetal intestine becomes exposed to amniotic fluid as a consequence of the in utero environment. In recent studies, we determined that the amniotic fluid can serve to limit the extent of TLR4 signaling within the intestine⁵⁰. Moreover, amniotic fluid was found to block TLR4 signaling in cultured enterocytes that had been exposed to bacterial products⁵⁰, resulting in a marked reduction in the degree of pro-inflammatory cytokine release. Given that amniotic fluid can block TLR4 signaling, and given that premature birth is characterized by an abrupt and necessary lack of exposure to amniotic fluid as a natural consequence of early delivery, we investigated whether the administration of amniotic fluid itself could

prevent the development of NEC. In support of this possibility, we recently determined that the administration of amniotic fluid to mice could reduce the incidence and severity of experimental NEC, and that the absence of the anti-TLR4 signaling effects of the amniotic fluid places the preterm infant at risk for NEC development. In identifying the specific factors within the amniotic fluid that could inhibit TLR4 within the intestinal epithelium of the newborn host, we further identified that the presence of the hormone epidermal growth factor within the amniotic fluid was largely responsible for the inhibitory effects of amniotic fluid on TLR4 signaling, as demonstrated by the fact that mice lacking the EGF receptor were not protected from the development of NEC after being administered amniotic fluid, while the administration of EGF not only inhibited the development of NEC – a finding consistent with prior studies – but also limited the degree of TLR4 signaling. Taken together, these findings illustrate that in the absence of the TLR4-inhibitory properties of amniotic fluid in the postnatal period, the premature infant may exhibit unrestrained TLR4 signaling within the intestinal mucosa upon its colonization by microbes within the neonatal intensive care unit, a situation that leads to NEC through disruption of the gut barrier. Given that several groups have reported that the administration of EGF or its homologue HB-EGF can treat experimental NEC^{51, 52}, it is tempting to speculate that these prior studies may be explained through an inhibitory effect of these growth factors on TLR4 signaling, in addition to their restorative and reparative effects on the intestinal mucosa.

9. Model: TLR4 signaling is required for gut barrier failure in the pathogenesis of NEC

Based upon the studies described above, we now propose the following model to explain the pathogenesis of necrotizing enterocolitis based upon our current understanding of the expression and function of TLR4 signaling in the intestinal mucosa of the premature infant (Figure 1). Under normal conditions, the full term infant is characterized by low levels of TLR expression, which in the setting of microbial colonization does not elicit a particular pro-inflammatory response, which allows for the normal adaptation of the small intestine to the colonizing commensal microbes. By contrast, under conditions of prematurity, TLR4 expression remains high, as a consequence in part of the developmental role of TLR4 in utero, in which TLR4 is important for the normal differentiation of the intestinal epithelium. When the premature infant intestine is colonized by flora within the neonatal intensive care unit, and in the absence of host-derived factors that normally serve to restrict TLR4 signaling including TLR9, NOD2 and Hsp70, TLR4 signaling within the gut becomes particularly exaggerated, which has three effects [1] an increase in the expression of NFkB and pro-inflammatory mediators; [2] an increase in cell death via TLR4-mediated apoptosis of the intestinal epithelium; and [3] a significant reduction in the ability of the intestinal mucosa to undergo spontaneous repair, due to the combined effects of reduced enterocyte proliferation and enterocyte migration. The net effect of these TLR4-mediated effects on the intestinal mucosa is to cause significant gut barrier failure, which leads to bacterial translocation, the development of systemic sepsis, and the clinical and pathologic features of NEC. Based upon these findings, we propose that an understanding of the early TLR4 mediated signaling events will not only allow for a greater understanding of the pathogenesis of NEC, but will also offer new and innovative approaches to its treatment.

10. Future directions: Development of novel biological therapeutics for necrotizing enterocolitis based upon an understanding of TLR4 signaling

Current approaches for the treatment of infants with NEC have included either non-specific management of feedings, broad spectrum antibiotics, and the judicious use of surgery to remove necrotic bowel. These approaches have been optimized over time, and are effective

in a large number of infants with NEC. However, the disturbing reality is that the overall survival of infants with NEC has not changed in the past quarter century. This statistic is a reflection of a lack of a complete understanding of the precise cellular mechanisms that lead to the development of NEC, and the lack of available precise biologic approaches to interfere with this pathogenesis. Given research that has shown that the development of NEC reflects in part a response to exaggerated TLR4 signaling in the intestinal mucosa, we now posit that the future of NEC therapy may involve cellular and molecular approaches to limit the extent of TLR4 signaling. Some of these approaches have been described above, and each shares the properties of effectively inhibiting TLR4 signaling within the gut. Importantly, each approach has effectively been shown to be able to limit the degree of severity of experimental NEC in mouse models, and include [1] the administration of the TLR9 agonist CpG-DNA, [2] the administration of the NOD2 ligand muramyl-di-peptide, [3] the administration of Celastrol, which increases Hsp70 within enterocytes; and [4] the administration of amniotic fluid, thereby partially restoring the in utero environment of the premature infant, and allowing for greater adaptation to the post-natal environment. It is possible that future treatment of children with NEC will include some or all of these agents in varying combinations, or through the use of other agents that may specifically and safely limit the degree of TLR4 signaling in the premature gut. Through these studies, it is our hope, and indeed the hope of all who work in the field of NEC and who care for infants with this disease, that these tiny patients be seen as they have never been seen before – as curable.

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Figure 1. The role of TLR4 activation on enterocytes in the pathogenesis of NEC

As described in the text, the lipopolysaccharide receptor toll like receptor 4 (TLR4) is expressed on the intestinal epithelium. In the premature host, the expression and signaling capacity of TLR4 are greatly exaggerated, due in part to the newly established role for TLR4 in regulation of intestinal differentiation. Upon colonization of the host by microbes, TLR4 activation within the gut of the premature infant results in damage to the intestinal barrier, through the combined effects of increased apoptosis and reduced proliferation and migration. These effects lead to bacterial translocation, the development of a systemic septic response, and the clinical manifestations of NEC. The host is endowed with counter-regulatory strategies that together serve to limit the extent of TLR4 signaling, and include toll like receptor 9 (TLR4), nod like receptor 2 (NOD2), as well as amniotic fluid, that limits the extent of TLR4 signaling in enterocytes via the effects of epidermal growth factor receptor (EGFR) activation. The balance between TLR4 activation and these counter-regulatory strategies is thought to play a pivotal role in the predisposition to NEC development.