The Altered Gut Microbiome and Necrotizing Enterocolitis

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KEYWORDS

- Necrotizing enterocolitis • Microbiome • Intestinal microbiota
- Noncultured based techniques • Sequencing

KEY POINTS

- The intestinal microbiota normally exist in a commensal or symbiotic relationship with the host, but in the neonate, and especially in the premature infant, this relationship needs to develop.
- Alterations of the intestinal microbiota may predispose the preterm infant to the development of necrotizing enterocolitis.
- Composition and quality of the intestinal microbiota depend on many factors, such as mode of birth, type of feeding, and use of antibiotics. There are many opportunities for physicians to provide interventions that could improve the adequate colonization of the neonatal gut.
- Culture-based techniques are highly limited, but the development of new molecular technologies to study previously unidentified organisms has enhanced our understanding of the microbial environment that may predispose to necrotizing enterocolitis.

INTRODUCTION

Necrotizing enterocolitis (NEC) is an enigmatic disease that has been recognized for more than a century, but with the advent of neonatal intensive care, it has become one of the most common and devastating diseases in neonates.[1–3] Among the reasons why NEC has been so difficult to understand and eradicate is that what has been termed NEC is certainly more than 1 disease with multiple causes.[2] For example, when an infant born at term who has a hypoplastic left ventricle presents with pneumatosis intestinalis at 2 days of age, the cause and pathophysiology of this baby's NEC is likely different from the 25-week gestation preterm who presents with pneumatosis intestinalis at 5 weeks of age. The first is more likely related to ischemic injury caused by hemodynamic insufficiency and hypoxic-ischemic injury[4] rather than

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a coalescence of factors that result in intestinal inflammation and injury largely as a result of intestinal immaturity, as in the preterm infant. In the latter case, sometimes referred to as classic NEC,[2] the interactions of a predisposing genetic background, an immature intestinal barrier, and a microbial environment that is conducive to the development of NEC are believed to play an interactive and critical role in pathogenesis.[2,3,5]

The linkage of NEC to bacterial colonization was recognized by Santulli and colleagues[6] more than 3 decades ago. Additional observations showing clusters of cases, outbreaks in institutions, the finding of pneumatosis intestinalis, which likely represents submucosal gas produced by bacterial fermentation, and the common findings of bacteremia and endotoxinemia in affected neonates support a microbial role in the pathogenesis of this disease.[5] Numerous bacteria have been related to NEC, but none of them has been found to fulfill Koch's postulates, because they are commonly found among patients without NEC.[7] Viruses have also been implicated in the pathogenesis of NEC, and coronavirus within fecal samples and resected intestinal segments were reported in patients with NEC,[8] but their role in the causation of the disease has not been substantiated.

Recently, the Human Microbiome Project has enabled development of novel technologies that should be highly instrumental in helping understand the contribution of the intestinal microbial ecology to the pathogenesis of NEC.[9] The realization that culture-based techniques are limited in delineating the vast array of microbes present in the human intestine along with the development of new technologies to study these previously unidentified organisms has enhanced our optimism for better describing the microbial environment that may predispose to classic NEC. Furthermore, an appreciation of the role of commensal microbes in protection of the intestine and the disease that may occur when the balance of these commensals is disrupted is opening a new era of thought in the understanding of NEC as well as diseases ranging from obesity to autism. Efforts by the National Institutes of Child Health and Human Development have also paved the way for new studies in NEC.[10] This review focuses on the role of the intestinal microbiota in the pathogenesis of classic NEC.

COMPLEMENTING TRADITIONAL CULTURE-BASED TECHNIQUES

The traditional view of the human is that they are composed of 10 trillion cells, which are the product of approximately 23,000 genes. However, in various niches of the human body, there reside several species of microbes and microbial genes that vastly outnumber those of the human host. In the past few years, emerging technologies derived largely from the Human Genome Project have been applied to evaluating the intestinal microbiota, and new discoveries using these techniques have prompted new initiatives such as the Human Microbiome Roadmap, designed to evaluate the role of the intestinal microbiome in health and disease.

