Published in final edited form as:
Pathophysiology. 2014 February ; 21(1): 81–93. doi:10.1016/j.pathophys.2013.11.007.

Toll-like Receptor Regulation of Intestinal Development and Inflammation in the Pathogenesis of Necrotizing Enterocolitis

Peng Lu[1] and **David J. Hackam**[1,2,*]

[1]Departments of Surgery, University of Pittsburgh School of Medicine

[2]Division of Pediatric Surgery, Children's Hospital of Pittsburgh of UPMC

Abstract

Toll-like receptors (TLRs) are a structurally related family of molecules that respond to a wide variety of endogenous and exogenous ligands, and which serve as important components of the innate immune system. While TLRs have established roles in host defense, these molecules have also been shown to play important roles in the development of various disease states. A particularly important example of the role of TLRs in disease induction includes necrotizing enterocolitis (NEC), which is the most common gastrointestinal disease in preterm infants, and which is associated with extremely high morbidity and mortality rates. The development of NEC is thought to reflect an abnormal interaction between microorganisms and the immature intestinal epithelium, and emerging evidence has clearly placed the spotlight on an important and exciting role for TLRs, particularly TLR4, in NEC pathogenesis. In premature infants, TLR4 signaling within the small intestinal epithelium regulates apoptosis, proliferation and migration of enterocytes, affects the differentiation of goblet cells, and reduces microcirculatory perfusion, which in combination result in the development of NEC. This review will explore the signaling properties of TLRs on hematopoietic and non-hematopoietic cells, and will examine the role of TLR4 signaling in the development of NEC. In addition, the effects of dampening TLR4 signaling using synthetic and endogenous TLR4 inhibitors and active components from amniotic fluid and human milk on NEC severity will be reviewed. In so doing, we hope to present a balanced approach to the understanding of the role of TLRs in both immunity and disease pathogenesis, and to dissect the precise roles for TLR4 in both the cause and therapeutic intervention of necrotizing enterocolitis.

Keywords

Toll-like receptor 4; necrotizing enterocolitis; intestinal inflammation; epithelium; endothelium; amniotic fluid

[*]Please address correspondence and reprint requests to: David J. Hackam, MD, PhD, Division of Pediatric Surgery, Children's Hospital of Pittsburgh of UPMC, One Children's Hospital Drive, 4401 Penn Avenue, Pittsburgh PA 15224, Tel: 412 692 8735, Fax: 412 692 8299, david.hackam@chp.edu.

Introduction

The recognition and clearance of microbial invasion depends on the coordinated activities of both the innate and adaptive immune systems. The Toll-like receptors (TLRs) play key roles in the production of innate inflammatory cytokines and also to regulate adaptive immune responses, and have thus emerged as important components of host defense. In addition to their signature role in host defense, TLRs have also been shown to play important roles in the induction and progression of various inflammatory diseases, in part through their signaling properties on both hematopoietic as well as non-hematopoietic cells. A particularly striking example of the role of TLRs in disease pathogenesis is necrotizing enterocolitis (NEC), a severe and often fatal disease that affects preterm infants whose development requires the activation of TLR4 signaling within the intestinal epithelium. In addition to the roles of TLR4 in driving a pro-inflammatory program, recent data also implicate TLR4 activation in the regulation of intestinal differentiation. We now evaluate the signaling properties of TLR4 within both hematopoietic and non-hematopoietic cells, and summarize the role of TLR4 in the pathogenesis of NEC. We further highlight recent findings for TLR4 in the regulation of intestinal development and inflammation in its pathogenesis. We close by discussing the exciting possibility of targeting TLR4 and its related downstream signaling cascades as novel potential strategies for this devastating disease and others.

General introduction to the TLRs

TLRs are mammalian homologues of the Toll protein identified from Drosophila [1] which was subsequently found to exert anti-fungi functions in a landmark paper by Hoffman and colleagues [2]. TLRs represent a large family of transmembrane molecules which play a pivotal role in innate immune responses against a broad range of pathogens including bacteria, mycobacteria, mycoplasma, fungi and virus [3]. Since the first human TLR was identified in 1997 by Janeway and colleagues [4], 10 TLRs have been identified in humans and 13 in mouse [5–7]. TLRs are expressed on various hematopoietic cells, including macrophages, dendritic cells, B cells [8] and T cells [9], and to a lesser extent, on non-immune cells, including epithelial cells, endothelial cells and fibroblasts [10, 11]. TLRs are present on the cell surface – as is the case for TLR1, TLR5, TLR6, TLR10 and TLR12 [3, 12], or intracellularly within endosomes – as is the case for TLR3, TLR7, TLR8 and TLR9 [12], or both on the cell surface and intracellularly – as is the case for TLR2 [13], TLR4 [14–18] and TLR11 [19], or in endosomes – as is the case for TLR13 [20]. Each TLR detects distinct ligands that may be exogenous and thus pathogen related – the so called pathogen-associated molecular patterns (PAMPs), and also endogenous ligands that are released during injured states, and are termed damage-associated molecular patterns (DAMPs). Examples of PAMPs include lipoproteins (TLR1, TLR2 and TLR6), pathogen nucleic acids (TLR3, TLR7, TLR8 and TLR9), lipopolysaccharide (TLR4), and flagellin (TLR5) [3, 10], while examples of DAMPs include proteins and peptides (TLR1, TLR2, TLR4, TLR7 and TLR8), fatty acids and lipoproteins (TLR2 and TLR4), proteoglycans and glycosaminoglycans (TLR2 and TLR4) and nucleic acids and protein-nucleic acids complexes (TLR3, TLR7, TLR8 and TLR9) [21]. After ligand binding, TLRs dimerize and recruit adaptor molecules in a myeloid differentiation factor 88 (MyD88)-dependent or TIR domain-containing adaptor protein inducing IFN-β (TRIF)-dependent manner [22, 23].

Subsequent downstream signaling leads to the translocation of nuclear factor-κB (NF-κB) from the cytoplasm to the nucleus, and the release of inflammatory cytokines, chemokines and type I interferons (IFNs) [24–27]. In parallel, TLR activation leads to the recruitment of neutrophils [28, 29] and to the activation of macrophages [30], resulting in specific innate and adaptive immunological responses. While TLRs have been well characterized on hematopoietic cells, they are also expressed on various non-hematopoietic cells, including epithelial cells, endothelial cells and fibroblasts, where they may contribute to both host defense and disease development. We will now review the important functions of TLRs in hematopoietic and non-hematopoietic cells, in order to understand the central roles that these molecules play both in host defense and in disease pathogenesis.

TLR signaling in hematopoietic cells

TLRs are highly expressed on hematopoietic cells, including macrophages, dendritic cells, neutrophils and lymphocytes, and TLR activation results in the initiation of both innate and adaptive responses [10, 11]. TLRs activate macrophages to produce pro-inflammatory cytokines and antimicrobial proteins and peptides [31], and to trigger the autophagy pathway to clear the ingested organism [30]. Engagement of hematopoietic cell TLRs also produces type I IFNs [3, 7], initiates phagocytosis of bacteria and viruses [32], and augments macrophage bactericidal activity [33]. TLRs induce dendritic cell maturation and activation, which migrate to the draining lymph nodes, presenting the antigen naïve T cells which induces regulatory T cell subsets [8, 34]. In parallel, the engagement of B cell TLRs induces T-independent antibody responses [35], and TLRs are involved in the reprogramming of regulatory T cells to T helper cells, which plays a role in modulating the adaptive immune defense against certain pathogens [34].

TLR signaling in non-hematopoietic cells

TLRs are also expressed on a variety of non-hematopoietic cells, including epithelial cells and endothelial cells. In the kidney, TLRs (TLR1–6 and TLR11) are found on renal epithelial cells [36–39], and TLRs contribute to the pathogenesis of a number of renal diseases [40–42]. Upon lipopolysaccharide (LPS) stimulation [37], renal tubular epithelial cells secrete CC-chemokines, leading to subsequent leukocyte infiltration and tubular injury during bacterial sepsis [36], and TLR4 on intrinsic renal cells is shown to be required for the initiation of antibacterial immunity during renal infection [42]. Moreover, in renal tubular epithelial cells, bacterial infections release β-defensins [40, 41], small antimicrobial peptides which are also endogenous ligands for TLR4 [43]. TLRs are also found on the renal endothelial cells, and renal vascular endothelial cells are shown to initiate early inflammatory responses in the setting of kidney injury [44]. TLR4 can regulate early endothelial activation during ischemic acute kidney injury, and the endogenous TLR4 ligand high-mobility group protein B1 (HMGB1) released by injured renal cells up-regulates the expression of adhesion molecules CD54 and CD62E on endothelium via TLR4, resulting in the production of cytokines and chemokines, and in tubular apoptosis [45].

In the lung, TLRs (TLR1–11) are shown to be expressed on airway epithelial cells [46], and activation of TLRs induces the production of cytokines [47], chemokines [48, 49], cell

surface adhesion molecules [50] and antimicrobial peptides [51], indicating a role for TLRs in the respiratory epithelium in the development of pulmonary inflammation. TLR9 activation in human bronchial epithelial cells augments interleukin-8 production [49]. TLR4 signaling in the respiratory epithelium is also proven to be critical in the response to inhaled LPS [52], and disruption of NF-κB translocation in the respiratory epithelium attenuates lung inflammation [53]. TLRs also contribute to host defense of the respiratory tract against bacterial, mycobacterial, fungal and viral pathogens [54]. For instance, TLR3, which is found to be constitutively expressed intracellularly in human alveolar and bronchial epithelial cells, is regulated by influenza A virus and by dsRNA, and TLR3 plays the vital role of the immune response of respiratory epithelial cells to influenza A virus and dsRNA [47]. The TLRs also function in respiratory endothelial cells. Endothelial TLR4 functions as the primary intravascular sentinel system for detection of bacteria [55], and LPS mediates endothelial activation in pulmonary endothelial cells [56].

