Published in final edited form as:

Pathophysiology. 2014 February ; 21(1): 55–65. doi:10.1016/j.pathophys.2013.11.010.

Inflammatory signaling in NEC: role of NFkB and cytokines

Catherine J. Hunter, MD⁺ and **Isabelle G. De Plaen, MD**

⁺Assistant Professor of Pediatric Surgery, Department of Surgery, Division of Pediatric Surgery, Northwestern University Feinberg School of Medicine, Ann and Robert H. Lurie Children's Hospital of Chicago

Associate Professor of Pediatrics, Department of Pediatrics, Division of Neonatology, Northwestern University Feinberg School of Medicine, Ann and Robert H. Lurie Children's Hospital of Chicago, 225 E Chicago Ave, Box 45, Chicago, IL 60611, Tel: (773) 755-6379

Introduction

The pathogenesis of necrotizing enterocolitis (NEC) is complex and the exact etiology remains unknown. Clinically, the presentation and progression of NEC is variable and often difficult to predict. The majority of affected infants (>90%) are premature, and the following factors may play a role in NEC pathogenesis: 1) The immaturity of the intestinal barrier, the mucus layer, decreased Immunoglobulin A (IgA) and defensins; 2) an abnormal intestinal capillary blood flow due to the incomplete development of the intestinal microvasculature or due to the immature regulation of its vascular tone, causing insufficient substrate and O₂ delivery to the intestinal epithelial cells; 3) abnormal bacterial colonization of the enteric tract triggering a mucosal pro-inflammatory response; and 4) immaturity of the immune system preventing normal control and killing of microbes, allowing them to penetrate the epithelium. Concomitantly, this immature immune system mounts an excessive production of inflammatory mediators which cause the recruitment of inflammatory cells such as neutrophils and subsequent tissue injury and necrosis.

In order to investigate the pathogenesis of NEC, correlative studies have been conducted measuring different inflammatory mediators such as cytokines in the plasma or in the tissues resected from patients with NEC. However, these tissues are obtained at late stages of the disease when these are commonly necrotic, and therefore may not yield information about the early pathogenic events leading to NEC. As mechanistic studies obviously cannot be conducted in humans, animal models have been used. Studies on rats and mice have contributed to the discovery of several potentially important inflammatory mediators in the pathogenesis of NEC. In this chapter, the current evidence for the role of these inflammatory

© 2013 Elsevier Ireland Ltd. All rights reserved.

Correspondence to: Isabelle G. De Plaen.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

mediators is presented and a current unifying hypothesis regarding NEC pathogenesis is proposed.

Initiation of the inflammatory cascade: Bacteria– Lipopolysaccharide- Toll-like receptors

During normal term birth, the neonatal intestine is exposed to bacteria present in the maternal birth canal and the environment, and colonization takes place. Breast milk feedings promotes the development of a rich balanced microflora and the specific growth of probiotic species including *bifidobacteria* and *lactobacillus*, which have been shown to have many protective properties for the neonatal intestine. Normal colonization is altered in premature infants which were found to have fewer species but more aggressive bacteria¹. This is facilitated by prolonged periods of fasting, lack of breast milk, the use of antibiotics and anti-acids² and the NICU environment. In addition, bacterial overgrowth is common in premature infants, facilitated by immaturities of intestinal digestive and motility functions, the intestinal barrier and the innate immunity. There is evidence that bacteria may play a role in the pathogenesis of acute intestinal injury as germ-free rats do not develop acute intestinal injury after platelet-activating factor (PAF) administration³. The intestinal microflora found in the early stage of human NEC was associated with a higher proportion of gram-negative bacteria⁴. In addition to other microbial products, called microbial-associated molecular patterns (MAMPs), gram-negative bacteria contain lipopolysaccharides (LPS or endotoxin) on their outer membranes, which is a potent activator of the host immune response. LPS is a potent activator of the transcription factor nuclear factor- κ B (NF- κ B)⁵, inducing the production of many cytokines, including IL1, IL6, chemokines and TNF⁶ and of PAF⁷, which all amplify the inflammatory response. Several pieces of evidence suggest that LPS may play a central role in NEC: 1) patients with NEC have been found to have higher levels of LPS in their plasma⁸; 2) intraperitoneal injection of LPS to rats and mice induces intestinal injury and shock⁹; 3) LPS is the ligand for toll-like receptor-4 (TLR-4), which has been found to mediate NEC^{10,11}.

TLRs are pattern-recognition receptors for MAMPs present on most cells. In humans, 10 different TLRs have been identified to date. The activation of TLR4 has been shown to contribute to NEC^{10,11}, while TLR9, a cell receptor for unmethylated CpG dinucleotides originated from bacterial DNA, has been shown to be protective¹². TLRs and their roles in the pathogenesis of NEC are discussed in chapter 3 of this issue.

MAMPs, generally from commensal bacteria, interact with TLRs located on the intestinal epithelium. This promotes epithelial cell proliferation, IgA production, tight junction integrity and antimicrobial peptide production, which all help to maintain a healthy intestinal barrier¹³. However, when MAMPs interact with TLRs present on underlying lamina propria immune cells, a pro-inflammatory response can be triggered¹³. The location of these interactions may influence the response: Apical exposure of intestinal epithelial cells (IECs) to CpG-DNA inhibits NF- κ B activation, while IEC basolateral exposure leads to its activation¹⁴. This suggests that those commensal bacteria unable to cross the barrier, thereby remaining on the apical site, elicit a homeostatic, anti-inflammatory response¹³.

while invasive bacteria which penetrate the epithelial barrier trigger NF- κ B activation and a pro-inflammatory response.

NF- κ B

Many pro-inflammatory cytokines, chemokines and leukocyte adhesion molecules^{15, 16} are upregulated during inflammation via the activation of the transcription factor NF- κ B, which consists of five subunits (p50, p65, p52, cRel and RelB). These subunits homo- or heterodimerize to form active NF- κ B^{15, 16}. p50-p50 and p50-p65 are the NF- κ B dimers mostly found in intestinal tissues^{17, 18}. NF- κ B is constitutively present in the cytoplasm of most cells, in an inactive state, as it is bound to inhibitory proteins I κ Bs (Fig. 1). When the upstream IKK complex¹⁵ [which consists of two catalytic subunits, IKK α and IKK β and a regulatory component, NEMO (NF- κ B essential modulator)¹⁹] is activated via TLR stimulation, it phosphorylates I κ B. I κ B is then ubiquitinated and degraded by the 26S proteasome, leaving NF- κ B free to translocate to the nucleus and to regulate the gene transcription of many inflammatory mediators^{15, 19}.

NF- κ B has been found to be constitutively present at low levels in the intestine of adult rats¹⁷ and to be activated in a model of acute bowel injury induced by PAF¹⁷. In addition, our lab has shown that the constitutive activation of NF- κ B in the intestine appears at 20 days of gestation in fetal rats (total length of gestation: 21 days)²⁰.

While physiological activation of NF- κ B has a protective role in the intestine, excessive activation may be detrimental and contribute to injury. Breast milk inhibits NF- κ B activation by increasing the production of I κ B α in IECs²¹ and probiotics inhibits NF- κ B activation through the proteasome²². Subsequent attenuation of NF- κ B activation in IEC may play a protective role against NEC^{21, 22}. Also, in a murine model of intestinal ischemia-reperfusion, when NF- κ B activation was blocked in IEC by IKK β deletion, the systemic inflammation and multiorgan dysfunction was ameliorated²³. However, in other *in vivo* models, the activation of IKK β and NF- κ B in IECs has been shown to be protective and to limit intestinal mucosal damage^{23–25}. Therefore, NF- κ B in IECs may play both protective and detrimental roles in the intestine.

NF- κ B activation and cytokine production are increased in immature IECs in response to flagellin²⁶ and in immature inflammatory cells in response to TNF- α ^{27, 28}, compared to mature cells. Also lower levels of specific I κ B genes were found in fetal enterocytes compared to adult cells²⁶.

Taken together, these data suggest that NF- κ B activation may be developmentally regulated.