Until the beginning of the last decade, culture-based techniques were the mainstay of evaluating intestinal microbes. However, most bacterial cells seen microscopically in feces cannot be cultured in the laboratory (**Fig. 1**).[11] Recently developed high-throughput molecular techniques analyze microbial DNA and RNA. There are 2 general approaches, both of which comprise several variants. One commonly used general approach is to use the 16S rRNA gene[12] and the other is a more complex metagenomic approach, in which community DNA is subject to shotgun (whole-genome) sequencing.[13] A comprehensive review of these techniques is beyond the scope of this article, but some of the more commonly used techniques are summarized in **Box 1**.

Both methods include the extraction of community DNA or RNA from feces or other samples of interest. The 16S rRNA is a part of the ribosomal RNA. It is commonly used

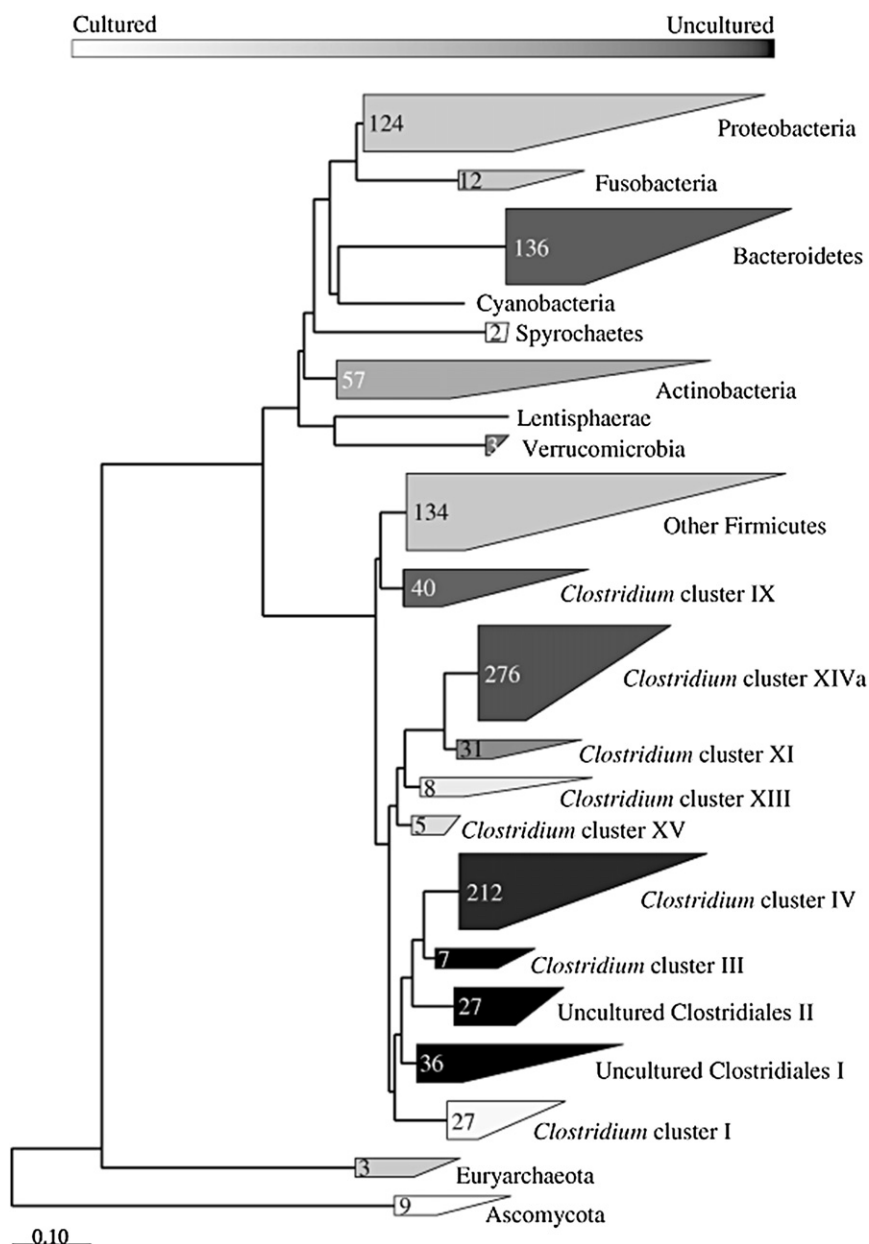**Fig. 1.** Intestinal microbiota remain mostly uncultured by traditional culture-based methods. Black fills indicate phylotypes detected in cultivation-independent studies and white indicates species detected in cultivation-based studies. (*From* Rajilic-Stojanovic M, Smidt H, de Vos WM. Diversity of the human gastrointestinal tract microbiota revisited. Environ Microbiol 2007;9:2125–36; with permission.)