In the intestine, TLRs are proven to be expressed on the intestinal epithelial cells [39, 57–60], intestinal endothelial cells [61, 62] and intestinal fibroblasts [63, 64]. Recent studies have proven that TLRs play a key role in a broad range of intestinal diseases, including inflammatory bowel diseases (IBD, *i.e.* Crohn's disease and ulcerative colitis) and NEC [39, 65, 66]. TLRs, especially TLR2, TLR3, TLR4, TLR5 and TLR9, regulates epithelial cell apoptosis, proliferation and migration, and secretion of IgA and antimicrobial peptides into the intestine lumen [67, 68]. TLR2 maintains tight junction regulation [69], and induces interleukin-11 protecting against lethal colitis in the intestine [70]. TLR2 knockout mice are hyper-susceptible to intestinal injury and inflammation [69]. The expression of TLR3 is down-regulated in active Crohn's disease [58], and TLR3 activation protects against dextran sodium sulfate-induced acute colitis [71]. TLR5 knockout mice develop spontaneous colitis with increased luminal bacteria density [72], and TLR5 activation by basolateral flagellin produces cytokines and chemokines, such as interleukin-8 and CC-chemokine ligand 20 [59, 73], showing a protective role against colitis. TLR9 knockout intestinal epithelial cells show a reduced NF-κB activation threshold, and TLR9 knockout mice are highly susceptible to colitis and NEC [74, 75]. The role of TLR4 in IBD is somewhat complicated. TLR4 is required to maintain intestinal homeostasis and protection against colonic injury [76, 77], and TLR4 is reported to limit the bacterial translocation [77]. However, other studies reveal that patients with IBD show increased expression of TLR4 in the intestinal mucosa [58, 78], and TLR4 induces intestinal damage and colitis [70]. TLR4 can also mediate phagocytosis and translocation of Gram-negative bacteria by enterocytes [14]. These apparently divergent findings suggest that the role of TLR4 may be influenced by the specific model that is used, the anatomic location and the degree of intestinal development [39, 79, 80].

Negative regulators of TLR signaling

A variety of studies have focused on pathways that restrict the extent of TLR signaling. Negative regulators of TLR4 signaling include Toll-interacting protein (TOLLIP) [81], single immunoglobulin IL-1R-related molecule (SIGIRR) [82, 83], IL-1R-associated kinases M (IRAK-M) [84], peroxisome proliferator activated receptor-γ (PPARγ) [85, 86] and A20 [87], which down-regulate the extent of TLR4 signaling, and reduce the production of inflammatory cytokines [60]. TOLLIP is an intracellular protein that inhibits TLR2 and

TLR4 signaling [88, 89] by reducing the MyD88-dependent NF-κB activation pathways [90]. Stimulation of intestinal epithelial cells with LPS and flagellin increases the expression of TOLLIP [91], while TOLLIP expression in intestinal epithelial cells isolated from inflamed IBD patients is not increased compared with that from non-inflamed IBD patients [92]. SIGIRR regulates colonic epithelial homeostasis via inhibition of TLR-induced NF-κB activation [83], and experimental colitis mediates the down-regulation of SIGIRR in intestinal epithelial cells [93]. SIGIRR knockout mice have normal susceptibility to systemic LPS toxicity but hyper-susceptibility to intestinal inflammation [94, 95], suggesting an intestinal specific role of SIGIRR in intestinal inflammation. IRAK-M is a negative regulator of TLR signaling [84], and IRAK-M is shown to down-regulate dextran sulfate sodium-induced colitis [96]. The colonic epithelial cells from ulcerative colitis patients express lower levels of PPARγ [85], while PPARγ ligand suppresses LPS-induced NF-κB promoter activity and dampens the inflammation in intestinal epithelial cells [97]. A20 is a deubiquitinating protein which inhibits NF-κB, and A20 knockout mice develop severe inflammation and tissue damage in multiple organs including intestine [98]. Enterocyte-specific A20 knockout mice do not show spontaneous intestinal inflammation, but exhibit increased susceptibility to experimental colitis and tumor necrosis factor α [99]. Another molecule that has been shown to negatively regulate TLR4 signaling and NF-κB activation are secretory leukocyte peptidase inhibitor (SLPI). SLPI is a negative regulator of NF-κB-mediated activation, and SLPI is up-regulated in Crohn's disease patients and detected by immunostaining in epithelial cells of inflamed tissue [100]. A recent study reported the important role of SLPI in the recovery from inflammation [101].

The role of TLR4 in the pathogenesis of NEC

NEC is the leading cause of morbidity and mortality from gastrointestinal disease in prematurely born infants (*i.e.* those born before 37 weeks gestational age) [102, 103]. NEC is characterized by coagulation necrosis of patchy areas of the ileum, and to a lesser extent, of the jejunum, colon and duodenum [103], and may rapidly progress to overwhelming sepsis, multisystem organ failure, and death [104–106]. Despite overall improvements in neonatal care, the survival for patients who require surgery remains only about 50% [107]. In addition to the early morbidity and mortality, those patients who survive NEC often experience long-term complications such as short bowel syndrome and neurodevelopmental impairment [108, 109]. Unfortunately, the lack of specific and effective treatment strategies for NEC contributes largely to the overall high morbidity and mortality in patients with this disease.

The precise pathogenesis of NEC remains incompletely understood, and is largely thought to be multifactorial. In the last decade, emerging evidence has pointed to a pivotal role for the interactions among a premature host, an inappropriate response to the colonization of the gastrointestinal tract by microorganisms, and the provision of formula feeding [110–113]. Several clinical observations highlight the importance of these features as potentially playing a role in disease pathogenesis. For instance, the development of NEC is almost exclusively restricted to infants born prematurely [114], and the onset of NEC usually occurs at 8–10 days after birth, the time when Gram-negative bacteria and other microorganisms colonize the premature intestine [112, 115, 116]. A successful and well

coordinated adaptation to colonization of the gut by microbes is essential, and infants with NEC show abnormal and inappropriate bacteria colonization as manifest by low microbiota diversity [117], increased Gram-negative pathogen colonization and high levels of pathogens in the peritoneal cavities [118] in NEC infants. Accordingly, administration of broad-spectrum antibiotics in newborn mice [66] and NEC infants [119, 120] decreases bacterial load and protects against the development of NEC.

Recent studies have identified a critical role for TLRs, particularly TLR4, in NEC pathogenesis [65]. Early studies reveal that TLR4 is expressed on the apical surface of enterocytes [121–123], and TLR4 mediates phagocytosis and translocation of Gram-negative bacteria by enterocytes [14]. Nanthakumar *et al.* and Fusunyan *et al.* show that human fetal enterocytes exhibit prominent TLR4 expression compared with adult enterocytes [124–126], and Lotz *et al.* shows that TLR4 signaling by microorganism colonization is decreased in vaginal delivered term mice but not in Caesarean sectioned preterm mice [127], which leads to the concept that TLR4 signaling triggered by earlier exposure to microorganisms might be exaggerated in preterm intestine. In line with the above observations, studies have shown that increased TLR4 expression and up-regulated TLR4 signaling are associated with NEC in mice, rat and premature infants [106, 128–131]. In addition, NEC is associated with increased TLR4 activator HMGB1 [132], co-receptor myeloid differentiation protein-2 (MD-2) [133] and co-receptor cluster of differentiation 14 [134]. Proof that TLR4 is required for the pathogenesis of NEC is found in studies by Jilling *et al.* [128], as well as from our group [106, 135], in which TLR4 mutant mice were protected from the development of NEC as compared to wild-type mice. Furthermore, TLR4 knockout mice show preservation of the small intestinal mucosa against NEC induction [66], and reduction of TLR4 expression by polyunsaturated fatty acids in rat is protective from the development of NEC [131]. Human NEC is also associated with increased circulating and luminal levels of platelet-activating factor, which up-regulates TLR4 expression [136], and platelet-activating factor inactivation decreases NEC in a neonatal rat model [137], further indicating the pathogenic role of TLR4 in the development of NEC. Moreover, NEC in humans is associated with significantly increased TLR4 in the intestine compared with patients that do not develop NEC, at similar gestational ages [106]. We will now explore in some detail the mechanisms by which TLR4 signaling within the intestinal epithelium leads to the development of NEC.

TLR4 regulation of intestinal injury and repair in the newborn gut

Jilling *et al.* reported in 2006 that the TLR4 mutant C3H/HeJ mice are protected from the development of NEC [128], but the precise mechanisms by which TLR4 acted to mediate NEC, and indeed, a direct link between TLR4 and the development of intestinal inflammation, remained unproven. We confirmed Jilling's findings in C3H/HeJ mice [106], as well as in TLR4 knockout mice [66, 135]. In seeking to understand the mechanisms involved, we demonstrated that TLR4 activation leads to an increase in enterocyte apoptosis and a reduction in enterocyte proliferation and migration in the premature intestine, which promotes intestinal injury and reduces the capacity of mucosal repair [106, 138, 139]. TLR4 activation was found to significantly inhibit enterocyte proliferation in the ileum of newborn mice, but not of adult mice [135]. In additional studies, we found that TLR4 signaling within

enterocytes leads to the phosphorylation of glycogen synthase kinase 3β and inhibition of the β-catenin signaling pathway, which ultimately reduced the extent of enterocyte proliferation [135]. In focusing on the important cells that mediate replenishment of the injured mucosa, we determined that TLR4 is expressed on the surface of Leucine-rich repeat-containing G protein-coupled receptor 5-positive intestinal stem cells, and more interestingly, TLR4 activation causes decreased proliferation and increased apoptosis in intestinal stem cells [138]. Such an impairment of intestinal stem cell proliferation and number by TLR4 was found to be dependent on the activation of p53-up-regulated modulator of apoptosis, a crucial mediator of p53-dependent and p53-independent apoptosis [140, 141], which prevented cell division [138].

In addition to deleterious effects on apoptosis and proliferation, TLR4 activation also causes a reduction in enterocyte migration [106, 142]. Enterocytes migrate up toward the villus tip or across a wound, providing a protective mechanism to repair minor mucosal damages [143]. On the contrary, disruption in enterocyte migration markedly reduces intestinal repair and regeneration [144], and exposure of enterocytes to LPS leads to significantly decreased enterocyte migration due to an increase in focal adhesion kinase-dependent cell matrix adhesiveness [106, 121, 142, 144]. In seeking to understand the mechanisms by which TLR4 activation can impair enterocyte migration, we have shown that TLR4 signaling leads to a marked induction of autophagy within the intestinal epithelium, which is required for the impaired migration to occur [126]. The premature intestine in human and mice showed increased autophagy genes compared with mature intestine, and the increased enterocyte autophagy is required for NEC development, as mice lacking the autophagy gene autophagy-related protein 7 from the intestinal epithelium did not develop NEC as compared to wild-type mice [126]. TLR4 induced autophagy causes impairment in enterocyte migration in a mechanism which requires activation of Rho-GTPase in enterocytes [126]. HMGB1, another TLR4 activator, affects cell-matrix adhesiveness in enterocytes, and HMGB1-induced TLR4 activation inhibits enterocyte migration through activation of the RhoA (Ras homolog gene family, member A) as indicated by increased phosphorylation of cofilin [139]. Taken together, these findings show that TLR4 signaling leads to impairment in enterocyte migration through effects on cell-matrix interactions, enterocyte apoptosis and cell adhesions.