NF κ B has been found to be strongly activated in the intestine of newborn rats at birth²⁰. After breast milk feeding this activation is downregulated within 24 hours²⁰. This contrasts with pups exposed to a NEC protocol in whom NF κ B remained elevated²⁰ and endogenous NF- κ B inhibitory proteins I κ B α and I κ B β were decreased²⁰. In neonatal rats, TLR-2 staining and NF- κ B activation in IEC correlated with NEC severity, IEC apoptosis and impaired proliferation²⁹. Our laboratory has shown that neonatal rats treated with NEMO-binding domain peptide (NBD peptide), a specific NF- κ B inhibitory peptide, were less

susceptible to bowel injury and had decreased mortality when exposed to the NEC model ²⁰, suggesting a central role for NF- κ B in NEC. NBD was shown to decrease LPS-induced chemokine CXCL2 (or MIP-2) gene expression in IECs³⁰ and LPS-induced interleukin-1 β (IL-1 β), IL-6 and TNF-alpha gene expression in macrophages in vitro (J774.1)³¹.

While NF- κ B activation is critical for host immunity against pathogens ³², an exaggerated and prolonged NF- κ B activation in premature infants may lead to an increased pro-inflammatory response with excessive levels of cytokines, contributing to intestinal tissue injury in NEC ²⁰. Although there have not been any human studies looking at inhibiting cytokines in NEC, many clinical trials have been conducted in IBD: anti-TNF therapies have been shown to make a significant difference to the health-related quality of life of many patients with IBD³³. Anti-IL-12/IL-23, IL-2R and IFN-g have been shown to have limited results in early phase clinical trials, and studies using antibodies against IL-6, IL-6R, IL-13, IL-17, IL-18 and IL-21 are now entering phase I³³.

Interleukin-6 (IL-6)

IL-6 is predominantly generated by activated macrophages, T-cells, and the endothelium, but is also expressed by enterocytes in response to infection³⁴. Its expression is under the control of NF- κ B³⁵. IL-6 stimulates the production of acute phase proteins in the liver, B cell proliferation and antibody production. IL-6 levels have been found to be elevated in the plasma and the stools of patients with NEC ³⁶ and to be correlated with the severity of disease ^{37,38}. In a study of 62 newborn infants with suspected sepsis or NEC, Interleukin-6 levels were five- to tenfold higher in infants with bacterial sepsis plus NEC at the onset of disease than in infants with bacterial sepsis alone ³⁷. In a study of 60 preterm infants there was a trend to higher levels of IL-6 with a greater degree of NEC³⁸. In an experimental model of NEC induced by *Cronobacter sakazakii* infection, increased pup serum IL-6 was noted in association with increased enterocyte apoptosis³⁴.

IL8

IL-8 is generally regarded as a pro-inflammatory chemokine predominantly produced by macrophages and endothelial cells. However, exposure to amniotic fluid containing IL-8 has been postulated to be important for promoting intestinal health³⁹. IL-8 is present in significant concentrations in human milk and, when human fetal and adult intestinal cells are treated with rh IL-8 *in vitro*, it stimulates cell migration, proliferation, and differentiation³⁹. IL-8 is a potent chemoattractant for neutrophils and an angiogenic factor. As with several other proinflammatory cytokines, elevated IL-8 levels have been associated with human NEC^{40,41} and with animal models of intestinal inflammation⁴². Furthermore, high IL-8 levels may correlate with human NEC severity⁴⁰. One recent study reports that IL-8 appears to be a promising biomarker for the extent of intestinal necrosis⁴¹. Cellular maturity may affect the response to bacterial challenge. Indeed, when compared to mature enterocytes, immature fetal intestinal cells have been shown to produce more IL-8 in response to LPS⁴³ and flagellin ²⁶. These developmental differences may predispose the premature intestine to inflammation.

IL-10

In contrast to IL-6 and IL-8, IL-10 is generally considered to be an anti-inflammatory cytokine. IL-10 modulates the innate and adaptive immune response in part by inhibiting cytokine production by macrophages and other antigen presenting cells⁴⁴. Along with several other cytokines, IL-10 is increased in infants with severe NEC^{40,45}. Several pieces of evidence suggest that this represents a protective mechanism to dampen the intestinal inflammatory response: IL-10 deficient rodents have been shown to develop colitis⁴⁶. Also, IL-10 knockout mice were found to have more profound histologic intestinal injury in a neonatal NEC model than controls⁴⁷; in these mice, intestinal injury was associated with decreased junctional adhesion molecule-1 and increased inducible nitric oxide synthase (iNOS) expression and with increased enterocyte apoptosis⁴⁷. Intraperitoneal administration of IL-10 has been shown to decrease the severity of intestinal injury⁴⁸ and the level of NO synthesized by inhibiting iNOS expression⁴⁸.

The breast milk of mothers of premature infants with NEC was more likely to lack IL-10 than those without NEC⁴⁹. Indeed, IL-10 levels were not detectable in 86% of breast milk samples obtained from women whose premature infants developed NEC compared to 40% obtained from women whose premature infants did not⁴⁹. When stimulated with TNF-alpha or LPS, mononuclear leukocytes obtained from premature infants had decreased IL-10 production compared to term infants⁵⁰. Decreased IL-10 levels may increase the susceptibility of premature infants to inflammation.

IL-11

As with IL-10, IL-11 is another cytokine thought to promote intestinal health. It is produced in the intestine by subepithelial myofibroblasts and downregulates the production of Th1 cytokines such as IL-12 and IFN γ by epithelial cells and lamina propria macrophages⁵¹. It has other functions such as the stimulation of megakaryocyte maturation⁵². In a study of 21 infants with NEC, IL-11 was found to be upregulated and those with elevated IL-11 mRNA appeared to have a decreased risk of pan necrosis⁵³. IL-11 has been shown to prevent mucosal atrophy and to enhance small intestine absorptive function after intestinal resection^{54,55}.

IL-12

IL-12 is secreted by neutrophils, macrophages, dendritic cells and B cells when stimulated with bacteria, viruses and their products. It activated macrophages and Th1 cells, inducing IFN γ . In a small human NEC study, patients with increased IL-12 appeared to have diminished risk of pan necrosis⁵³. However, conflicting data exist on the role of IL-12 in experimental NEC: In one study, IL-12 was found to be elevated in pups exposed to experimental NEC and the increase was correlated with tissue damage⁵⁶, while in another study, IL-12 was found to be downregulated⁵⁷. Therefore further investigation is needed to establish the role of IL-12 in NEC.

IL-18

IL-18 is a cytokine produced by macrophages, dendritic cells and IECs, and together with IL-12, induces cell-mediated immunity following activation by MAMPs. It induces the production of IFN γ , IL-1 β , IL-8 and TNF-alpha by immune cells. IL-18 production is increased in the ileum of neonatal rats with NEC^{56, 58} and mice deficient in IL-18 have a decreased incidence of NEC, fewer ileal macrophages and higher level of IkappaB-alpha and beta, suggesting decreased NF- κ B activation^{56, 58}. Infants with NEC were more likely found to have IL-18 polymorphisms⁵⁹.

TNF α

An increase in TNF α mRNA⁶⁰ and protein⁶¹ was detected in intestinal tissues taken from patients with NEC. When examined by *in situ* hybridization, human NEC tissues were found to have a marked increase in TNF-alpha mRNA in Paneth cells, as well as in infiltrating eosinophils and macrophages⁶². However, TNF α does not appear consistently elevated in serum samples of patients with NEC^{36, 37, 45, 63}. Increased intestinal TNF-alpha levels were found in hypoxia/reoxygenation-induced bowel injury in neonatal rats⁶⁴ but not in a neonatal rat NEC model⁵⁷ and in an acute intestinal injury model (Our lab unpublished data, 2008).

TNF-alpha has been shown to cause a marked loss of mucus-containing goblet cells in immature mice⁶⁵. TNF-alpha causes apoptosis in IECs by inducing mitochondrial ROS (reactive oxygen species) production and by activating the JNK/p38 signaling pathway⁶¹.

While a role of TNF-alpha in inflammatory bowel disease has been clearly established⁶⁶, the evidence for TNF α implication in the pathogenesis of NEC is less certain. Anti-TNF α therapy has been shown in some studies to decrease the degree of experimental NEC^{67, 68}. However, we did not identify similar findings within our own experimental models when we compared rats treated with anti-TNF antibodies versus control immunoglobulin (2008, unpublished data).

IFN gamma

While interferon gamma (or type II interferon)(IFN γ) is mostly released by T cells and natural killer cells, it is also produced by B cells, NK-T cells, dendritic cells and macrophages⁶⁹.

Early during infection, macrophages produced IFN γ in response to IL-12 and IL-18 stimulation⁶⁹. IFN γ activates several signaling pathways, including STAT1, PI-3 kinase/Akt and MAPKs which control the expression of more than 500 genes known to regulate apoptosis, proliferation, leukocyte migration and epithelial permeability⁶⁹.