Box 1
Nonculture techniques of intestinal microbiota identification

16s rRNA sequencing V3/V4 and V4/V5 regions

Shotgun approach: Illumina (Illumina, Inc, San Diego, CA, USA) or 454 Titanium (454 Life Sciences Corporation, Branford, CT, USA) for longer sequence reads

Fingerprinting: DGGE/TGGE: separates individual rRNA genes and provides a fingerprint of the complexity of the intestinal microbiota

T-RFLP: rapid comparative analysis and very sensitive

FISH: best for enumeration of species in the intestinal tract

PhyloChip (Affymetrix Corporation, Lawrence Berkeley Lab (LBNL), San Francisco, CA, USA): DNA microarray for multiple bacterial identification

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for phylogenetic studies, because some regions of the gene encoding it are highly conserved and can act as primer binding sites, whereas other regions containing phylogenetic information are highly variable and enable their use for taxonomic classification. Using this approach, polymerase chain reaction (PCR) amplicons are sequenced using novel high-throughput pyrosequencing technology. Based on the degree of nucleotide similarity (usually between 95% and 99%), sequences can be binned into operational taxonomic units that form the basis for comparisons of microbiota composition and diversity between samples.

Although most of the initial efforts for taxonomic evaluation of the microbiome involved use of the 16S rRNA, more recent work has focused on sequencing of the genomes of the entire community using shotgun techniques that sequence the entire genome. Using this approach, Venter and colleagues[14] identified a vast number of microbes that had not previously been recognized to exist in the oceans. Over the past few years, similar techniques are being applied to the human intestinal microbiome, and with future refinement of the technology, bioinformatics, statistical analyses, and decreases in cost of the analyses should yield important new information about the microbes present in the intestine and how they relate to health and disease.

ADJUNCTS TO MICROBIOME ANALYSIS: FUNCTIONAL CHARACTERIZATION

Even if the taxonomy of a particular microbial community is identified, the functional expression as it relates to physiology and interaction with the host is not clarified. Simple identification of individual microbes or microbial genotypes often does not clarify their phenotypic expression. Thus, in addition to microbiome identification-based technologies, it is important to clarify the function of the microbial communities of interest within a particular niche. It thus is of interest to identify the mRNA and protein expression of the genes as well as the metabolites that result before and after interaction of the microbial gene products with the host. Additional "-omic" disciplines are thus being applied to augment studies of the microbiome (**Box 2**). Metatranscriptomics, metaproteinomics, and metabolomics identify gene expression products (mRNA), proteins, and metabolites resulting from the genes within a complex microbial community, such as that found in fecal sample.

Several metatranscriptome and metaproteome studies describing the human intestinal microbiota have confirmed the importance of bacterial functions related to carbohydrate metabolism in the colon. Similar results were seen in a transcriptional analysis of fecal samples from a monozygotic, obese twin pair[15] and metatranscriptomic

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Box 2
Studies of functional expression of the intestinal microbiome

Metagenomics (profiling intestinal microbiota, DNA): comparison with known functional expression of similar sequences

Metabolomics: metabolic profiles (metabolites) associated with microbiota

Metaproteomics: catalytic potential of microbiota (proteins)

Metatranscriptomics: microbiota responses to environmental changes (RNA)

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analysis of fecal samples from 10 healthy volunteers.[16] Metatranscriptomic data from the less studied small intestinal microbiota showed enrichment in sugar phospho-transferase (PTS) and other carbohydrate transport systems, as well as energy, central metabolic, and amino acid conversion pathways compared with the metagenome.[17] This finding suggests rapid uptake and fermentation of available simple sugars by the small intestinal microbiota, compared with the degradation of more complex carbohydrates by the bacteria in the colon. The importance of carbohydrate metabolism is also evident from the enormous amount of carbohydrate-active enzymes (CAZymes) present in the gut microbiome. Another recent study using fecal samples from studies of adult humans found that most assigned transcripts belonged to the metabolism cluster (26% of all sequences), underlining that even at the end of the intestinal tract the microbiota are still very active.[18]