TLR4 signaling in epithelial and endothelial cells is required for the development of NEC

As described above, TLR4 is expressed on hematopoietic cells [10, 11], as well as non-hematopoietic cells including epithelial cells [124] and endothelial cells [145]. To investigate the precise role of TLR4 in NEC development in epithelial cells and endothelial cells, we generated mice lacking TLR4 selectively within the intestinal epithelium ($TLR4^{\Delta IEC}$ mice) [66] and within endothelium ($TLR4^{\Delta endoth}$ mice) [146]. TLR4 activation in the intestinal epithelium was found to lead to NEC in part through the induction of mucosal injury via increased apoptosis, as well as through reduced mucosal repair, and through reduced proliferation and migration [106, 135, 139]. In addition, TLR4 activation in the intestinal epithelium was found to play a previously unrecognized role in intestinal

differentiation, as TLR4$^{\Delta IEC}$ mice showed significantly increased goblet cell differentiation as well as reduced NEC severity which is comparable to that seen in TLR4 knockout mice, which lack TLR4 in all cells [66]. In seeking to understand the mechanisms by which TLR4 regulates enterocyte differentiation, TLR4 deletion was found to suppress Notch signaling in the small intestinal epithelium, which resulted in increased goblet cell differentiation and decreased NEC severity [66]. Interestingly, bile acids, which are significantly increased during the development of NEC [147] and are associated with reduced goblet cells and decreased mucin Muc2 expression in premature ileum [148], are significantly reduced in TLR4$^{\Delta IEC}$ mice and TLR4 knockout mice [66].

TLR4 activation in the endothelium was found to be required for the development of NEC as NEC severity was significantly reduced in TLR4$^{\Delta endoth}$ mice [146]. Detailed analysis revealed that TLR4 activation in the endothelium leads to a reduction in endothelial nitric oxide synthase (eNOS), while the expression of eNOS is significantly reduced in NEC. In addition, eNOS knockout mice developed severe NEC [146]. ENOS is responsible for the generation of nitric oxide [149], which regulates microcirculatory perfusion in intestine [150]. Strikingly, TLR4 activation significantly reduced microcirculatory perfusion in the immature intestine in TLR4 knockout mice, while it does not impair perfusion of the intestinal microcirculation in TLR4$^{\Delta endoth}$ mice [146]. The above observations strongly suggest that TLR4 activation in the endothelium leads to the development of NEC via the reduction of eNOS and intestinal microcirculatory perfusion. Importantly, the administration of the nitric oxide donor sodium nitrate to mice that had been administered formula was found to restore mesenteric perfusion in the face of persistent endothelial TLR4 signaling, and thus to reverse the deleterious effects of formula administration on NEC development [146]. Consistent with these findings, the administration of the phosphodiesterase-5 inhibitor sildenafil to maintain intraluminal nitric oxide activity [151] also markedly reduces NEC severity [146], while eNOS deficient mice demonstrated a significantly increased NEC phenotype. These findings confirm the importance of endothelial nitric oxide regulation in NEC pathogenesis, and may explain why infant formula – which is deficient in nitrate – may predispose to the development of this disease.

Cellular regulation of TLR4 signaling as a therapeutic target in NEC

Having shown that TLR4 activation plays a pivotal role in the development of NEC, the question emerges as to whether TLR4 and its associated downstream pathways can serve as preventive or therapeutic targets against NEC. Below, we will summarize recent studies that have tried to answer this question.

i. Intracellular inhibitor, heat shock protein 70 (HSP70)

HSP70 is an important member of the heat shock protein family, which is strongly up-regulated by various environmental stressors including heat, but also reactive oxygen species [152, 153], ultraviolet light [154], γ-irradiation [155] and chemicals [156]. We have recently shown that HSP70 is down-regulated in the inflamed intestine in NEC [157], while others have reported that HSP70 is decreased in IBD [158]. In seeking to understand the role of HSP70 down-regulation in NEC, we showed that intracellular HSP70 induction in enterocytes up-regulates co-chaperone carboxyl-terminus of HSP70 interacting protein-

mediated ubiquitination of TLR4, which leads to the proteasomal degradation of TLR4, and subsequently a down-regulation of NEC severity [157]. Importantly, we showed that the administration of the cell permeable triterpenoid antioxidant celastrol led to the pharmacologic induction of intracellular HSP70 [159, 160], which inhibited TLR4 signaling in the intestinal epithelium and attenuated NEC severity [157]. Glutamine, one of the non-essential amino acids, protects against gut injury and promotes mucosal healing through HSP70 induction [161], and glutamine treatment is associated with TLR4 down-regulation [162]. Interestingly, glutamine supplementation was shown to reduces the histologic evidence of hypoxia-reoxygenation-induced intestinal injury in experimental NEC [163], and was suggested to have beneficial effects on intestinal integrity and the overall incidence of NEC in preterm infants [164].

ii. Inhibition of Notch signaling

It has been suggested that increased luminal bile acids [165], abnormal bile acid metabolism [147] and dysregulated bile acid transporters [166, 167] contribute to NEC via dampening goblet cell differentiation and decreasing mucin Muc2 expression in premature ileum [148]. We further showed that bile acids activated Notch signaling plays a role in the development of NEC [66]. These findings suggest that the inhibition of Notch signaling could serve as a preventive or therapeutic target in patients with NEC. In support of this possibility, the administration of dibenzazepine, a pharmacologic inhibitor of γ-secretase and Notch signaling [168], was found to markedly increase the number of goblet cells in the premature intestine and to therefore dramatically reduce NEC severity [66]. These findings raise the possibility that small molecule approaches that regulate Notch signaling could prevent or treat NEC in part through effects on goblet cell differentiation.

iii. Crosstalk between TLR4 and other innate immune receptors (TLR9 and NOD2)

Several studies have indicated that the crosstalk between TLR4 and other innate immune receptors play essential roles in the extent and severity of the innate immune response in the gut in the pathogenesis of NEC. For instance, the TLR4 homologue TLR9, which recognizes the CpG-DNA from bacteria, mycobacteria, parasite *plasmodium* and viruses [10], has been shown to limit the extent of TLR4 signaling in enterocytes [75]. NEC in both mice and humans was associated with decreased expression of TLR9 in the intestine, and TLR9 knockout mice exhibited increased NEC severity [75]. Although TLR9 can induce inflammatory signaling when signaling in hematopoietic cells [169], TLR9 activation with CpG-DNA inhibited TLR4 signaling in enterocytes both *in vitro* and in the premature intestine in a mechanism dependent upon the inhibitory molecule IRAK-M [75], a negative regulator of TLR4 signaling [84]. Importantly, the administration of CpG-DNA dramatically reduced NEC development through reduced enterocyte apoptosis and bacterial translocation [75]. Another innate immune receptor, nucleotide-binding oligomerization domain-2 (NOD2), is a member of nucleotide oligomerization domain-like receptor family, which regulates the host innate immune response through recognition of muramyl-di-peptide (MDP) which is present on gram positive bacteria [170]. NOD2 activation by the bacterial ligand MDP was found to inhibit TLR4 signaling in enterocytes, resulting in reduced enterocyte apoptosis [171]. Administration of MDP down-regulated the apoptosis regulatory protein SMAC-DIABLO (second mitochondria-derived activator of caspase/direct inhibitor

of apoptosis-binding protein with low PI) [171], a mitochondrial protein which promotes caspase activation by binding to inhibitor of apoptosis proteins and removing their inhibitory activity [172], and markedly attenuates the degree of intestinal injury and NEC severity [171]. These findings suggest that that modulation of the crosstalk between various innate immune receptors within the intestine of the premature infant may be harnessed in order to reduce NEC severity.

Reduction in NEC severity through the administration of amniotic fluid

The developing fetus continuously swallows amniotic fluid, which then passes through the premature gastrointestinal tract. Although amniotic fluid is not the nutritional mainstay of the fetus [173], amniotic fluid acts as a source of growth factors, and is particularly abundant in epidermal growth factor [174, 175]. Given that the premature infant is not exposed to amniotic fluid as a necessary consequence of early delivery, we sought to investigate whether amniotic fluid supplementation could attenuate NEC severity, and if so, whether amniotic fluid could inhibit TLR4 signaling within the developing gut. In support of this possibility, we showed that the administration of amniotic fluid reduced the severity of NEC in mice [176], a finding that was subsequently confirmed in piglet models by others [177]. Importantly, we showed that amniotic fluid reduced TLR4 signaling in the gut through the activation of epidermal growth factor receptor, which directly reduced the extent of TLR4 signaling through up-regulation of the transcription factor PPAR-γ [176], which exerts anti-inflammatory effects within the intestine [178]. These findings reveal that the susceptibility of premature infants to NEC may stem in part from exaggerated TLR4 signaling in the absence of amniotic fluid, and also suggest that the administration of amniotic fluid – either natural or synthetically manufactured – could play a protective or therapeutic role in NEC [176].

In silico search for small molecules that could prevent or treat NEC

Having shown the importance of TLR4 signaling in the pathogenesis of NEC, we sought to identify novel TLR4 inhibitors that could have therapeutic benefit. To do so, we performed an *in silico* similarity search based on the structure of E5564, a synthetic lipid A analogue which was shown to inhibit LPS signaling [179, 180]. As a result of these studies, we identified and subsequently synthesized a novel TLR4 inhibitor – which we have termed C34 [181]. C34 is an aminomonosaccharide which docks into the hydrophobic pocket in MD-2, resulting inhibition of TLR4 signaling. Administration of C34 in experimental NEC attenuated NEC severity, and strikingly, C34 inhibited TLR4 signaling in human tissue *ex vivo* obtained from infants with NEC, indicating the possibility to use C34 as a new therapeutic option in NEC [181]. Importantly, C34 is an aminomonosaccharide, which shares molecular similarities with other oligosaccharides that are present in human breast milk. Human milk is well known to be the most important nutritional strategy to attenuate NEC development, although the protective properties of NEC are incompletely understood [182–184]. This suggests the possibility that other identified human milk oligosaccharides, such as disialyllacto-N-tetraose, could act in the protection of NEC through previously unrecognized inhibitory effects on TLR4 signaling.

Lactobacillus reuteri reduce NEC incidence and severity via modulation of TLR4

Probiotics, beneficial live microorganisms when administered to the host, has been suggested to be associated with reduced NEC incidence and severity in both animal NEC models [185–188] and infants [189–191]. *L. reuteri* strains DSM 17938 and ATCC PTA 4659 were proven to decrease the expression of TLR4 and NF-κB, inhibit phosphorylation of NF-κB inhibitor IκB, and down-regulate the expression of inflammatory cytokines TNF-α, IL-6 and IL-1β in experimental NEC rat models [191]. Moreover, prophylactic initiation of *L. reuteri* DSM 17938 showed a significant decreasing on feeding tolerance and duration of hospitalization in premature infants with birth weight less than 1500 grams [192] and a significant benefit for prevention of NEC in infants with birth weight less than 1000 grams [191].