IFN γ synthesis is inhibited by IL-4, IL-10, TGF β and glucocorticoids⁶⁹.

A role for IFN γ in NEC has been suggested by the evidence that knock-out mice are protected against experimental NEC and have improved epithelial cell restitution compared

to wild-type controls when exposed to the NEC model⁷⁰. IFN γ was found to inhibit enterocyte migration by reversibly displacing connexin 43 from lipid rafts⁷¹.

PAF

Platelet-activating factor (PAF), an endogenous phospholipid mediator is released by many cells, including platelets, neutrophils, mast cells, eosinophils, macrophages and endothelial cells. Bacteria such as *Escherichia coli* and *Helicobacter pylori* have also been found to produce PAF^{72–75}. The receptor for PAF (PAF-R) is a G protein-coupled receptor which is mainly expressed in the ileum, but also abundantly present in the jejunum and the spleen⁷⁶. PAF-R is not only present on inflammatory cells including neutrophils, macrophages and eosinophils^{76, 77} but also on epithelial⁷⁷ and endothelial cells⁷⁸. *In vivo*, free PAF is rapidly degraded by PAF-acetylhydrolase (PAF-AH), a PAF degrading enzyme. PAF-R activation leads to the activation of several transduction pathways including the activation of NF- κ B¹⁷ and of PI3kinase/Akt⁷⁹, and the production of endogenous PAF (by phospholipase A2 (PLA2))⁸⁰, oxygen radicals by xanthine oxidase⁸¹ and TNF- α ⁸² which cause lasting effects *in vivo*. Several pieces of evidence suggest an important role for PAF in NEC: 1) Circulating PAF levels⁸³ are increased and plasma PAF-acetylhydrolase⁸⁴ levels are decreased in infants with NEC compared to age-matched controls; 2) human milk contains PAF-AH, the enzyme degrading PAF⁸⁵; 3) experimental NEC is prevented by PAF-receptor antagonists in a neonatal rat model⁸⁶ and in a piglet model⁸⁷; 4) intravenous infusion of recombinant PAF-AH prevents NEC in rats^{88, 89}; 5) PAF-AH knock-out mice are more susceptible to NEC when exposed to the NEC protocol⁹⁰, although they have a decreased mortality; and 5) the ileum, the site of predilection of NEC, has the highest amounts of PAF-R⁷⁶. PAF also plays a role in the intestinal injury induced by hypoxia/reperfusion⁹¹, TNF- α ⁹² and LPS⁹³. Exogenous administration of PAF causes systemic hypotension, increased vascular permeability, hemoconcentration, and tissue necrosis⁹⁴ which is limited to the intestine and predominates in the small intestine (frequently the ileum) as seen in NEC. This is why PAF administration has been used as a model of acute bowel necrosis and NEC⁹⁴. PAF-induced bowel injury is worsened by the intraperitoneal administration of LPS⁹. Furthermore, PAF has been shown to mediate LPS⁹⁵ and hypoxia-induced bowel necrosis⁹⁵. We have shown that the intravenous injection of PAF, even at a dose below that which causes bowel necrosis¹⁷, causes a very rapid activation of NF- κ B in the intestine (within 5 minutes, peaking at 30 minutes), and induces the gene expression of PLA2⁸⁰, TNF- α ⁸², NF- κ B p50 precursor p105⁹⁶, PAF-R⁷⁶ and TLR4 in IECs⁹⁷ and the release of leukotriene C4⁹⁸. PAF plays an important role in the allergic and inflammatory response causing neutrophil and platelet aggregation, systemic and mesenteric vasodilation and increased vascular permeability⁹⁹.

Nitric oxide (NO)

NO is a free radical that is implicated in many homeostatic and pathologic processes. It has been implicated in regulation of the vascular tone, inflammation, neurotransmission and tissue restitution and repair¹⁰⁰. NO is formed by the action of nitric oxide synthase (NOS) on oxygen and arginine. There are three isoforms of NOS, each encoded by a separate gene; endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS)(Fig 2). eNOS

and nNOS are typically expressed at low (picomolar) constitutive levels. iNOS is not expressed under normal conditions. When iNOS is upregulated, it generates high concentrations of NO (micromolar). NO is soluble in both lipids and water, and is able to diffuse rapidly to neighboring tissues and cells without reliance on channels or transporters. At low levels NO appears to be effectively cleared, however at increased concentrations it reacts with a superoxide anion producing peroxynitrite, a highly toxic oxidation intermediate¹⁰¹. Peroxynitrite disrupts protein conformations and alters cellular process and reactions¹⁰². Although low levels of NO may be beneficial¹⁰¹, by increasing intestinal water absorption¹⁰³ and controlling intestinal barrier permeability¹⁰⁴, high levels appear clearly detrimental increasing enterocyte apoptosis¹⁰⁵ and impairing cell proliferation¹⁰⁶(Fig 2). NO released by activated macrophages has been found to inhibit enterocyte migration¹⁰⁷. NO mediates dendritic cell apoptosis¹⁰⁸, while protecting against apoptosis in other cell types¹⁰⁹.

iNOS was first implicated in the pathogenesis of NEC, after elevated levels of iNOS induced NO were found in the IEC of resected intestinal samples taken from infants undergoing bowel resection secondary to NEC¹¹⁰. Animal studies also have shown that iNOS is upregulated in a neonatal rat NEC model⁵⁷ and that iNOS mediates LPS-induced increase in intestinal permeability¹¹¹. In the intestine of neonatal rats fed with formula eNOS and nNOS were downregulated while iNOS was upregulated when compared with dam fed controls¹¹² and in piglets, formula feeding downregulated eNOS¹¹³. When L-NAME, a NO synthase inhibitor, was administered intravenously in a neonatal piglet model of NEC, it worsened the intestinal injury while L-arginine, a NO synthase substrate, attenuates intestinal injury¹¹⁴. Under physiological conditions, we found that nNOS suppresses iNOS gene expression in the small intestine of rats through NF- κ B downregulation¹¹⁵. When nNOS was inhibited, I κ B alpha was degraded, NF- κ B was activated, and iNOS expression induced¹¹⁵. In a mouse model of DSS colitis, eNOS and iNOS were found to be detrimental while nNOS was found to be beneficial¹¹⁶. However, in other studies using a similar model, eNOS was found to be protective against intestinal inflammation^{117, 118}.

ROS

Under physiological conditions, when bacteria interact with intestinal epithelial cells, reactive oxygen species are reversibly produced which, among other functions, modulate neddylation of cullin-1, leading to downregulation of the proteasome pathway and subsequent NF- κ B pathway suppression^{119, 120} and beta-catenin stabilization^{119, 120} leading to increased intestinal cell proliferation¹²¹. However, during inflammation, reactive oxygen species (ROS) may be a major effector of PAF and many other cytokines. The xanthine oxidase/dehydrogenase system is one of the main producers of ROS in the intestine. Indeed, the enzyme xanthine dehydrogenase is constitutively expressed in large amounts in the intestinal villous epithelium¹²² and catalyzes the transformation of xanthine into uric acid ($\text{Xanthine} + \text{H}_2\text{O} + \text{NAD} \rightarrow \text{Uric acid} + \text{NADH} + \text{H}^+$). However, during ischemia, xanthine dehydrogenase is converted into xanthine oxidase, which leads to the transformation of xanthine into uric acid and the production of superoxide ($\text{Xanthine} + \text{H}_2\text{O} + \text{O}_2 \rightarrow \text{Uric acid} + 2\text{O}_2^- + 2\text{H}^+$).

ROS activates the intestinal mitochondrial apoptotic signaling pathway during oxidative stress¹²³ and leads to IEC apoptosis via the activation of p38 MAPK¹²⁴. Xanthine oxidase and superoxide have been found to mediate intestinal reperfusion injury¹²⁵ and PAF-induced bowel necrosis⁸¹, as allopurinol, a xanthine oxidase inhibitor, has been shown to be protective in these models. Together, these studies suggest a central role for XO and ROS in the pathogenesis of intestinal injury.