Metaproteomics is the study of proteins collectively expressed within microbial communities that usually use mass spectrometry-based analyses to detect proteins associated with the microbiota identified within a given niche. These studies usually also use a systems-biology approach to evaluate the likely functions of the proteins produced by the microbiota. A recent study used a nontargeted, shotgun mass spectrometry-based whole-community proteomics, or metaproteomics, approach for the first deep proteome measurements of thousands of proteins in human fecal samples.[19] The resulting metaproteomes had a distribution that was unexpected relative to the metagenome, with more proteins for translation, energy production, and carbohydrate metabolism when compared with what was earlier predicted from metagenomics. Human proteins, including antimicrobial peptides, were also identified, providing a nontargeted glimpse of the host response to the microbiota. Several unknown proteins represented previously undescribed microbial pathways or host immune responses, revealing a novel complex interplay between the human host and its associated microbes.

The intestinal microbiota are involved in the regulation of multiple host metabolic pathways, giving rise to interactive host-microbiota metabolic, signaling, and immune-inflammatory processes connecting the intestine, liver, muscle, and brain.

These interactions begin at birth and likely even during fetal life. The microbiota shape the development of the immune system, and the immune system in turn shapes the composition of the microbiota through a cross-talk between the microbes and the host immune system. The signaling processes, together with direct chemical interactions between the microbe and host, act on multiple organs such as the gut, liver, muscle, and brain.

Fig. 2 summarizes some of the gut bacteria and the metabolites they contribute. Notable among these metabolites are the short-chain fatty acids (acetate, propionate, and butyrate), choline, bile acids, vitamin K, and polyamines, all believed to be important for optimal functioning of the gastrointestinal tract as well as the entire human. The production of these metabolites by microbes contributes to the host
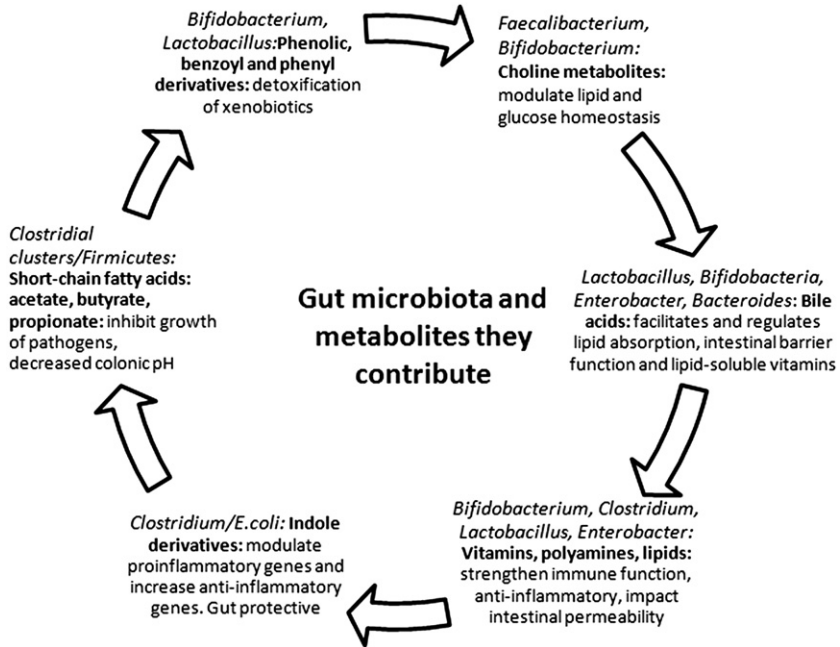
Fig. 2. Intestinal microbiota and the potential biologic functions and metabolites.

metabolic phenotype and hence to disease risk. Despite the putative importance of these metabolic functions, few studies are available as they pertain to NEC in the neonate.