Putting it all together: summary and future directions

Given the high morbidity and mortality that is seen in patients with NEC, it is clear that more effective preventative and therapeutic strategies are desperately needed. We have identified a critical role for TLR4 signaling in the intestinal epithelium in the development of NEC, as TLR4 signaling is elevated in humans who develop NEC, while mice lacking TLR4 are protected from disease development. The premature infant is particularly susceptible to NEC development as TLR4 expression is significantly increased in the premature intestine as compared to the full term intestine, which reflects the recently recognized role of TLR4 in regulating normal intestinal differentiation. We and others have shown that in the post-natal period, TLR4 regulates the balance between injury and repair in the premature intestinal tract as well as the extent of perfusion of the intestine, while the crosstalk between different innate immune receptors and negative regulatory factors can influence the extent of TLR4 activation that occurs in response to microbial colonization of the premature infant GI tract. Insights from the use of biologic fluids such as amniotic fluid, as well as from small molecules that are found within breast milk, may expand our understanding of the pathogenesis of NEC, while also suggesting novel therapeutic strategies for this disease. In fact, based upon the studies summarized above, there is reason to believe that a variety of new and promising therapies against NEC may be on the horizon, including celastrol, which induces intracellular HSP70 expression, dibenzazepine, which inhibit Notch signaling and increases goblet cell differentiation, CpG-DNA, which activates TLR9 and reduces TLR4 signaling in the gut, MDP, which stimulates NOD2 signaling and negatively regulates TLR4, epidermal growth factor, which increases PPAR-γ and reduces the extent of TLR4-induced inflammation, sodium nitrate and sildenafil which preserve intestinal perfusion in the face of tonic endothelial TLR4 signaling, novel molecules such as the amino-monosaccharide C34, which docks with and prevents downstream signaling of TLR4 co-receptor MD-2, and probiotics *L. reuteri*, which inhibit TLR4 and NF-κB, thereby significantly inhibiting TLR4 activation and downstream signaling cascades and markedly reducing NEC severity. Future studies on understanding the precise point at which TLR4 signaling becomes injurious to the premature infant, and factors that influence disease susceptibility and therefore may serve as important biomarkers to predict disease

development will enhance our understanding of how to more effectively treat these fragile patients, and in so doing, will hopefully reduce the severity of this devastating disease.

Acknowledgments

Research support

DJH is supported by The Hartwell Foundation, Memphis, TN and R01GM078238, RO1DK08752 and P50GM053789 from the NIH.

Abbreviations

TLR	Toll-like receptor
NEC	necrotizing enterocolitis
PAMP	pathogen-associated molecular pattern
DAMP	damage-associated molecular pattern
MyD88	myeloid differentiation factor 88
TRIF	TIR domain-containing adaptor protein inducing IFN-β
NF-κB	nuclear factor-κB
IFN	interferon
LPS	lipopolysaccharide
HMGB1	high-mobility group protein B1
IBD	inflammatory bowel diseases
TOLLIP	Toll-interacting protein
SIGIRR	single immunoglobulin IL-1R-related molecule
IRAK-M	IL-1R-associated kinases M
PPARγ	peroxisome proliferator activated receptor-γ
SLPI	secretory leukocyte peptidase inhibitor
MD-2	myeloid differentiation protein-2
RhoA	Ras homolog gene family, member A
eNOS	endothelial nitric oxide synthase
HSP70	heat shock protein 70
NOD2	nucleotide-binding oligomerization domain-2
MDP	muramyl-di-peptide
SMAC-DIABLO	second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low PI

References

1. Anderson KV, Jurgens G, Nusslein-Volhard C. Establishment of dorsal-ventral polarity in the Drosophila embryo: genetic studies on the role of the Toll gene product. Cell. 1985; 42:779–789. [PubMed: 3931918]
2. Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell. 1996; 86:973–983. [PubMed: 8808632]
3. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity. 2011; 34:637–650. [PubMed: 21616434]
4. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature. 1997; 388:394–397. [PubMed: 9237759]
5. Roach JC, Glusman G, Rowen L, Kaur A, Purcell MK, Smith KD, Hood LE, Aderem A. The evolution of vertebrate Toll-like receptors. Proceedings of the National Academy of Sciences of the United States of America. 2005; 102:9577–9582. [PubMed: 15976025]
6. Kawai T, Akira S. TLR signaling. Cell death and differentiation. 2006; 13:816–825. [PubMed: 16410796]
7. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nature immunology. 2010; 11:373–384. [PubMed: 20404851]
8. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nature immunology. 2004; 5:987–995. [PubMed: 15454922]
9. Dai J, Liu B, Li Z. Regulatory T cells and Toll-like receptors: what is the missing link? International immunopharmacology. 2009; 9:528–533. [PubMed: 19539562]
10. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006; 124:783–801. [PubMed: 16497588]
11. Miyake K. Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors. Seminars in immunology. 2007; 19:3–10. [PubMed: 17275324]
12. Blasius AL, Beutler B. Intracellular toll-like receptors. Immunity. 2010; 32:305–315. [PubMed: 20346772]
13. Uronen-Hansson H, Allen J, Osman M, Squires G, Klein N, Callard RE. Toll-like receptor 2 (TLR2) and TLR4 are present inside human dendritic cells, associated with microtubules and the Golgi apparatus but are not detectable on the cell surface: integrity of microtubules is required for interleukin-12 production in response to internalized bacteria. Immunology. 2004; 111:173–178. [PubMed: 15027902]
14. Neal MD, Leaphart C, Levy R, Prince J, Billiar TR, Watkins S, Li J, Cetin S, Ford H, Schreiber A, Hackam DJ. Enterocyte TLR4 mediates phagocytosis and translocation of bacteria across the intestinal barrier. J Immunol. 2006; 176:3070–3079. [PubMed: 16493066]
15. Dunzendorfer S, Lee HK, Soldau K, Tobias PS. Toll-like receptor 4 functions intracellularly in human coronary artery endothelial cells: roles of LBP and sCD14 in mediating LPS responses. Faseb J. 2004; 18:1117–1119. [PubMed: 15132988]
16. Hornef MW, Frisan T, Vandewalle A, Normark S, Richter-Dahlfors A. Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. The Journal of experimental medicine. 2002; 195:559–570. [PubMed: 11877479]
17. Hornef MW, Normark BH, Vandewalle A, Normark S. Intracellular recognition of lipopolysaccharide by toll-like receptor 4 in intestinal epithelial cells. The Journal of experimental medicine. 2003; 198:1225–1235. [PubMed: 14568981]
18. Latz E, Visintin A, Lien E, Fitzgerald KA, Monks BG, Kurt-Jones EA, Golenbock DT, Espevik T. Lipopolysaccharide rapidly traffics to and from the Golgi apparatus with the toll-like receptor 4-MD-2-CD14 complex in a process that is distinct from the initiation of signal transduction. The Journal of biological chemistry. 2002; 277:47834–47843. [PubMed: 12324469]
19. Pifer R, Benson A, Sturge CR, Yarovinsky F. UNC93B1 is essential for TLR11 activation and IL-12-dependent host resistance to Toxoplasma gondii. The Journal of biological chemistry. 2011; 286:3307–3314. [PubMed: 21097503]

20. Brinkmann MM, Spooner E, Hoebe K, Beutler B, Ploegh HL, Kim YM. The interaction between the ER membrane protein UNC93B and TLR3, 7, and 9 is crucial for TLR signaling. The Journal of cell biology. 2007; 177:265–275. [PubMed: 17452530]
21. Piccinini AM, Midwood KS. DAMPening inflammation by modulating TLR signalling. Mediators of inflammation. 2010; 2010
22. Yamamoto, M.; Sato, S.; Hemmi, H.; Hoshino, K.; Kaisho, T.; Sanjo, H.; Takeuchi, O.; Sugiyama, M.; Okabe, M.; Takeda, K.; Akira, S. Science. Vol. 301. New York, N.Y: 2003. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway; p. 640-643.
23. O'Neill LA, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol. 2007; 7:353–364. [PubMed: 17457343]
24. Takeda K, Akira S. TLR signaling pathways. Seminars in immunology. 2004; 16:3–9. [PubMed: 14751757]
25. Barton GM, Kagan JC. A cell biological view of Toll-like receptor function: regulation through compartmentalization. Nat Rev Immunol. 2009; 9:535–542. [PubMed: 19556980]
26. Takeda K, Akira S. Roles of Toll-like receptors in innate immune responses. Genes Cells. 2001; 6:733–742. [PubMed: 11554921]
27. Palsson-McDermott EM, O'Neill LA. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. Immunology. 2004; 113:153–162. [PubMed: 15379975]
28. Hayashi F, Means TK, Luster AD. Toll-like receptors stimulate human neutrophil function. Blood. 2003; 102:2660–2669. [PubMed: 12829592]
29. Parker LC, Whyte MK, Dower SK, Sabroe I. The expression and roles of Toll-like receptors in the biology of the human neutrophil. Journal of leukocyte biology. 2005; 77:886–892. [PubMed: 15728244]
30. Sanjuan MA, Dillon CP, Tait SW, Moshiach S, Dorsey F, Connell S, Komatsu M, Tanaka K, Cleveland JL, Withoff S, Green DR. Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. Nature. 2007; 450:1253–1257. [PubMed: 18097414]
31. Medzhitov R. Recognition of microorganisms and activation of the immune response. Nature. 2007; 449:819–826. [PubMed: 17943118]
32. Blander JM. Coupling Toll-like receptor signaling with phagocytosis: potentiation of antigen presentation. Trends in immunology. 2007; 28:19–25. [PubMed: 17126600]
33. West AP, Brodsky IE, Rahner C, Woo DK, Erdjument-Bromage H, Tempst P, Walsh MC, Choi Y, Shadel GS, Ghosh S. TLR signalling augments macrophage bactericidal activity through mitochondrial ROS. Nature. 2011; 472:476–480. [PubMed: 21525932]
34. Jin B, Sun T, Yu XH, Yang YX, Yeo AE. The effects of TLR activation on T-cell development and differentiation. Clinical & developmental immunology. 2012; 2012:836485. [PubMed: 22737174]
35. Pone EJ, Xu Z, White CA, Zan H, Casali P. B cell TLRs and induction of immunoglobulin class-switch DNA recombination. Front Biosci. 2012; 17:2594–2615.
36. Tsuboi N, Yoshikai Y, Matsuo S, Kikuchi T, Iwami K, Nagai Y, Takeuchi O, Akira S, Matsuguchi T. Roles of toll-like receptors in C-C chemokine production by renal tubular epithelial cells. J Immunol. 2002; 169:2026–2033. [PubMed: 12165529]
37. Wolfs TG, Buurman WA, van Schadewijk A, de Vries B, Daemen MA, Hiemstra PS, van 't Veer C. In vivo expression of Toll-like receptor 2 and 4 by renal epithelial cells: IFN-gamma and TNF-alpha mediated up-regulation during inflammation. J Immunol. 2002; 168:1286–1293. [PubMed: 11801667]
38. Anders HJ, Banas B, Schlondorff D. Signaling danger: toll-like receptors and their potential roles in kidney disease. J Am Soc Nephrol. 2004; 15:854–867. [PubMed: 15034087]
39. Gribar SC, Richardson WM, Sodhi CP, Hackam DJ. No longer an innocent bystander: epithelial toll-like receptor signaling in the development of mucosal inflammation. Molecular medicine, (Cambridge, Mass. 2008; 14:645–659.
40. Nitschke M, Wiehl S, Baer PC, Kreft B. Bactericidal activity of renal tubular cells: the putative role of human beta-defensins. Experimental nephrology. 2002; 10:332–337. [PubMed: 12381917]
41. Lehmann J, Retz M, Harder J, Krams M, Kellner U, Hartmann J, Hohgrawe K, Raffenberg U, Gerber M, Loch T, Weichert-Jacobsen K, Stockle M. Expression of human beta-defensins 1 and 2

in kidneys with chronic bacterial infection. BMC infectious diseases. 2002; 2:20. [PubMed: 12238953]