Role of immune cells

Several evidences suggest that neutrophils may play an important role in NEC: 1) Neutropenic rats are protected against PAF-induced bowel injury¹²⁶ and have decreased intestinal NF- κ B activation¹⁷; 2) P-selectin deficient mice¹²⁷ are protected against PAF-induced bowel injury, as are mice treated with antibody against beta-2 integrin¹²⁸ (P-selectin and beta-2 integrin are adhesion molecules necessary for neutrophils to roll and adhere to the endothelium); 3) mice treated with antibodies against CXCL2, a major chemokine for neutrophils, have decreased systemic inflammation and are protected against PAF-induced acute intestinal injury¹²⁹. However, when NEC was induced by *Cronobacter sakazakii* infection, PMN and macrophages from the lamina propria were responsible for *C. sakazaki* killing and clearance, and PMN and macrophage depletion exacerbated cytokine production and bowel injury¹³⁰. In this model, *C. sakazakii* induced epithelial damage was secondary to dendritic cells (DCs) recruitment into the intestine¹³¹ with subsequent TGF- β production, iNOS production, apoptosis and epithelial cell damage¹³¹. Other investigators have found that, in utero, intestinal macrophages progressively acquire a non-inflammatory profile due to increased mucosal TGF- β (2) production and macrophages with a hyper-inflammatory phenotype reside in the premature intestine, increasing its susceptibility to injury¹³². Activated macrophages have been found to inhibit enterocyte gap junction formation and IEC restitution via the release of nitric oxide¹⁰⁷.

Systemic inflammation

In NEC, the inflammatory response is not limited to the intestine, but liver and brain inflammation might be present. A study done on neonatal rats suggests that the liver may amplify the inflammation during NEC¹³³. Indeed, the number of Kupffer cells (KC) and the level of hepatic IL-18 and TNF-alpha were found to be elevated in experimental NEC and to correlate with the severity of the intestinal damage¹³³. When KC were inhibited with gadolinium chloride, the levels of TNF-alpha found in the intestinal lumen of rats with NEC were significantly decreased¹³³.

It is recognized that high cytokine levels in preterm infants may contribute to neurologic injury, and poor long-term outcomes¹³⁴. In a study of 84 very low birth weight infants in a neonatal intensive care unit (NICU), 22% developed NEC. White matter injury was found 2.2 times more frequently in those infants with NEC¹³⁵. Higher plasma levels of interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor alpha (TNF α) were found in those infants. Whether the cytokines exhibit a direct effect on the oligodendrocytes remains inconclusive.

SUMMARY – CURRENT HYPOTHESIS

SUMMARY - Current Hypothesis

(Fig 3)

While we don't clearly understand NEC pathogenesis, studies have helped elaborate the current hypothesis, which is that NEC results from a local intestinal inflammation initiated by perinatal stress. Upon introduction of feedings, intestinal bacteria proliferate, favored by the immaturity of the neonatal mucosal innate immune system. Intestinal microbes and their products (MAMP) adhere to the epithelium, breach the immature and fragile intestinal mucosal barrier and activate NF- κ B in lamina propria immunocytes, causing them to secrete pro-inflammatory mediators, chemokines (CXCL2), cytokines (TNF, IL), prostanoids, platelet-activating factor, and nitric oxide. (We hypothesized that the bowel injury in NEC results from inappropriately elevated and prolonged NF- κ B activity in inflammatory cells). These inflammatory agents attract further inflammatory cells, in particular neutrophils, induce the production of reactive oxygen species, and inflict further damage to the intestinal barrier resulting in increased bacterial translocation, intestinal epithelial damage, impaired epithelial cell restitution, apoptosis and mucosal necrosis. Thus, a vicious cycle characteristic of severe NEC is created by bacterial invasion, immune activation, uncontrolled inflammation with production of reactive oxygen and nitrogen species, vasoconstriction followed by ischemia-reperfusion injury, gut barrier failure, intestinal necrosis, sepsis and shock.

References

1. Wang Y, Hoenig JD, Malin KJ, Qamar S, Petrof EO, Sun J, Antonopoulos DA, Chang EB, Claud EC. 16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis. *The ISME journal*. 2009; 3:944–954. [PubMed: 19369970]
2. Dinsmore JE, Jackson RJ, Smith SD. The protective role of gastric acidity in neonatal bacterial translocation. *J Pediatr Surg*. 1997; 32:1014–1016. [PubMed: 9247224]
3. Rozenfeld RA, Liu X, DePlaen I, Hsueh W. Role of gut flora on intestinal group II phospholipase A2 activity and intestinal injury in shock. *Am J Physiol Gastrointest Liver Physiol*. 2001; 281:G957–G963. [PubMed: 11557516]
4. Carlisle EM, Poroyko V, Caplan MS, Alverdy JA, Liu D. Gram negative bacteria are associated with the early stages of necrotizing enterocolitis. *PloS one*. 2011; 6:e18084. [PubMed: 21445365]
5. Essani NA, McGuire GM, Manning AM, Jaeschke H. Endotoxin-induced activation of the nuclear transcription factor kappa B and expression of E-selectin messenger RNA in hepatocytes, Kupffer cells, and endothelial cells in vivo. *J Immunol*. 1996; 156:2956–2963. [PubMed: 8609416]
6. Hawiger J. Innate immunity and inflammation: a transcriptional paradigm. *Immunologic research*. 2001; 23:99–109. [PubMed: 11444396]
7. Ferraris L, Karmeli F, Eliakim R, Klein J, Fiocchi C, Rachmilewitz D. Intestinal epithelial cells contribute to the enhanced generation of platelet activating factor in ulcerative colitis. *Gut*. 1993; 34:665–668. [PubMed: 8504968]
8. Sharma R, Tepas JJ III, Hudak ML, Mollitt DL, Wludyka PS, Teng RJ, Premachandra BR. Neonatal gut barrier and multiple organ failure: role of endotoxin and proinflammatory cytokines in sepsis and necrotizing enterocolitis. *J Pediatr Surg*. 2007; 42:454–461. [PubMed: 17336180]
9. Gonzalez-Crussi F, Hsueh W. Experimental model of ischemic bowel necrosis. The role of platelet-activating factor and endotoxin. *Am J Pathol*. 1983; 112:127–135. [PubMed: 6859226]