ALTERATIONS OF THE INTESTINAL MICROBIOTA IN NEC

The intestinal microbiota normally exist in a commensal or symbiotic relationship with the host,[20] but in the neonate and especially in the premature infant, this relationship needs to develop, and many factors define its delicate equilibrium with the capability to modulate immune responses and promote health (**Fig. 3**).[21] As mentioned earlier, a specific pathogen that fulfills Koch's postulates for the cause of NEC has not been found. Whether the use of new-generation sequencing technologies will help in this search is unknown, although some preliminary studies are offering clues.

In a study by Wang and colleagues,[22] fecal samples from 20 preterm infants, 10 with NEC and 10 matched controls (including 4 twin pairs), were obtained from patients in a single-site level III neonatal intensive care unit. Bacterial DNA was subjected to terminal restriction fragment length polymorphism analysis and library sequencing of the 16S rRNA gene. The distribution of samples from patients with NEC distinctly clustered separately from controls. Intestinal bacterial colonization in all preterm infants was notable for low diversity. Patients with NEC had even less diversity, an increase in abundance of Gammaproteobacteria, a decrease in other bacteria species, and had received a higher mean number of previous days of antibiotics. These results suggested a relationship with previous use of antibiotics in patients with NEC. Whether the differences in clustering were because the samples were obtained at the time of NEC when these babies were receiving antibiotics is not clear.

In another study by Mai and colleagues,[23] starting with the first stool and continuing until discharge, weekly stool specimens were collected prospectively from infants
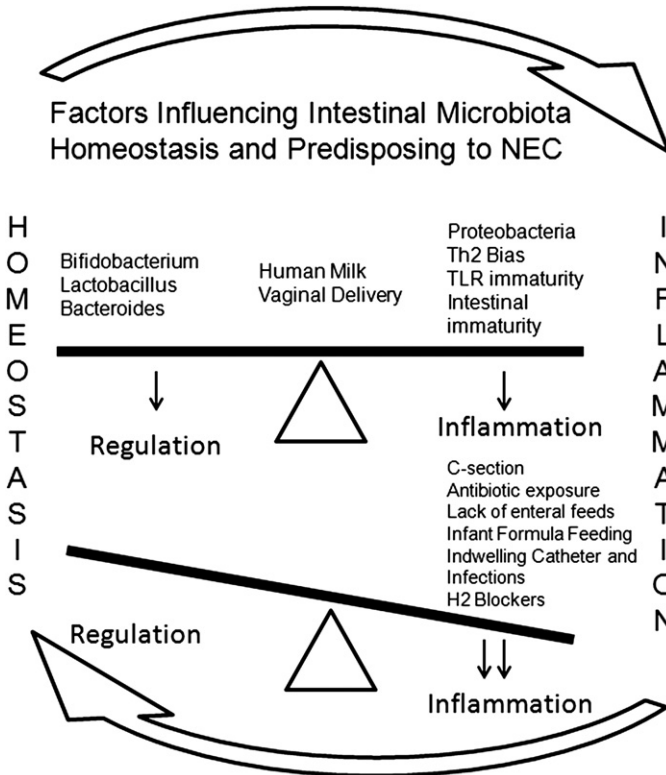
Fig. 3. Factors influencing intestinal microbiota homeostasis and predisposing to NEC. The intestinal microbiota are in perfect equilibrium with bacteria that regulate and others that have the potential to cause inflammation. Disruption of this equilibrium (dysbiosis) for different factors leads to inflammation.

with gestational ages 32 completed weeks or less or birth weights 1250 g or less. High-throughput 16S rRNA sequencing was used to compare the diversity of microbiota and the prevalence of specific bacterial signatures in 9 NEC infants and in 9 matched controls. A bloom (34% increase) of Proteobacteria and a decrease (32%) in Firmicutes in NEC cases between 1 week and less than 72 hours was detected. No significant change was identified in the controls. Several molecular signatures were identified to be increased in NEC cases 1 week before and within 72 hours of NEC development. One of the bacterial signatures detected more frequently in NEC cases ($P<.01$) matched closest to γ-Proteobacteria (similar to the Wang study[22]). Although this sequence grouped to the well-studied Enterobacteriaceae family, it did not match any sequence in GenBank by more than 97%. These observations suggest that abnormal patterns of microbiota and potentially a novel pathogen contribute to the cause of NEC.