42. Patole PS, Schubert S, Hildinger K, Khandoga S, Khandoga A, Segerer S, Henger A, Kretzler M, Werner M, Krombach F, Schlondorff D, Anders HJ. Toll-like receptor-4: renal cells and bone marrow cells signal for neutrophil recruitment during pyelonephritis. Kidney international. 2005; 68:2582–2587. [PubMed: 16316333]
43. Biragyn, A.; Ruffini, PA.; Leifer, CA.; Klyushnenkova, E.; Shakhov, A.; Chertov, O.; Shirakawa, AK.; Farber, JM.; Segal, DM.; Oppenheim, JJ.; Kwak, LW. Science. Vol. 298. New York, N.Y: 2002. Toll-like receptor 4-dependent activation of dendritic cells by beta-defensin 2; p. 1025-1029.
44. Akcay A, Nguyen Q, Edelstein CL. Mediators of inflammation in acute kidney injury. Mediators of inflammation. 2009; 2009:137072. [PubMed: 20182538]
45. Chen J, John R, Richardson JA, Shelton JM, Zhou XJ, Wang Y, Wu QQ, Hartono JR, Winterberg PD, Lu CY. Toll-like receptor 4 regulates early endothelial activation during ischemic acute kidney injury. Kidney international. 2011; 79:288–299. [PubMed: 20927041]
46. Greene CM, McElvaney NG. Toll-like receptor expression and function in airway epithelial cells. Archivum immunologiae et therapiae experimentalis. 2005; 53:418–427. [PubMed: 16314825]
47. Guillot L, Le Goffic R, Bloch S, Escriou N, Akira S, Chignard M, Si-Tahar M. Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. The Journal of biological chemistry. 2005; 280:5571–5580. [PubMed: 15579900]
48. Sha Q, Truong-Tran AQ, Plitt JR, Beck LA, Schleimer RP. Activation of airway epithelial cells by toll-like receptor agonists. American journal of respiratory cell and molecular biology. 2004; 31:358–364. [PubMed: 15191912]
49. Parilla NW, Hughes VS, Lierl KM, Wong HR, Page K. CpG DNA modulates interleukin 1beta-induced interleukin-8 expression in human bronchial epithelial (16HBE14o-) cells. Respiratory research. 2006; 7:84. [PubMed: 16740161]
50. Greene CM, Carroll TP, Smith SG, Taggart CC, Devaney J, Griffin S, O'Neill J, McElvaney SNG. TLR-induced inflammation in cystic fibrosis and non-cystic fibrosis airway epithelial cells. J Immunol. 2005; 174:1638–1646. [PubMed: 15661927]
51. Hertz CJ, Wu Q, Porter EM, Zhang YJ, Weismuller KH, Godowski PJ, Ganz T, Randell SH, Modlin RL. Activation of Toll-like receptor 2 on human tracheobronchial epithelial cells induces the antimicrobial peptide human beta defensin-2. J Immunol. 2003; 171:6820–6826. [PubMed: 14662888]
52. Noulin N, Quesniaux VF, Schnyder-Candrian S, Schnyder B, Maillet I, Robert T, Vargaftig BB, Ryffel B, Couillin I. Both hemopoietic and resident cells are required for MyD88-dependent pulmonary inflammatory response to inhaled endotoxin. J Immunol. 2005; 175:6861–6869. [PubMed: 16272344]
53. Skerrett SJ, Liggitt HD, Hajjar AM, Ernst RK, Miller SI, Wilson CB. Respiratory epithelial cells regulate lung inflammation in response to inhaled endotoxin. Am J Physiol Lung Cell Mol Physiol. 2004; 287:L143–L152. [PubMed: 15047567]
54. Kovach MA, Standiford TJ. Toll like receptors in diseases of the lung. International immunopharmacology. 2011; 11:1399–1406. [PubMed: 21624505]
55. Andonegui G, Zhou H, Bullard D, Kelly MM, Mullaly SC, McDonald B, Long EM, Robbins SM, Kubes P. Mice that exclusively express TLR4 on endothelial cells can efficiently clear a lethal systemic Gram-negative bacterial infection. The Journal of clinical investigation. 2009; 119:1921–1930. [PubMed: 19603547]
56. Menden H, Tate E, Hogg N, Sampath V. LPS-mediated endothelial activation in pulmonary endothelial cells: role of Nox2-dependent IKK-beta phosphorylation. Am J Physiol Lung Cell Mol Physiol. 2012; 304:L445–L455. [PubMed: 23333803]
57. Cario E, Brown D, McKee M, Lynch-Devaney K, Gerken G, Podolsky DK. Commensal-associated molecular patterns induce selective toll-like receptor-trafficking from apical membrane to cytoplasmic compartments in polarized intestinal epithelium. The American journal of pathology. 2002; 160:165–173. [PubMed: 11786410]

58. Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. Infection and immunity. 2000; 68:7010–7017. [PubMed: 11083826]
59. Gewirtz AT, Navas TA, Lyons S, Godowski PJ, Madara JL. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. J Immunol. 2001; 167:1882–1885. [PubMed: 11489966]
60. Gopal R, Birdsell D, Monroy FP. Regulation of toll-like receptors in intestinal epithelial cells by stress and Toxoplasma gondii infection. Parasite immunology. 2008; 30:563–576. [PubMed: 19067837]
61. Ogawa H, Rafiee P, Heidemann J, Fisher PJ, Johnson NA, Otterson MF, Kalyanaraman B, Pritchard KA Jr, Binion DG. Mechanisms of endotoxin tolerance in human intestinal microvascular endothelial cells. J Immunol. 2003; 170:5956–5964. [PubMed: 12794122]
62. Maaser C, Heidemann J, von Eiff C, Lugering A, Spahn TW, Binion DG, Domschke W, Lugering N, Kucharzik T. Human intestinal microvascular endothelial cells express Toll-like receptor 5: a binding partner for bacterial flagellin. J Immunol. 2004; 172:5056–5062. [PubMed: 15067088]
63. Otte JM, Rosenberg IM, Podolsky DK. Intestinal myofibroblasts in innate immune responses of the intestine. Gastroenterology. 2003; 124:1866–1878. [PubMed: 12806620]
64. Burke JP, Cunningham MF, Watson RW, Docherty NG, Coffey JC, O'Connell PR. Bacterial lipopolysaccharide promotes profibrotic activation of intestinal fibroblasts. The British journal of surgery. 2010; 97:1126–1134. [PubMed: 20632282]
65. Afrazi A, Sodhi CP, Richardson W, Neal M, Good M, Siggers R, Hackam DJ. New insights into the pathogenesis and treatment of necrotizing enterocolitis: Toll-like receptors and beyond. Pediatric research. 2011; 69:183–188. [PubMed: 21135755]
66. Sodhi CP, Neal MD, Siggers R, Sho S, Ma C, Branca MF, Prindle T Jr, Russo AM, Afrazi A, Good M, Brower-Sinning R, Firek B, Morowitz MJ, Ozolek JA, Gittes GK, Billiar TR, Hackam DJ. Intestinal epithelial Toll-like receptor 4 regulates goblet cell development and is required for necrotizing enterocolitis in mice. Gastroenterology. 2012; 143:708–718. e701–e705. [PubMed: 22796522]
67. 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]
68. Artis D. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. Nat Rev Immunol. 2008; 8:411–420. [PubMed: 18469830]
69. Cario E, Gerken G, Podolsky DK. Toll-like receptor 2 controls mucosal inflammation by regulating epithelial barrier function. Gastroenterology. 2007; 132:1359–1374. [PubMed: 17408640]
70. Gibson DL, Montero M, Ropeleski MJ, Bergstrom KS, Ma C, Ghosh S, Merkens H, Huang J, Mansson LE, Sham HP, McNagny KM, Vallance BA. Interleukin-11 reduces TLR4-induced colitis in TLR2-deficient mice and restores intestinal STAT3 signaling. Gastroenterology. 2010; 139:1277–1288. [PubMed: 20600022]
71. Vijay-Kumar M, Wu H, Aitken J, Kolachala VL, Neish AS, Sitaraman SV, Gewirtz AT. Activation of toll-like receptor 3 protects against DSS-induced acute colitis. Inflammatory bowel diseases. 2007; 13:856–864. [PubMed: 17393379]
72. Vijay-Kumar M, Sanders CJ, Taylor RT, Kumar A, Aitken JD, Sitaraman SV, Neish AS, Uematsu S, Akira S, Williams IR, Gewirtz AT. Deletion of TLR5 results in spontaneous colitis in mice. The Journal of clinical investigation. 2007; 117:3909–3921. [PubMed: 18008007]
73. Rhee SH, Im E, Riegler M, Kokkotou E, O'Brien M, Pothoulakis C. Pathophysiological role of Toll-like receptor 5 engagement by bacterial flagellin in colonic inflammation. Proceedings of the National Academy of Sciences of the United States of America. 2005; 102:13610–13615. [PubMed: 16157881]
74. 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. Nature cell biology. 2006; 8:1327–1336.