10. Jilling T, Simon D, Lu J, Meng FJ, Li D, Schy R, Thomson RB, Soliman A, Arditi M, Caplan MS. The roles of bacteria and TLR4 in rat and murine models of necrotizing enterocolitis. *J Immunol.* 2006; 177:3273–3282. [PubMed: 16920968]
11. Leaphart CL, Cavallo J, Gribar SC, Cetin S, Li J, Branca MF, Dubowski TD, Sodhi CP, Hackam DJ. A critical role for TLR4 in the pathogenesis of necrotizing enterocolitis by modulating intestinal injury and repair. *J Immunol.* 2007; 179:4808–4820. [PubMed: 17878380]
12. Gribar SC, Sodhi CP, Richardson WM, Anand RJ, Gittes GK, Branca MF, Jakub A, Shi XH, Shah S, Ozolek JA, Hackam DJ. Reciprocal expression and signaling of TLR4 and TLR9 in the pathogenesis and treatment of necrotizing enterocolitis. *J Immunol.* 2009; 182:636–646. [PubMed: 19109197]
13. Abreu MT. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nat Rev Immunol.* 2010; 10:131–144. [PubMed: 20098461]
14. Lee J, Mo JH, Katakura K, Alkalay I, Rucker AN, Liu YT, Lee HK, Shen C, Cojocaru G, Shenouda S, Kagnoff M, Eckmann L, Ben-Neriah Y, Raz E. Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. *Nat cell biol.* 2006; 8:1327–1336. [PubMed: 17128265]
15. Hayden MS, Ghosh S. Signaling to NF-kappaB. *Genes Dev.* 2004; 18:2195–2224. [PubMed: 15371334]
16. Li Q, Verma IM. NF-kappaB regulation in the immune system. *Nat Rev Immunol.* 2002; 2:725–734. [PubMed: 12360211]
17. De Plaen IG, Tan XD, Chang H, Qu XW, Liu QP, Hsueh W. Intestinal NF-kappaB is activated, mainly as p50 homodimers, by platelet-activating factor. *Biochim Biophys Acta.* 1998; 1392:185–192. [PubMed: 9630621]
18. De Plaen IG, Tan XD, Chang H, Wang L, Remick DG, Hsueh W. Lipopolysaccharide activates nuclear factor kappaB in rat intestine: role of endogenous platelet-activating factor and tumour necrosis factor. *Br J Pharmacol.* 2000; 129:307–314. [PubMed: 10694237]
19. DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M. A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. *Nature.* 1997; 388:548–554. [PubMed: 9252186]
20. De Plaen IG, Liu SX, Tian R, Neequaye I, May MJ, Han XB, Hsueh W, Jilling T, Lu J, Caplan MS. Inhibition of nuclear factor-kappaB ameliorates bowel injury and prolongs survival in a neonatal rat model of necrotizing enterocolitis. *Pediatr Res.* 2007; 61:716–721. [PubMed: 17426653]
21. Minekawa R, Takeda T, Sakata M, Hayashi M, Isobe A, Yamamoto T, Tasaka K, Murata Y. Human breast milk suppresses the transcriptional regulation of IL-1beta-induced NF-kappaB signaling in human intestinal cells. *Am J Physiol Cell Physiol.* 2004; 287:C1404–C1411. [PubMed: 15229109]
22. Petrof EO, Kojima K, Ropeleski MJ, Musch MW, Tao Y, De Simone C, Chang EB. Probiotics inhibit nuclear factor-kappaB and induce heat shock proteins in colonic epithelial cells through proteasome inhibition. *Gastroenterology.* 2004; 127:1474–1487. [PubMed: 15521016]
23. Chen LW, Egan L, Li ZW, Greten FR, Kagnoff MF, Karin M. The two faces of IKK and NF-kappaB inhibition: prevention of systemic inflammation but increased local injury following intestinal ischemia-reperfusion. *Nat Med.* 2003; 9:575–581. [PubMed: 12692538]
24. Egan LJ, Eckmann L, Greten FR, Chae S, Li ZW, Myhre GM, Robine S, Karin M, Kagnoff MF. IkappaB-kinasebeta-dependent NF-kappaB activation provides radioprotection to the intestinal epithelium. *Proc Natl Acad Sci USA.* 2004; 101:2452–2457. [PubMed: 14983030]
25. Chae S, Eckmann L, Miyamoto Y, Pothoulakis C, Karin M, Kagnoff MF. Epithelial cell I kappa B-kinase beta has an important protective role in *Clostridium difficile* toxin A-induced mucosal injury. *J Immunol.* 2006; 177:1214–1220. [PubMed: 16818780]
26. Claud EC, Lu L, Anton PM, Savidge T, Walker WA, Cherayil BJ. Developmentally regulated IkappaB expression in intestinal epithelium and susceptibility to flagellin-induced inflammation. *Proc Natl Acad Sci USA.* 2004; 101:7404–7408. [PubMed: 15123821]

27. Vancurova I, Bellani P, Davidson D. Activation of nuclear factor-kappaB and its suppression by dexamethasone in polymorphonuclear leukocytes: newborn versus adult. *Pediatr Res.* 2001; 49:257–262. [PubMed: 11158523]
28. Kilpinen S, Henttinen T, Lahdenpohja N, Hulkkonen J, Hurme M. Signals leading to the activation of NF-kappa B transcription factor are stronger in neonatal than adult T lymphocytes. *Scand J Immunol.* 1996; 44:85–88. [PubMed: 8693296]
29. Le Mandat Schultz A, Bonnard A, Barreau F, Aigrain Y, Pierre-Louis C, Berrebi D, Peuchmaur M. Expression of TLR-2, TLR-4, NOD2 and pNF-kappaB in a neonatal rat model of necrotizing enterocolitis. *PloS one.* 2007; 2:e1102. [PubMed: 17971865]
30. De Plaen IG, Han XB, Liu X, Hsueh W, Ghosh S, May MJ. Lipopolysaccharide induces CXCL2/macrophage inflammatory protein-2 gene expression in enterocytes via NF-kappaB activation: independence from endogenous TNF-alpha and platelet-activating factor. *Immunology.* 2006; 118:153–163. [PubMed: 16771850]
31. Shibata W, Maeda S, Hikiba Y, Yanai A, Ohmae T, Sakamoto K, Nakagawa H, Ogura K, Omata M. Cutting edge: The IkappaB kinase (IKK) inhibitor, NEMO-binding domain peptide, blocks inflammatory injury in murine colitis. *J Immunol.* 2007; 179:2681–2685. [PubMed: 17709478]
32. Kelly D, Conway S. Bacterial modulation of mucosal innate immunity. *Mol Immunol.* 2005; 42:895–901. [PubMed: 15829279]
33. Danese S. New therapies for inflammatory bowel disease: from the bench to the bedside. *Gut.* 2012; 61:918–932. [PubMed: 22115827]
34. Hunter CJ, Singamsetty VK, Chokshi NK, Boyle P, Camerini V, Grishin AV, Upperman JS, Ford HR, Prasadaraio NV. Enterobacter sakazakii enhances epithelial cell injury by inducing apoptosis in a rat model of necrotizing enterocolitis. *The Journal of infectious diseases.* 2008; 198:586–593. [PubMed: 18588483]
35. Shimizu H, Mitomo K, Watanabe T, Okamoto S, Yamamoto K. Involvement of a NF-kappa B-like transcription factor in the activation of the interleukin-6 gene by inflammatory lymphokines. *Molecular and cellular biology.* 1990; 10:561–568. [PubMed: 2405250]
36. Morecroft JA, Spitz L, Hamilton PA, Holmes SJ. Plasma cytokine levels in necrotizing enterocolitis. *Acta Paediatr Suppl.* 1994; 396:18–20. [PubMed: 8086674]
37. Harris MC, Costarino AT Jr, Sullivan JS, Dulkerian S, McCawley L, Corcoran L, Butler S, Kilpatrick L. Cytokine elevations in critically ill infants with sepsis and necrotizing enterocolitis. *J Pediatr.* 1994; 124:105–111. [PubMed: 8283358]
38. Duffy LC, Zielezny MA, Carrion V, Griffiths E, Dryja D, Hilty M, Rook C, Morin F 3rd. Concordance of bacterial cultures with endotoxin and interleukin-6 in necrotizing enterocolitis. *Digestive diseases and sciences.* 1997; 42:359–365. [PubMed: 9052520]
39. Maheshwari A, Lu W, Lacson A, Barileycorn AA, Nolan S, Christensen RD, Calhoun DA. Effects of interleukin-8 on the developing human intestine. *Cytokine.* 2002; 20:256–267. [PubMed: 12633567]
40. Edelson MB, Bagwell CE, Rozycki HJ. Circulating pro- and counterinflammatory cytokine levels and severity in necrotizing enterocolitis. *Pediatrics.* 1999; 103:766–771. [PubMed: 10103300]
41. Benkoe T, Reck C, Gleiss A, Kettner S, Repa A, Horcher E, Rebhandl W. Interleukin 8 correlates with intestinal involvement in surgically treated infants with necrotizing enterocolitis. *Journal of pediatric surgery.* 2012; 47:1548–1554. [PubMed: 22901915]
42. Chen Y, Hung WT, Chen SM, Tseng SH. Suppression of elevated plasma interleukin-8 levels due to total ischemia and reperfusion of the small intestine by luminal perfusion with fetal bovine serum. *Pediatric surgery international.* 2002; 18:107–109. [PubMed: 11956773]
43. Nanthakumar NN, Fusunyan RD, Sanderson I, Walker WA. Inflammation in the developing human intestine: A possible pathophysiologic contribution to necrotizing enterocolitis. *Proceedings of the National Academy of Sciences of the United States of America.* 2000; 97:6043–6048. [PubMed: 10823949]
44. Paul G, Khare V, Gasche C. Inflamed gut mucosa: downstream of interleukin-10. *European journal of clinical investigation.* 2012; 42:95–109. [PubMed: 21631466]