FACTORS AFFECTING THE INTESTINAL MICROBIOTA
Prenatal

Thus, at this juncture, it seems that the microbiota of babies who subsequently develop NEC are different from those who do not. It will be important to dissect

the factors that contribute to these differences, which may begin in fetal life (see **Fig. 3**). Recent PCR-based studies estimate the prevalence of microbial invasion of the amniotic cavity to be 30% to 50% higher than that detected by cultivation-based methods.[24] These studies have shown that cultivation-resistant anaerobes belonging to the family Fusobacteriaceae (particularly *Sneathia sanguinegens* and *Leptotrichia* spp) are also commonly found in amniotic fluid. Other diverse microbes detected by PCR of amniotic fluid include as-yet uncultivated and uncharacterized species. A causal relationship between diverse microbes, as detected by PCR, and preterm birth is supported by types of association (eg, space, time, and dose) proposed as alternatives to Koch's postulates for inferring causality from molecular findings. Whether this colonization affects the gastrointestinal tract of the fetus is not known, but the fact that the fetus swallows large quantities of amniotic fluid during the last trimester of pregnancy and the recent finding that microbial DNA is present in meconium suggest that the fetal intestine is exposed to the amniotic fluid microbes. The high sensitivity of the fetal intestine to inflammatory mediators such as lipopolysaccharide (LPS) suggests that in utero microbes may trigger intestinal inflammation in utero[25] Furthermore, it can be speculated that exposure of the intestinal tract to toll-like receptor (TLR) agonists such as LPS, flagellin, or microbial CpG in low quantities may also make the intestine tolerant to further inflammatory stimuli.[26]

Postnatal

Postnatally, many factors can influence intestinal bacterial colonization as well as the responses to colonization. Some studies have shown that mode of delivery (vaginal vs cesarean section), type of milk (human vs formula), and use of antibiotics influence the intestinal flora.[27–29] Those infants born via cesarean section, fed formula milk, and exposed to antibiotics have a decrease in diversity of intestinal microbiota and abnormal patterns of colonization, with suppression of healthy bacteria such as Lactobacillus and bifidobacteria.

Cesarean Section Versus Vaginal Delivery

Epidemiologic data showing a relationship between increased rate of cesarean section and risk for development of subsequent diseases was recently reviewed.[30] In the United States, the rate of cesarean delivery has increased 48% since 1996, reaching a level of 31.8% in 2007. This trend is reflected in many parts of the world, with the most populous country in the world, China, approaching 50% and some private clinics in Brazil approaching 80%. Concurrent with the trend of increasing deliveries by cesarean section, there has been an epidemic of both autoimmune diseases, such as type 1 diabetes, Crohn disease, and multiple sclerosis, and allergic diseases, such as asthma, allergic rhinitis, and atopic dermatitis. The occurrence of these diseases is higher in more affluent, Western, industrialized countries. As reviewed in Ref,[30] several previous studies have shown differences in microbial colonization after cesarean section and vaginal deliveries. A more recent study,[31] in which nonculture-based sequencing technology was used, examined the early stages of the colonization of the body by microbes. Babies born vaginally were colonized predominantly by *Lactobacillus*, whereas babies delivered by cesarean section were colonized by a mixture of potentially pathogenic bacteria typically found on the skin and in hospitals, such as *Staphylococcus* and *Acinetobacter*, suggesting that babies born by cesarean delivery were colonized with skin flora in lieu of the traditionally vaginal type of bacterium.[32] The data relating these changes to later outcomes such as celiac disease, allergies, and atopy are compelling, but a relationship of cesarean

section versus vaginal delivery, intestinal colonization, and the development of NEC has not been found.