75. 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]
76. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell. 2004; 118:229–241. [PubMed: 15260992]
77. Fukata M, Michelsen KS, Eri R, Thomas LS, Hu B, Lukasek K, Nast CC, Lechago J, Xu R, Naiki Y, Soliman A, Arditi M, Abreu MT. Toll-like receptor-4 is required for intestinal response to epithelial injury and limiting bacterial translocation in a murine model of acute colitis. American journal of physiology. 2005; 288:G1055–G1065. [PubMed: 15826931]
78. Szebeni B, Veres G, Dezsofi A, Rusai K, Vannay A, Mraz M, Majorova E, Arato A. Increased expression of Toll-like receptor (TLR) 2 and TLR4 in the colonic mucosa of children with inflammatory bowel disease. Clinical and experimental immunology. 2008; 151:34–41. [PubMed: 17991289]
79. Siggers RH, Hackam DJ. The role of innate immune-stimulated epithelial apoptosis during gastrointestinal inflammatory diseases. Cell Mol Life Sci. 2011; 68:3623–3634. [PubMed: 21986983]
80. Rakoff-Nahoum S, Hao L, Medzhitov R. Role of toll-like receptors in spontaneous commensal-dependent colitis. Immunity. 2006; 25:319–329. [PubMed: 16879997]
81. Shah JA, Vary JC, Chau TT, Bang ND, Yen NT, Farrar JJ, Dunstan SJ, Hawn TR. Human TOLLIP regulates TLR2 and TLR4 signaling and its polymorphisms are associated with susceptibility to tuberculosis. J Immunol. 2012; 189:1737–1746. [PubMed: 22778396]
82. Wald D, Qin J, Zhao Z, Qian Y, Naramura M, Tian L, Towne J, Sims JE, Stark GR, Li X. SIGIRR, a negative regulator of Toll-like receptor-interleukin 1 receptor signaling. Nature immunology. 2003; 4:920–927. [PubMed: 12925853]
83. Qin J, Qian Y, Yao J, Grace C, Li X. SIGIRR inhibits interleukin-1 receptor- and toll-like receptor 4-mediated signaling through different mechanisms. The Journal of biological chemistry. 2005; 280:25233–25241. [PubMed: 15866876]
84. Kobayashi K, Hernandez LD, Galan JE, Janeway CA Jr, Medzhitov R, Flavell RA. IRAK-M is a negative regulator of Toll-like receptor signaling. Cell. 2002; 110:191–202. [PubMed: 12150927]
85. Dubuquoy L, Jansson EA, Deeb S, Rakotobe S, Karoui M, Colombel JF, Auwerx J, Pettersson S, Desreumaux P. Impaired expression of peroxisome proliferator-activated receptor gamma in ulcerative colitis. Gastroenterology. 2003; 124:1265–1276. [PubMed: 12730867]
86. Appel S, Mirakaj V, Bringmann A, Weck MM, Grunebach F, Brossart P. PPAR-gamma agonists inhibit toll-like receptor-mediated activation of dendritic cells via the MAP kinase and NF-kappaB pathways. Blood. 2005; 106:3888–3894. [PubMed: 16105976]
87. Gon Y, Asai Y, Hashimoto S, Mizumura K, Jibiki I, Machino T, Ra C, Horie T. A20 inhibits toll-like receptor 2- and 4-mediated interleukin-8 synthesis in airway epithelial cells. American journal of respiratory cell and molecular biology. 2004; 31:330–336. [PubMed: 15142865]
88. Burns K, Clatworthy J, Martin L, Martinon F, Plumpton C, Maschera B, Lewis A, Ray K, Tschopp J, Volpe F. Tollip, a new component of the IL-1RI pathway, links IRAK to the IL-1 receptor. Nature cell biology. 2000; 2:346–351.
89. Zhang G, Ghosh S. Negative regulation of toll-like receptor-mediated signaling by Tollip. The Journal of biological chemistry. 2002; 277:7059–7065. [PubMed: 11751856]
90. Capelluto DG. Tollip: a multitasking protein in innate immunity and protein trafficking. Microbes and infection / Institut Pasteur. 2012; 14:140–147. [PubMed: 21930231]
91. Oshima N, Ishihara S, Rumi MA, Aziz MM, Mishima Y, Kadota C, Moriyama I, Ishimura N, Amano Y, Kinoshita Y. A20 is an early responding negative regulator of Toll-like receptor 5 signalling in intestinal epithelial cells during inflammation. Clinical and experimental immunology. 2010; 159:185–198. [PubMed: 19912257]
92. Steenholdt C, Andresen L, Pedersen G, Hansen A, Brynskov J. Expression and function of toll-like receptor 8 and Tollip in colonic epithelial cells from patients with inflammatory bowel disease. Scandinavian journal of gastroenterology. 2009; 44:195–204. [PubMed: 18985539]

93. Kadota C, Ishihara S, Aziz MM, Rumi MA, Oshima N, Mishima Y, Moriyama I, Yuki T, Amano Y, Kinoshita Y. Down-regulation of single immunoglobulin interleukin-1R-related molecule (SIGIRR)/TIR8 expression in intestinal epithelial cells during inflammation. Clinical and experimental immunology. 2010; 162:348–361. [PubMed: 21077278]

94. Garlanda C, Riva F, Polentarutti N, Buracchi C, Sironi M, De Bortoli M, Muzio M, Bergottini R, Scanziani E, Vecchi A, Hirsch E, Mantovani A. Intestinal inflammation in mice deficient in Tir8, an inhibitory member of the IL-1 receptor family. Proceedings of the National Academy of Sciences of the United States of America. 2004; 101:3522–3526. [PubMed: 14993616]

95. Xiao H, Gulen MF, Qin J, Yao J, Bulek K, Kish D, Altuntas CZ, Wald D, Ma C, Zhou H, Tuohy VK, Fairchild RL, de la Motte C, Cua D, Vallance BA, Li X. The Toll-interleukin-1 receptor member SIGIRR regulates colonic epithelial homeostasis, inflammation, and tumorigenesis. Immunity. 2007; 26:461–475. [PubMed: 17398123]

96. Berglund M, Melgar S, Kobayashi KS, Flavell RA, Hornquist EH, Hultgren OH. IL-1 receptor-associated kinase M downregulates DSS-induced colitis. Inflammatory bowel diseases. 2010; 16:1778–1786. [PubMed: 20848470]

97. Eun CS, Han DS, Lee SH, Paik CH, Chung YW, Lee J, Hahm JS. Attenuation of colonic inflammation by PPARgamma in intestinal epithelial cells: effect on Toll-like receptor pathway. Digestive diseases and sciences. 2006; 51:693–697. [PubMed: 16614990]

98. Lee, EG.; Boone, DL.; Chai, S.; Libby, SL.; Chien, M.; Lodolce, JP.; Ma, A. Science. Vol. 289. New York, N.Y: 2000. Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice; p. 2350-2354.

99. Vereecke L, Sze M, Mc Guire C, Rogiers B, Chu Y, Schmidt-Supprian M, Pasparakis M, Beyaert R, van Loo G. Enterocyte-specific A20 deficiency sensitizes to tumor necrosis factor-induced toxicity and experimental colitis. The Journal of experimental medicine. 2010; 207:1513–1523. [PubMed: 20530205]

100. Schmid M, Fellermann K, Fritz P, Wiedow O, Stange EF, Wehkamp J. Attenuated induction of epithelial and leukocyte serine antiproteases elafin and secretory leukocyte protease inhibitor in Crohn's disease. Journal of leukocyte biology. 2007; 81:907–915. [PubMed: 17200145]

101. Reardon C, Lechmann M, Brustle A, Gareau MG, Shuman N, Philpott D, Ziegler SF, Mak TW. Thymic stromal lymphopoetin-induced expression of the endogenous inhibitory enzyme SLPI mediates recovery from colonic inflammation. Immunity. 2011; 35:223–235. [PubMed: 21820333]

102. Neu J, Walker WA. Necrotizing enterocolitis. N Engl J Med. 2011; 364:255–264. [PubMed: 21247316]

103. Berman L, Moss RL. Necrotizing enterocolitis: An update. Semin Fetal Neonatal Med. 2011; 16:145–150. [PubMed: 21514258]

104. 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]

105. Hackam DJ, Upperman JS, Grishin A, Ford HR. Disordered enterocyte signaling and intestinal barrier dysfunction in the pathogenesis of necrotizing enterocolitis. Seminars in pediatric surgery. 2005; 14:49–57. [PubMed: 15770588]

106. 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]

107. Blakely ML, Lally KP, McDonald S, Brown RL, Barnhart DC, Ricketts RR, Thompson WR, Scherer LR, Klein MD, Letton RW, Chwals WJ, Touloukian RJ, Kurkchubasche AG, Skinner MA, Moss RL, Hilfiker ML. Postoperative outcomes of extremely low birth-weight infants with necrotizing enterocolitis or isolated intestinal perforation: a prospective cohort study by the NICHD Neonatal Research Network. Annals of surgery. 2005; 241:984–989. discussion 989–994. [PubMed: 15912048]

108. Hintz SR, Kendrick DE, Stoll BJ, Vohr BR, Fanaroff AA, Donovan EF, Poole WK, Blakely ML, Wright L, Higgins R. Neurodevelopmental and growth outcomes of extremely low birth weight infants after necrotizing enterocolitis. Pediatrics. 2005; 115:696–703. [PubMed: 15741374]

109. Lainwala S, Perritt R, Poole K, Vohr B. Neurodevelopmental and growth outcomes of extremely low birth weight infants who are transferred from neonatal intensive care units to level I or II nurseries. Pediatrics. 2007; 119:e1079–e1087. [PubMed: 17403821]
110. Guner YS, Franklin AL, Chokshi NK, Castle SL, Pontarelli E, Wang J, Wang L, Prasadarao NV, Upperman JS, Grishin AV, Ford HR. P-glycoprotein induction by breast milk attenuates intestinal inflammation in experimental necrotizing enterocolitis. Laboratory investigation; a journal of technical methods and pathology. 2011; 91:1668–1679.
111. Gregory KE, Deforge CE, Natale KM, Phillips M, Van Marter LJ. Necrotizing enterocolitis in the premature infant: neonatal nursing assessment, disease pathogenesis, and clinical presentation. Adv Neonatal Care. 2011; 11:155–164. quiz 165-156. [PubMed: 21730907]
112. Claud EC, Walker WA. Hypothesis: inappropriate colonization of the premature intestine can cause neonatal necrotizing enterocolitis. Faseb J. 2001; 15:1398–1403. [PubMed: 11387237]
113. Hackam DJ, Good M, Sodhi CP. Mechanisms of gut barrier failure in the pathogenesis of necrotizing enterocolitis: Toll-like receptors throw the switch. Seminars in pediatric surgery. 2013; 22:76–82. [PubMed: 23611610]
114. Lin PW, Stoll BJ. Necrotising enterocolitis. Lancet. 2006; 368:1271–1283. [PubMed: 17027734]
115. Blakey JL, Lubitz L, Campbell NT, Gillam GL, Bishop RF, Barnes GL. Enteric colonization in sporadic neonatal necrotizing enterocolitis. Journal of pediatric gastroenterology and nutrition. 1985; 4:591–595. [PubMed: 2863341]
116. Hentges DJ. The anaerobic microflora of the human body. Clin Infect Dis. 1993; 16(Suppl 4):S175–S180. [PubMed: 8324114]
117. 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]
118. Coates EW, Karlowicz MG, Croitoru DP, Buescher ES. Distinctive distribution of pathogens associated with peritonitis in neonates with focal intestinal perforation compared with necrotizing enterocolitis. Pediatrics. 2005; 116:e241–e246. [PubMed: 15995004]
119. Bury RG, Tudehope D. Enteral antibiotics for preventing necrotizing enterocolitis in low birthweight or preterm infants. Cochrane database of systematic reviews (Online). 2001:CD000405.
120. Krediet TG, van Lelyveld N, Vijlbrief DC, Brouwers HA, Kramer WL, Fleer A, Gerards LJ. Microbiological factors associated with neonatal necrotizing enterocolitis: protective effect of early antibiotic treatment. Acta Paediatr. 2003; 92:1180–1182. [PubMed: 14632335]
121. Cetin S, Ford HR, Sysko LR, Agarwal C, Wang J, Neal MD, Baty C, Apodaca G, Hackam DJ. Endotoxin inhibits intestinal epithelial restitution through activation of Rho-GTPase and increased focal adhesions. The Journal of biological chemistry. 2004; 279:24592–24600. [PubMed: 15169791]
122. Cario E, Rosenberg IM, Brandwein SL, Beck PL, Reinecker HC, Podolsky DK. Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. J Immunol. 2000; 164:966–972. [PubMed: 10623846]
123. Otte JM, Cario E, Podolsky DK. Mechanisms of cross hyporesponsiveness to Toll-like receptor bacterial ligands in intestinal epithelial cells. Gastroenterology. 2004; 126:1054–1070. [PubMed: 15057745]
124. Nanthakumar N, Meng D, Goldstein AM, Zhu W, Lu L, Uauy R, Llanos A, Claud EC, Walker WA. The mechanism of excessive intestinal inflammation in necrotizing enterocolitis: an immature innate immune response. PloS one. 2011; 6:e17776. [PubMed: 21445298]
125. Fusunyan RD, Nanthakumar NN, Baldeon ME, Walker WA. Evidence for an innate immune response in the immature human intestine: toll-like receptors on fetal enterocytes. Pediatric research. 2001; 49:589–593. [PubMed: 11264445]
126. Neal MD, Sodhi CP, Dyer M, Craig BT, Good M, Jia H, Yazji I, Afrazi A, Richardson WM, Beer-Stolz D, Ma C, Prindle T, Grant Z, Branca MF, Ozolek J, Hackam DJ. A Critical Role for TLR4 Induction of Autophagy in the Regulation of Enterocyte Migration and the Pathogenesis of Necrotizing Enterocolitis. J Immunol. 2007; 190:3541–3551. [PubMed: 23455503]