45. Benkoe T, Baumann S, Weninger M, Pones M, Reck C, Rebhandl W, Oehler R. Comprehensive evaluation of 11 cytokines in premature infants with surgical necrotizing enterocolitis. *PloS one*. 2013; 8:e58720. [PubMed: 23472217]
46. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell*. 1993; 75:263–274. [PubMed: 8402911]
47. Emami CN, Chokshi N, Wang J, Hunter C, Guner Y, Goth K, Wang L, Grishin A, Ford HR. Role of interleukin-10 in the pathogenesis of necrotizing enterocolitis. *American journal of surgery*. 2012; 203:428–435. [PubMed: 22450025]
48. Kling KM, Kirby L, Kwan KY, Kim F, McFadden DW. Interleukin-10 inhibits inducible nitric oxide synthase in an animal model of necrotizing enterocolitis. *International journal of surgical investigation*. 1999; 1:337–342. [PubMed: 12774458]
49. Fituch CC, Palkowetz KH, Goldman AS, Schanler RJ. Concentrations of IL-10 in preterm human milk and in milk from mothers of infants with necrotizing enterocolitis. *Acta paediatrica (Oslo Norway : 1992)*. 2004; 93:1496–1500.
50. Chheda S, Palkowetz KH, Garofalo R, Rassin DK, Goldman AS. Decreased interleukin-10 production by neonatal monocytes and T cells: relationship to decreased production and expression of tumor necrosis factor-alpha and its receptors. *Pediatric research*. 1996; 40:475–483. [PubMed: 8865287]
51. Leng SX, Elias JA. Interleukin-11 inhibits macrophage interleukin-12 production. *Journal of immunology (Baltimore Md : 1950)*. 1997; 159:2161–2168.
52. Ciurea SO, Hoffman R. Cytokines for the treatment of thrombocytopenia. *Seminars in hematology*. 2007; 44:166–182. [PubMed: 17631181]
53. Nadler EP, Stanford A, Zhang XR, Schall LC, Alber SM, Watkins SC, Ford HR. Intestinal cytokine gene expression in infants with acute necrotizing enterocolitis: interleukin-11 mRNA expression inversely correlates with extent of disease. *Journal of pediatric surgery*. 2001; 36:1122–1129. [PubMed: 11479840]
54. Dickinson EC, Tuncer R, Nadler EP, Koltuksuz U, Boyle P, Alber SM, Watkins SC, Ford HR. Recombinant human interleukin-11 prevents mucosal atrophy and bowel shortening in the defunctionalized intestine. *Journal of pediatric surgery*. 2000; 35:1079–1083. [PubMed: 10917300]
55. Alavi K, Prasad R, Lundgren K, Schwartz MZ. Interleukin-11 enhances small intestine absorptive function and mucosal mass after intestinal adaptation. *Journal of pediatric surgery*. 2000; 35:371–374. [PubMed: 10693699]
56. Halpern MD, Holubec H, Dominguez JA, Williams CS, Meza YG, McWilliam DL, Payne CM, McCuskey RS, Besselsen DG, Dvorak B. Up-regulation of IL-18 and IL-12 in the ileum of neonatal rats with necrotizing enterocolitis. *Pediatric research*. 2002; 51:733–739. [PubMed: 12032269]
57. Nadler EP, Dickinson E, Knisely A, Zhang XR, Boyle P, Beer-Stolz D, Watkins SC, Ford HR. Expression of inducible nitric oxide synthase and interleukin-12 in experimental necrotizing enterocolitis. *J Surg Res*. 2000; 92:71–77. [PubMed: 10864485]
58. Xu J, Treem WR, Roman C, Anderson V, Rubenstein R, Schwarz SM. Ileal immune dysregulation in necrotizing enterocolitis: role of CD40/CD40L in the pathogenesis of disease. *Journal of pediatric gastroenterology and nutrition*. 2011; 52:140–146. [PubMed: 21240009]
59. Heninger E, Treszl A, Kocsis I, Derfalvi B, Tulassay T, Vasarhelyi B. Genetic variants of the interleukin-18 promoter region (-607) influence the course of necrotising enterocolitis in very low birth weight neonates. *European journal of pediatrics*. 2002; 161:410–411. [PubMed: 12174825]
60. Viscardi RM, Lyon NH, Sun CC, Hebel JR, Hasday JD. Inflammatory cytokine mRNAs in surgical specimens of necrotizing enterocolitis and normal newborn intestine. *Pediatric pathology & laboratory medicine : journal of the Society for Pediatric Pathology affiliated with the International Paediatric Pathology Association*. 1997; 17:547–559.
61. Baregamian N, Song J, Bailey CE, Papaconstantinou J, Evers BM, Chung DH. Tumor necrosis factor-alpha and apoptosis signal-regulating kinase 1 control reactive oxygen species release, mitochondrial autophagy, and c-Jun N-terminal kinase/p38 phosphorylation during necrotizing enterocolitis. *Oxid Med Cell Longev*. 2009; 2:297–306. [PubMed: 20716917]

62. Tan X, Hsueh W, Gonzalez-Crussi F. Cellular localization of tumor necrosis factor (TNF)-alpha transcripts in normal bowel and in necrotizing enterocolitis. TNF gene expression by Paneth cells, intestinal eosinophils, and macrophages, *Am J Pathol.* 1993; 142:1858–1865.
63. Morecroft JA, Spitz L, Hamilton PA, Holmes SJ. Plasma interleukin-6 and tumour necrosis factor levels as predictors of disease severity and outcome in necrotizing enterocolitis. *J Pediatr Surg.* 1994; 29:798–800. [PubMed: 8078025]
64. Akisu M, Baka M, Yalaz M, Huseyinov A, Kultursay N. Supplementation with *Saccharomyces boulardii* ameliorates hypoxia/reoxygenation-induced necrotizing enterocolitis in young mice. *European journal of pediatric surgery : official journal of Austrian Association of Pediatric Surgery [et al] = Zeitschrift fur Kinderchirurgie.* 2003; 13:319–323.
65. McElroy SJ, Prince LS, Weitkamp JH, Reese J, Slaughter JC, Polk DB. Tumor necrosis factor receptor 1-dependent depletion of mucus in immature small intestine: a potential role in neonatal necrotizing enterocolitis. *Am J Physiol Gastrointest Liver Physiol.* 2011; 301:G656–G666. [PubMed: 21737776]
66. van Dullemen HM, van Deventer SJ, Hommes DW, Bijl HA, Jansen J, Tytgat GN, Woody J. Treatment of Crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2). *Gastroenterology.* 1995; 109:129–135. [PubMed: 7797011]
67. Halpern MD, Clark JA, Saunders TA, Doelle SM, Hosseini DM, Stagner AM, Dvorak B. Reduction of experimental necrotizing enterocolitis with anti-TNF-alpha. *Am J Physiol Gastrointest Liver Physiol.* 2006; 290:G757–G764. [PubMed: 16269520]
68. Seitz G, Warmann SW, Guglielmetti A, Heitmann H, Ruck P, Kreis ME, Fuchs J. Protective effect of tumor necrosis factor alpha antibody on experimental necrotizing enterocolitis in the rat. *J Pediatr Surg.* 2005; 40:1440–1445. [PubMed: 16150346]
69. Beaupaire C, Smyth D, McKay DM. Interferon-gamma regulation of intestinal epithelial permeability. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research.* 2009; 29:133–144.
70. Leapheart CL, Qureshi F, Cetin S, Li J, Dubowski T, Baty C, Beer-Stolz D, Guo F, Murray SA, Hackam DJ. Interferon-gamma inhibits intestinal restitution by preventing gap junction communication between enterocytes. *Gastroenterology.* 2007; 132:2395–2411. [PubMed: 17570214]
71. Leapheart CL, Dai S, Gripar SC, Richardson W, Ozolek J, Shi XH, Bruns JR, Branca M, Li J, Weisz OA, Sodhi C, Hackam DJ. Interferon-gamma inhibits enterocyte migration by reversibly displacing connexin43 from lipid rafts. *American journal of physiology Gastrointestinal and liver physiology.* 2008; 295:G559–569. [PubMed: 18635599]
72. Denizot Y, Dassa E, Kim HY, Bossant MJ, Salem N Jr, Thomas Y, Benveniste J. Synthesis of paf-acether from exogenous precursors by the prokaryote *Escherichia coli*. *FEBS Lett.* 1989; 243:13–16. [PubMed: 2646144]
73. Denizot Y, Dassa E, Thomas Y, Benveniste J. Production of paf-acether by various bacterial strains. *Gastroenterol Clin Biol.* 1990; 14:681–682. [PubMed: 2227243]
74. Benveniste J, Chignard M, Le Couedic JP, Vargaftig BB. Biosynthesis of platelet-activating factor (PAF-acether). II Involvement of phospholipase A2 in the formation of PAF-acether and lyso-PAF-acether from rabbit platelets. *Thromb Res.* 1982; 25:375–385.
75. Denizot Y, Sobhani I, Rambaud JC, Lewin M, Thomas Y, Benveniste J. Paf-acether synthesis by *Helicobacter pylori*. *Gut.* 1990; 31:1242–1245. [PubMed: 2253906]
76. Wang H, Tan X, Chang H, Gonzalez-Crussi F, Remick DG, Hsueh W. Regulation of platelet-activating factor receptor gene expression in vivo by endotoxin, platelet-activating factor and endogenous tumour necrosis factor. *Biochem J.* 1997; 322 (Pt 2):603–608. [PubMed: 9065783]
77. Wang H, Tan X, Chang H, Huang W, Gonzalez-Crussi F, Hsueh W. Platelet-activating factor receptor mRNA is localized in eosinophils and epithelial cells in rat small intestine: regulation by dexamethasone and gut flora. *Immunology.* 1999; 97:447–454. [PubMed: 10447766]
78. Balestrieri ML, Giovane A, Milone L, Servillo L. Endothelial progenitor cells express PAF receptor and respond to PAF via Ca(2+)-dependent signaling. *Biochim Biophys Acta.* 2010; 1801:1123–1132. [PubMed: 20637897]

79. Lu J, Caplan MS, Li D, Jilling T. Polyunsaturated fatty acids block platelet-activating factor-induced phosphatidylinositol 3 kinase/Akt-mediated apoptosis in intestinal epithelial cells. *American journal of physiology Gastrointestinal and liver physiology*. 2008; 294:G1181–1190. [PubMed: 18356536]
80. Tan XD, Wang H, Gonzalez-Crussi FX, Chang H, Gonzalez-Crussi F, Hsueh W. Platelet activating factor and endotoxin increase the enzyme activity and gene expression of type II phospholipase A2 in the rat intestine. Role of polymorphonuclear leukocytes, *J Immunol*. 1996; 156:2985–2990.
81. Qu XW, Rozenfeld RA, Huang W, Bulkley GB, Hsueh W. The role of xanthine oxidase in platelet activating factor induced intestinal injury in the rat. *Gut*. 1999; 44:203–211. [PubMed: 9895379]
82. Huang L, Tan X, Crawford SE, Hsueh W. Platelet-activating factor and endotoxin induce tumour necrosis factor gene expression in rat intestine and liver. *Immunology*. 1994; 83:65–69. [PubMed: 7821968]
83. MacKendrick W, Hill N, Hsueh W, Caplan M. Increase in plasma platelet-activating factor levels in enterally fed preterm infants. *Biol Neonate*. 1993; 64:89–95. [PubMed: 8260551]
84. Caplan M, Hsueh W, Kelly A, Donovan M. Serum PAF acetylhydrolase increases during neonatal maturation. *Prostaglandins*. 1990; 39:705–714. [PubMed: 2371412]
85. Furukawa M, Narahara H, Yasuda K, Johnston JM. Presence of platelet-activating factor-acetylhydrolase in milk. *Journal of lipid research*. 1993; 34:1603–1609. [PubMed: 8228643]
86. Caplan MS, Hedlund E, Adler L, Lickerman M, Hsueh W. The platelet-activating factor receptor antagonist WEB 2170 prevents neonatal necrotizing enterocolitis in rats. *J Pediatr Gastroenterol Nutr*. 1997; 24:296–301. [PubMed: 9138176]
87. Ewer AK, Al-Salti W, Coney AM, Marshall JM, Ramani P, Booth IW. The role of platelet activating factor in a neonatal piglet model of necrotising enterocolitis. *Gut*. 2004; 53:207–213. [PubMed: 14724152]
88. Caplan MS, Lickerman M, Adler L, Dietsch GN, Yu A. The role of recombinant platelet-activating factor acetylhydrolase in a neonatal rat model of necrotizing enterocolitis. *Pediatric research*. 1997; 42:779–783. [PubMed: 9396557]
89. Muguruma K, Gray PW, Tjoelker LW, Johnston JM. The central role of PAF in necrotizing enterocolitis development. *Adv Exp Med Biol*. 1997; 407:379–382. [PubMed: 9321979]
90. Lu J, Pierce M, Franklin A, Jilling T, Stafforini DM, Caplan M. Dual roles of endogenous platelet-activating factor acetylhydrolase in a murine model of necrotizing enterocolitis. *Pediatric research*. 2010; 68:225–230. [PubMed: 20531249]
91. Caplan MS, Sun XM, Hsueh W. Hypoxia causes ischemic bowel necrosis in rats: the role of platelet-activating factor (PAF-acether). *Gastroenterology*. 1990; 99:979–986. [PubMed: 2394352]
92. Sun XM, Hsueh W. Bowel necrosis induced by tumor necrosis factor in rats is mediated by platelet-activating factor. *J Clin Invest*. 1988; 81:1328–1331. [PubMed: 3366898]
93. Hsueh W, Gonzalez-Crussi F, Arroyave JL. Platelet-activating factor: an endogenous mediator for bowel necrosis in endotoxemia. *FASEB J*. 1987; 1:403–405. [PubMed: 3678700]
94. Hsueh W, Gonzalez-Crussi F, Arroyave JL. Platelet-activating factor-induced ischemic bowel necrosis. An investigation of secondary mediators in its pathogenesis, *AmJPathol*. 1986; 122:231–239.
95. Caplan MS, Kelly A, Hsueh W. Endotoxin and hypoxia-induced intestinal necrosis in rats: the role of platelet activating factor. *Pediatr Res*. 1992; 31:428–434. [PubMed: 1603619]
96. Tan X, Sun X, Gonzalez-Crussi FX, Gonzalez-Crussi F, Hsueh W. PAF and TNF increase the precursor of NF-kappa B p50 mRNA in mouse intestine: quantitative analysis by competitive PCR. *Biochim Biophys Acta*. 1994; 1215:157–162. [PubMed: 7947998]
97. Soliman A, Michelsen KS, Karahashi H, Lu J, Meng FJ, Qu X, Crother TR, Rabizadeh S, Chen S, Caplan MS, Ardit M, Jilling T. Platelet-activating factor induces TLR4 expression in intestinal epithelial cells: implication for the pathogenesis of necrotizing enterocolitis. *PloS one*. 2010; 5:e15044. [PubMed: 20976181]
98. Hsueh W, Gonzalez-Crussi F, Arroyave JL. Release of leukotriene C4 by isolated, perfused rat small intestine in response to platelet-activating factor. *J Clin Invest*. 1986; 78:108–114. [PubMed: 3459734]