127. Lotz M, Gutle D, Walther S, Menard S, Bogdan C, Hornef MW. Postnatal acquisition of endotoxin tolerance in intestinal epithelial cells. The Journal of experimental medicine. 2006; 203:973–984. [PubMed: 16606665]
128. 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]
129. Chan KL, Wong KF, Luk JM. Role of LPS/CD14/TLR4-mediated inflammation in necrotizing enterocolitis: pathogenesis and therapeutic implications. World J Gastroenterol. 2009; 15:4745–4752. [PubMed: 19824106]
130. Liu Y, Zhu L, Fatheree NY, Liu X, Pacheco SE, Tatevian N, Rhoads JM. Changes in intestinal Toll-like receptors and cytokines precede histological injury in a rat model of necrotizing enterocolitis. American journal of physiology. 2009; 297:G442–G450. [PubMed: 19608731]
131. Lu J, Jilling T, Li D, Caplan MS. Polyunsaturated fatty acid supplementation alters proinflammatory gene expression and reduces the incidence of necrotizing enterocolitis in a neonatal rat model. Pediatric research. 2007; 61:427–432. [PubMed: 17515866]
132. Zamora R, Grishin A, Wong C, Boyle P, Wang J, Hackam D, Upperman JS, Tracey KJ, Ford HR. High-mobility group box 1 protein is an inflammatory mediator in necrotizing enterocolitis: protective effect of the macrophage deactivator semapimod. American journal of physiology. 2005; 289:G643–G652. [PubMed: 15947118]
133. Caplan MS, Simon D, Jilling T. The role of PAF, TLR, and the inflammatory response in neonatal necrotizing enterocolitis. Seminars in pediatric surgery. 2005; 14:145–151. [PubMed: 16084401]
134. Mollen KP, Gribar SC, Anand RJ, Kaczorowski DJ, Kohler JW, Branca MF, Dubowski TD, Sodhi CP, Hackam DJ. Increased expression and internalization of the endotoxin coreceptor CD14 in enterocytes occur as an early event in the development of experimental necrotizing enterocolitis. Journal of pediatric surgery. 2008; 43:1175–1181. [PubMed: 18558203]
135. Sodhi CP, Shi XH, Richardson WM, Grant ZS, Shapiro RA, Prindle T Jr, Branca M, Russo A, Gribar SC, Ma C, Hackam DJ. Toll-like receptor-4 inhibits enterocyte proliferation via impaired beta-catenin signaling in necrotizing enterocolitis. Gastroenterology. 2010; 138:185–196. [PubMed: 19786028]
136. Soliman A, Michelsen KS, Karahashi H, Lu J, Meng FJ, Qu X, Crother TR, Rabizadeh S, Chen S, Caplan MS, Arditi 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]
137. 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]
138. Neal MD, Sodhi CP, Jia H, Dyer M, Egan CE, Yazji I, Good M, Afrazi A, Marino R, Slagle D, Ma C, Branca MF, Prindle T Jr, Grant Z, Ozolek J, Hackam DJ. Toll-like receptor 4 is expressed on intestinal stem cells and regulates their proliferation and apoptosis via the p53 up-regulated modulator of apoptosis. The Journal of biological chemistry. 2012; 287:37296–37308. [PubMed: 22955282]
139. Dai S, Sodhi C, Cetin S, Richardson W, Branca M, Neal MD, Prindle T, Ma C, Shapiro RA, Li B, Wang JH, Hackam DJ. Extracellular high mobility group box-1 (HMGB1) inhibits enterocyte migration via activation of Toll-like receptor-4 and increased cell-matrix adhesiveness. The Journal of biological chemistry. 2010; 285:4995–5002. [PubMed: 20007974]
140. Yu J, Zhang L. PUMA, a potent killer with or without p53. Oncogene. 2008; 27(Suppl 1):S71–S83. [PubMed: 19641508]
141. Wu B, Qiu W, Wang P, Yu H, Cheng T, Zambetti GP, Zhang L, Yu J. p53 independent induction of PUMA mediates intestinal apoptosis in response to ischaemia-reperfusion. Gut. 2007; 56:645–654. [PubMed: 17127703]
142. Qureshi FG, Leaphart C, Cetin S, Li J, Grishin A, Watkins S, Ford HR, Hackam DJ. Increased expression and function of integrins in enterocytes by endotoxin impairs epithelial restitution. Gastroenterology. 2005; 128:1012–1022. [PubMed: 15825082]

143. Mammen JM, Matthews JB. Mucosal repair in the gastrointestinal tract. Critical care medicine. 2003; 31:S532–S537. [PubMed: 12907883]

144. Hackam DJ, Ford HR. Cellular, biochemical, and clinical aspects of wound healing. Surgical infections. 2002; 3(Suppl 1):S23–S35. [PubMed: 12573037]

145. Ibrahim, A.; Mbodji, K.; Hassan, A.; Aziz, M.; Boukhettala, N.; Coeffier, M.; Savoye, G.; Dechelotte, P.; Marion-Letellier, R. Clinical nutrition. Vol. 30. Edinburgh, Scotland: 2011. Anti-inflammatory and anti-angiogenic effect of long chain n-3 polyunsaturated fatty acids in intestinal microvascular endothelium; p. 678-687.

146. Yazji I, Sodhi CP, Lee EK, Good M, Egan CE, Afrazi A, Neal MD, Jia H, Lin J, Ma C, Branca MF, Prindle T, Richardson WM, Ozolek J, Billiar TR, Binion DG, Gladwin MT, Hackam DJ. Endothelial TLR4 activation impairs intestinal microcirculatory perfusion in necrotizing enterocolitis via eNOS-NO-nitrite signaling. Proceedings of the National Academy of Sciences of the United States of America. 2013

147. Halpern MD, Dvorak B. Does abnormal bile acid metabolism contribute to NEC? Seminars in perinatology. 2008; 32:114–121. [PubMed: 18346535]

148. Martin NA, Mount Patrick SK, Estrada TE, Frisk HA, Rogan DT, Dvorak B, Halpern MD. Active transport of bile acids decreases mucin 2 in neonatal ileum: implications for development of necrotizing enterocolitis. PloS one. 2011; 6:e27191. [PubMed: 22162748]

149. Forstermann U, Sessa WC. Nitric oxide synthases: regulation and function. European heart journal. 2012; 33:829–837. 837a–837d. [PubMed: 21890489]

150. Marletta MA. Nitric oxide synthase structure and mechanism. The Journal of biological chemistry. 1993; 268:12231–12234. [PubMed: 7685338]

151. Kloner RA. Pharmacology and drug interaction effects of the phosphodiesterase 5 inhibitors: focus on alpha-blocker interactions. The American journal of cardiology. 2005; 96:42M–46M. [PubMed: 15979430]

152. Jornot L, Mirault ME, Junod AF. Differential expression of hsp70 stress proteins in human endothelial cells exposed to heat shock and hydrogen peroxide. American journal of respiratory cell and molecular biology. 1991; 5:265–275. [PubMed: 1910812]

153. Madamanchi NR, Li S, Patterson C, Runge MS. Reactive oxygen species regulate heat-shock protein 70 via the JAK/STAT pathway. Arteriosclerosis, thrombosis, and vascular biology. 2001; 21:321–326.

154. Muramatsu T, Tada H, Kobayashi N, Yamji M, Shirai T, Ohnishi T. Induction of the 72-kD heat shock protein in organ-cultured normal human skin. The Journal of investigative dermatology. 1992; 98:786–790. [PubMed: 1569328]

155. Melkonyan HS, Ushakova TE, Umansky SR. Hsp70 gene expression in mouse lung cells upon chronic gamma-irradiation. International journal of radiation biology. 1995; 68:277–280. [PubMed: 7561387]

156. Liu RY, Corry PM, Lee YJ. Regulation of chemical stress-induced hsp70 gene expression in murine L929 cells. Journal of cell science. 1994; 107(Pt 8):2209–2214. [PubMed: 7983179]

157. Afrazi A, Sodhi CP, Good M, Jia H, Siggers R, Yazji I, Ma C, Neal MD, Prindle T, Grant ZS, Branca MF, Ozolek J, Chang EB, Hackam DJ. Intracellular heat shock protein-70 negatively regulates TLR4 signaling in the newborn intestinal epithelium. J Immunol. 2012; 188:4543–4557. [PubMed: 22461698]

158. Hu S, Ciancio MJ, Lahav M, Fujiya M, Lichtenstein L, Anant S, Musch MW, Chang EB. Translational inhibition of colonic epithelial heat shock proteins by IFN-gamma and TNF-alpha in intestinal inflammation. Gastroenterology. 2007; 133:1893–1904. [PubMed: 18054561]

159. Paimela T, Hyttinen JM, Viiri J, Ryhanen T, Karjalainen RO, Salminen A, Kaarniranta K. Celastrol regulates innate immunity response via NF-kappaB and Hsp70 in human retinal pigment epithelial cells. Pharmacol Res. 2011; 64:501–508. [PubMed: 21683142]

160. Kalmar B, Greensmith L. Activation of the heat shock response in a primary cellular model of motoneuron neurodegeneration-evidence for neuroprotective and neurotoxic effects. Cellular & molecular biology letters. 2009; 14:319–335. [PubMed: 19183864]

161. Wischmeyer PE, Musch MW, Madonna MB, Thisted R, Chang EB. Glutamine protects intestinal epithelial cells: role of inducible HSP70. The American journal of physiology. 1997; 272:G879–G884. [PubMed: 9142921]

162. Kessel A, Toubi E, Pavlotzky E, Mogilner J, Coran AG, Lurie M, Karry R, Sukhotnik I. Treatment with glutamine is associated with down-regulation of Toll-like receptor-4 and myeloid differentiation factor 88 expression and decrease in intestinal mucosal injury caused by lipopolysaccharide endotoxaemia in a rat. Clinical and experimental immunology. 2008; 151:341–347. [PubMed: 18070149]

163. Akisu M, Baka M, Huseyinov A, Kultursay N. The role of dietary supplementation with L-glutamine in inflammatory mediator release and intestinal injury in hypoxia/reoxygenation-induced experimental necrotizing enterocolitis. Annals of nutrition & metabolism. 2003; 47:262–266. [PubMed: 14520021]

164. Sevastiadou S, Malamitsi-Puchner A, Costalos C, Skouroliakou M, Briana DD, Antsaklis A, Roma-Giannikou E. The impact of oral glutamine supplementation on the intestinal permeability and incidence of necrotizing enterocolitis/septicemia in premature neonates. J Matern Fetal Neonatal Med. 2011; 24:1294–1300. [PubMed: 21463215]

165. Halpern MD, Holubec H, Saunders TA, Dvorak K, Clark JA, Doelle SM, Ballatori N, Dvorak B. Bile acids induce ileal damage during experimental necrotizing enterocolitis. Gastroenterology. 2006; 130:359–372. [PubMed: 16472592]

166. Halpern MD, Weitkamp JH, Mount Patrick SK, Dobrenen HJ, Khailova L, Correa H, Dvorak B. Apical sodium-dependent bile acid transporter upregulation is associated with necrotizing enterocolitis. American journal of physiology. 2010; 299:G623–G631. [PubMed: 20616306]

167. Cherrington NJ, Estrada TE, Frisk HA, Canet MJ, Hardwick RN, Dvorak B, Lux K, Halpern MD. The hepatic bile acid transporters Ntcp and Mrp2 are downregulated in experimental necrotizing enterocolitis. American journal of physiology. 2012; 304:G48–G56. [PubMed: 23125159]

168. Milano J, McKay J, Dagenais C, Foster-Brown L, Pognan F, Gadient R, Jacobs RT, Zacco A, Greenberg B, Ciaccio PJ. Modulation of notch processing by gamma-secretase inhibitors causes intestinal goblet cell metaplasia and induction of genes known to specify gut secretory lineage differentiation. Toxicol Sci. 2004; 82:341–358. [PubMed: 15319485]

169. Wu Y, Zhu X, Li N, Chen T, Yang M, Yao M, Liu X, Jin B, Wang X, Cao X. CMRF-35-like molecule 3 preferentially promotes TLR9-triggered proinflammatory cytokine production in macrophages by enhancing TNF receptor-associated factor 6 ubiquitination. J Immunol. 2011; 187:4881–4889. [PubMed: 21940676]

170. Franchi L, Warner N, Viani K, Nunez G. Function of Nod-like receptors in microbial recognition and host defense. Immunological reviews. 2009; 227:106–128. [PubMed: 19120480]

171. Richardson WM, Sodhi CP, Russo A, Siggers RH, Afrazi A, Gribar SC, Neal MD, Dai S, Prindle T Jr, Branca M, Ma C, Ozolek J, Hackam DJ. Nucleotide-binding oligomerization domain-2 inhibits toll-like receptor-4 signaling in the intestinal epithelium. Gastroenterology. 2010; 139:904–917. 917 e901–906. [PubMed: 20580721]

172. Du C, Fang M, Li Y, Li L, Wang X. Smac a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell. 2000; 102:33–42. [PubMed: 10929711]

173. Mulvihill SJ, Stone MM, Debas HT, Fonkalsrud EW. The role of amniotic fluid in fetal nutrition. Journal of pediatric surgery. 1985; 20:668–672. [PubMed: 4087096]

174. Scott SM, Buenaflor GG, Orth DN. Immunoreactive human epidermal growth factor concentrations in amniotic fluid, umbilical artery and vein serum, and placenta in full-term and preterm infants. Biology of the neonate. 1989; 56:246–251. [PubMed: 2605277]

175. Barka, T.; van der Noen, H.; Gresik, EW.; Kerenyi, T. The Mount Sinai journal of medicine. Vol. 45. New York: 1978. Immunoreactive epidermal growth factor in human amniotic fluid; p. 679-684.

176. Good M, Siggers RH, Sodhi CP, Afrazi A, Alkhudari F, Egan CE, Neal MD, Yazji I, Jia H, Lin J, Branca MF, Ma C, Prindle T, Grant Z, Shah S, Slagle D 2nd, Paredes J, Ozolek J, Gittes GK, Hackam DJ. Amniotic fluid inhibits Toll-like receptor 4 signaling in the fetal and neonatal intestinal epithelium. Proceedings of the National Academy of Sciences of the United States of America. 2012; 109:11330–11335. [PubMed: 22733781]

177. Siggers JL, Ostergaard MV, Siggers RH, Skovgaard K, Molbak L, Thymann T, Schmidt M, Moller HK, Purup S, Fink LN, Frokiaer H, Boye M, Sangild PT, Bering SB. Postnatal amniotic fluid intake reduces gut inflammatory responses and necrotizing enterocolitis in preterm neonates. American journal of physiology. 2013

178. Dubuquoy L, Rousseaux C, Thuru X, Peyrin-Biroulet L, Romano O, Chavatte P, Chamaillard M, Desreumaux P. PPARgamma as a new therapeutic target in inflammatory bowel diseases. Gut. 2006; 55:1341–1349. [PubMed: 16905700]

179. Fort MM, Mozaffarian A, Stover AG, Correia Jda S, Johnson DA, Crane RT, Ulevitch RJ, Persing DH, Bielefeldt-Ohmann H, Probst P, Jeffery E, Fling SP, Hershberg RM. A synthetic TLR4 antagonist has anti-inflammatory effects in two murine models of inflammatory bowel disease. J Immunol. 2005; 174:6416–6423. [PubMed: 15879143]

180. Barochia A, Solomon S, Cui X, Natanson C, Eichacker PQ. Eritoran tetrasodium (E5564) treatment for sepsis: review of preclinical and clinical studies. Expert opinion on drug metabolism & toxicology. 2011; 7:479–494. [PubMed: 21323610]

181. Neal MD, Jia H, Eyer B, Good M, Guerriero CJ, Sodhi CP, Afrazi A, Prindle T Jr, Ma C, Branca M, Ozolek J, Brodsky JL, Wipf P, Hackam DJ. Discovery and Validation of a New Class of Small Molecule Toll-Like Receptor 4 (TLR4) Inhibitors. PloS one. 2013; 8:e65779. [PubMed: 23776545]

182. Kosloske AM. Breast milk decreases the risk of neonatal necrotizing enterocolitis. Advances in nutritional research. 2001; 10:123–137. [PubMed: 11795037]

183. Meinzen-Derr J, Poindexter B, Wrage L, Morrow AL, Stoll B, Donovan EF. Role of human milk in extremely low birth weight infants' risk of necrotizing enterocolitis or death. J Perinatol. 2009; 29:57–62. [PubMed: 18716628]

184. Quigley MA, Henderson G, Anthony MY, McGuire W. Formula milk versus donor breast milk for feeding preterm or low birth weight infants. Cochrane database of systematic reviews (Online). 2007:CD002971.

185. Hunter CJ, Williams M, Petrosyan M, Guner Y, Mittal R, Mock D, Upperman JS, Ford HR, Prasadarao NV. Lactobacillus bulgaricus prevents intestinal epithelial cell injury caused by Enterobacter sakazakii-induced nitric oxide both in vitro and in the newborn rat model of necrotizing enterocolitis. Infection and immunity. 2009; 77:1031–1043. [PubMed: 19075027]

186. Khailova L, Dvorak K, Arganbright KM, Halpern MD, Kinouchi T, Yajima M, Dvorak B. Bifidobacterium bifidum improves intestinal integrity in a rat model of necrotizing enterocolitis. American journal of physiology. 2009; 297:G940–G949. [PubMed: 20501441]

187. Khailova L, Mount Patrick SK, Arganbright KM, Halpern MD, Kinouchi T, Dvorak B. Bifidobacterium bifidum reduces apoptosis in the intestinal epithelium in necrotizing enterocolitis. American journal of physiology. 2010; 299:G1118–G1127. [PubMed: 20705904]

188. Liu Y, Fatheree NY, Mangalat N, Rhoads JM. Lactobacillus reuteri strains reduce incidence and severity of experimental necrotizing enterocolitis via modulation of TLR4 and NF-kappaB signaling in the intestine. American journal of physiology. 2012; 302:G608–G617. [PubMed: 22207578]

189. Deshpande G, Rao S, Patole S, Bulsara M. Updated meta-analysis of probiotics for preventing necrotizing enterocolitis in preterm neonates. Pediatrics. 2010; 125:921–930. [PubMed: 20403939]

190. Caplan M, Frost B. Myth: necrotizing enterocolitis: probiotics will end the disease, and surgical intervention improves the outcome. Seminars in fetal & neonatal medicine. 2011; 16:264–268. [PubMed: 21482212]

191. Hunter C, Dimaguila MA, Gal P, Wimmer JE Jr, Ransom JL, Carlos RQ, Smith M, Davanzo CC. Effect of routine probiotic, Lactobacillus reuteri DSM 17938, use on rates of necrotizing enterocolitis in neonates with birthweight< 1000 grams: a sequential analysis. BMC pediatrics. 2012; 12:142. [PubMed: 22947597]

192. Rojas MA, Lozano JM, Rojas MX, Rodriguez VA, Rondon MA, Bastidas JA, Perez LA, Rojas C, Ovalle O, Garcia-Harker JE, Tamayo ME, Ruiz GC, Ballesteros A, Archila MM, Arevalo M. Prophylactic probiotics to prevent death and nosocomial infection in preterm infants. Pediatrics. 2012; 130:e1113–e1120. [PubMed: 23071204]