99. Ford-Hutchinson AW. Neutrophil aggregating properties of PAF-acether and leukotriene B₄. *International journal of immunopharmacology*. 1983; 5:17–21. [PubMed: 6302012]
100. Chokshi NK, Guner YS, Hunter CJ, Upperman JS, Grishin A, Ford HR. The role of nitric oxide in intestinal epithelial injury and restitution in neonatal necrotizing enterocolitis. *Seminars in perinatology*. 2008; 32:92–99. [PubMed: 18346532]
101. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *The American journal of physiology*. 1996; 271:C1424–1437. [PubMed: 8944624]
102. Alvarez B, Radi R. Peroxynitrite reactivity with amino acids and proteins. *Amino acids*. 2003; 25:295–311. [PubMed: 14661092]
103. Barry MK, Aloisi JD, Pickering SP, Yeo CJ. Nitric oxide modulates water and electrolyte transport in the ileum. *Annals of surgery*. 1994; 219:382–388. [PubMed: 8161264]
104. Payne D, Kubes P. Nitric oxide donors reduce the rise in reperfusion-induced intestinal mucosal permeability. *Am J Physiol*. 1993; 265:G189–G195. [PubMed: 8393297]
105. Potoka DA, Upperman JS, Nadler EP, Wong CT, Zhou X, Zhang XR, Ford HR. NF-kappaB inhibition enhances peroxynitrite-induced enterocyte apoptosis. *The Journal of surgical research*. 2002; 106:7–14. [PubMed: 12127801]
106. Potoka DA, Upperman JS, Zhang XR, Kaplan JR, Corey SJ, Grishin A, Zamora R, Ford HR. Peroxynitrite inhibits enterocyte proliferation and modulates Src kinase activity in vitro. *American journal of physiology Gastrointestinal and liver physiology*. 2003; 285:G861–869. [PubMed: 12842830]
107. Anand RJ, Dai S, Rippel C, Leaphart C, Qureshi F, Gribar SC, Kohler JW, Li J, Stolz DB, Sodhi C, Hackam DJ. Activated macrophages inhibit enterocyte gap junctions via the release of nitric oxide. *American journal of physiology Gastrointestinal and liver physiology*. 2008; 294:G109–119. [PubMed: 17975131]
108. Stanford A, Chen Y, Zhang XR, Hoffman R, Zamora R, Ford HR. Nitric oxide mediates dendritic cell apoptosis by downregulating inhibitors of apoptosis proteins and upregulating effector caspase activity. *Surgery*. 2001; 130:326–332. [PubMed: 11490367]
109. Chen Y, Stanford A, Simmons RL, Ford HR, Hoffman RA. Nitric oxide protects thymocytes from gamma-irradiation-induced apoptosis in correlation with inhibition of p53 upregulation and mitochondrial damage. *Cellular immunology*. 2001; 214:72–80. [PubMed: 11902831]
110. Ford H, Watkins S, Reblock K, Rowe M. The role of inflammatory cytokines and nitric oxide in the pathogenesis of necrotizing enterocolitis. *J Pediatr Surg*. 1997; 32:275–282. [PubMed: 9044137]
111. Chen K, Inoue M, Okada A. Expression of inducible nitric oxide synthase mRNA in rat digestive tissues after endotoxin and its role in intestinal mucosal injury. *Biochemical and biophysical research communications*. 1996; 224:703–708. [PubMed: 8713110]
112. D'Souza A, Fordjour L, Ahmad A, Cai C, Kumar D, Valencia G, Aranda JV, Beharry KD. Effects of probiotics, prebiotics, and synbiotics on messenger RNA expression of caveolin-1, NOS, and genes regulating oxidative stress in the terminal ileum of formula-fed neonatal rats. *Pediatric research*. 2010; 67:526–531. [PubMed: 20101198]
113. van Haver ER, Oste M, Thymann T, Sys SU, Lamers WH, Weyns AL, Sangild PT, van Ginneken CJ. Enteral feeding reduces endothelial nitric oxide synthase in the caudal intestinal microvasculature of preterm piglets. *Pediatric research*. 2008; 63:137–142. [PubMed: 18091345]
114. Di Lorenzo M, Bass J, Krantis A. Use of L-arginine in the treatment of experimental necrotizing enterocolitis. *J Pediatr Surg*. 1995; 30:235–240. [PubMed: 7537808]
115. Qu XW, Wang H, De Plaen IG, Rozenfeld RA, Hsueh W. Neuronal nitric oxide synthase (NOS) regulates the expression of inducible NOS in rat small intestine via modulation of nuclear factor kappa B. *FASEB journal*. 2001; 15:439–446. [PubMed: 11156959]
116. Beck PL, Xavier R, Wong J, Ezedi I, Mashimo H, Mizoguchi A, Mizoguchi E, Bhan AK, Podolsky DK. Paradoxical roles of different nitric oxide synthase isoforms in colonic injury. *American journal of physiology Gastrointestinal and liver physiology*. 2004; 286:G137–147. [PubMed: 14665440]

117. Sasaki M, Bharwani S, Jordan P, Elrod JW, Grisham MB, Jackson TH, Lefer DJ, Alexander JS. Increased disease activity in eNOS-deficient mice in experimental colitis. *Free radical biology & medicine*. 2003; 35:1679–1687. [PubMed: 14680690]
118. Vallance BA, Dijkstra G, Qiu B, van der Waaij LA, van Goor H, Jansen PL, Mashimo H, Collins SM. Relative contributions of NOS isoforms during experimental colitis: endothelial-derived NOS maintains mucosal integrity. *American journal of physiology Gastrointestinal and liver physiology*. 2004; 287:G865–874. [PubMed: 15217783]
119. Kumar A, Wu H, Collier-Hyams LS, Kwon YM, Hanson JM, Neish AS. The bacterial fermentation product butyrate influences epithelial signaling via reactive oxygen species-mediated changes in cullin-1 neddylation. *Journal of immunology (Baltimore Md : 1950)*. 2009; 182:538–546.
120. Kumar A, Wu H, Collier-Hyams LS, Hansen JM, Li T, Yamoah K, Pan ZQ, Jones DP, Neish AS. Commensal bacteria modulate cullin-dependent signaling via generation of reactive oxygen species. *The EMBO journal*. 2007; 26:4457–4466. [PubMed: 17914462]
121. Lee WJ. Bacterial-modulated signaling pathways in gut homeostasis. *Science signaling*. 2008; 1:pe24. [PubMed: 18506033]
122. Hsueh W, Caplan MS, Qu XW, Tan XD, De Plaen IG, Gonzalez-Crussi F. Neonatal necrotizing enterocolitis: clinical considerations and pathogenetic concepts. *Pediatr Dev Pathol*. 2003; 6:6–23. [PubMed: 12424605]
123. Baregamian N, Song J, Papaconstantinou J, Hawkins HK, Evers BM, Chung DH. Intestinal mitochondrial apoptotic signaling is activated during oxidative stress. *Pediatric surgery international*. 2011; 27:871–877. [PubMed: 21400030]
124. Zhou Y, Wang Q, Mark Evers B, Chung DH. Oxidative stress-induced intestinal epithelial cell apoptosis is mediated by p38 MAPK. *Biochemical and biophysical research communications*. 2006; 350:860–865. [PubMed: 17034759]
125. Parks DA, Bulkley GB, Granger DN, Hamilton SR, McCord JM. Ischemic injury in the cat small intestine: role of superoxide radicals. *Gastroenterology*. 1982; 82:9–15. [PubMed: 6273253]
126. Musemeche C, Caplan M, Hsueh W, Sun X, Kelly A. Experimental necrotizing enterocolitis: the role of polymorphonuclear neutrophils. *J Pediatr Surg*. 1991; 26:1047–1049. [PubMed: 1941482]
127. Sun X, Rozenfeld RA, Qu X, Huang W, Gonzalez-Crussi F, Hsueh W. P-selectin-deficient mice are protected from PAF-induced shock, intestinal injury, and lethality. *Am J Physiol*. 1997; 273:G56–G61. [PubMed: 9252509]
128. Sun XM, Qu XW, Huang W, Granger DN, Bree M, Hsueh W. Role of leukocyte beta 2-integrin in PAF-induced shock and intestinal injury. *Am J Physiol*. 1996; 270:G184–G190. [PubMed: 8772517]
129. Han XB, Liu X, Hsueh W, De Plaen IG. Macrophage inflammatory protein-2 mediates the bowel injury induced by platelet-activating factor. *Am J Physiol Gastrointest Liver Physiol*. 2004; 287:G1220–G1226. [PubMed: 15319184]
130. Emami CN, Mittal R, Wang L, Ford HR, Prasadara NV. Role of neutrophils and macrophages in the pathogenesis of necrotizing enterocolitis caused by *Cronobacter sakazakii*. *The Journal of surgical research*. 2012; 172:18–28. [PubMed: 21601887]
131. Emami CN, Mittal R, Wang L, Ford HR, Prasadara NV. Recruitment of dendritic cells is responsible for intestinal epithelial damage in the pathogenesis of necrotizing enterocolitis by *Cronobacter sakazakii*. *Journal of immunology (Baltimore Md : 1950)*. 2011; 186:7067–7079.
132. Maheshwari A, Kelly DR, Nicola T, Ambalavanan N, Jain SK, Murphy-Ullrich J, Athar M, Shimamura M, Bhandari V, Aprahamian C, Dimmitt RA, Serra R, Ohls RK. TGF-beta2 suppresses macrophage cytokine production and mucosal inflammatory responses in the developing intestine. *Gastroenterology*. 2011; 140:242–253. [PubMed: 20875417]
133. Halpern MD, Holubec H, Dominguez JA, Meza YG, Williams CS, Ruth MC, McCuskey RS, Dvorak B. Hepatic inflammatory mediators contribute to intestinal damage in necrotizing enterocolitis. *Am J Physiol Gastrointest Liver Physiol*. 2003; 284:G695–G702. [PubMed: 12529262]
134. Lodha A, Asztalos E, Moore AM. Cytokine levels in neonatal necrotizing enterocolitis and long-term growth and neurodevelopment. *Acta paediatrica (Oslo Norway : 1992)*. 2010; 99:338–343.

135. Procianoy RS, Silveira RC. Association between high cytokine levels with white matter injury in preterm infants with sepsis. *Pediatric critical care medicine : a journal of the Society of Critical Care Medicine and the World Federation of Pediatric Intensive and Critical Care Societies*. 2012; 13:183–187.

Fig. 1.
Signaling events leading to NF-κB activation

Fig. 2.
Role of nitric oxide *in vivo*

Fig. 3.
Schematic representation of the pathogenetic events leading to NEC