Human Milk Versus Infant Formula

Animal and human studies have shown that human milk decreases the incidence of NEC.[33–35] Beneficial factors of breast milk include immunoglobulins, cytokines, lactoferrin, lysozyme, and growth factors, but more importantly, healthy bacteria are promoted by human milk oligosaccharides (HMO).[27,36] HMOs contain a lactose core and act as prebiotics stimulating growth of *Bifidobacterium* species.[37,38] At birth, the rapid colonization of intestinal flora develops in the newborn first with aerobic or facultative anaerobic bacteria such as enterobacteria, enterococci, and staphylococci, and then during growth, they consume oxygen, allowing the proliferation of anaerobic bacteria such as *Bacteroides*, *Bifidobacterium*, and *Clostridium* species.[39,40] In the formula-fed infant, this transition tends to not occur that way and the newborn intestinal flora differs in its pattern of colonization with respect to breast-fed infants with predominance of gram-negatives and fewer anaerobes.[41,42] If they are grouped by phyla, breast-fed infants have predominance of Firmicutes mainly *Lactobacillus*, *Bacteroides* and Actinobacteria (*Bifidobacterium*) compared with formula-fed infants, who have predominance of Proteobacteria such as *Escherichia coli* and Firmicutes, some whom have pathogenic characteristics such as *Clostridium* and *Staphylococcus*. In 1 study, it was speculated that the abnormal pattern of colonization early at birth with delayed bloom of more pathogenic bacteria late in preterm infants could potentially explain those patients who develop NEC.[23] Sullivan and colleagues[33] in 1 multicenter study using donor human milk showed that an exclusively human milk-based diet is associated with significantly lower rates of NEC and surgical NEC when compared with a mother's milk-based diet that also includes bovine milk-based products. The reduction in NEC using strategies of only human milk was of 50% for clinical NEC and almost 90% for surgical NEC. The number needed to treat to prevent 1 case of NEC was estimated to be 10. In a recent policy statement by the American Academy of Pediatrics regarding breastfeeding and the use of human milk, the recommendation was to offer donor milk to all preterm infants in whom mother's own milk is unavailable.[43]

Antibiotic Exposure

The use of antibiotics is widespread in the neonatal intensive care unit (NICU). Often, their use is justified, but sometimes, it is the product of fear of infections in the preterm infant. Nevertheless, the use of antibiotics in neonates and especially in the preterm infant has unintended and sometimes devastating consequences. Antibiotic exposure may reduce the diversity of intestinal microbiota, delay the colonization of beneficial bacteria, and predispose preterm neonates to NEC. Previous studies[44–46] have shown that duration of antibiotic exposure is associated with an increased risk of NEC among neonates without previous sepsis. One study[47] found that overgrowth of pathogenic species is increased after 3 days of antimicrobial exposure. In the United States, most mothers giving birth prematurely are treated with antibiotics and many very-low-birth-weight infants are treated with a course of broad-spectrum antibiotics such as ampicillin and gentamicin. Studies[28] have shown the detrimental effects of antibiotics to the intestinal flora, even after 1 dose of antibiotics with alterations in the intestinal microbiota that could take years to recover. One study[48] showed a decrease in diversity, a predominance of less desirable bacteria and highly resistant clones with abundance of specific resistance genes as a result of antibiotic exposure that did not recover after 2 years after exposure. Cotten and colleagues[49] found that

each empiric treatment day with antibiotic was associated with increased odds of death, NEC, and the composite measure of NEC or death. These investigators concluded that prolonged initial empiric antibiotic therapy may be associated with increased risk of NEC or death and should be used with caution.

Extreme caution should then be exerted at the moment of deciding to start antibiotics in preterm infants. Long-lasting consequences and life-threatening situations could result from the indiscriminate use of antibiotics.

INTESTINAL MICROBIOTA ALTERATION INDUCES INTESTINAL INFLAMMATION AND CYTOKINES

Immature intestinal response and alterations in the intestinal microbiota predispose the intestine of neonates to inflammation and to a cascade of proinflammatory and counterinflammatory cytokines response. Proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin 1 (IL-1), IL-6, IL-8, endothelin 1, and platelet-activating factor are all increased in patients that develop NEC.[50–52]

The intestinal flora in the neonate have important functions in metabolism, nutrition, immune system, and defense against pathogens.[53,54] The immune system is developmentally regulated; therefore, in preterm babies, this immune system is still immature. Normally, it recognizes and fights harmful bacteria but does not react against healthy species of bacteria.[55,56] To avoid triggering pathologic immune responses, commensal bacteria must not express key virulence factors. For example, LPS is pentacylated in *Bacteroides* species, which are abundant in the intestinal flora of breast-fed, healthy infants. This modification makes lipid A in commensal *Bacteroides* a poor agonist of TLRs.[57] Conversely, lipid A of Proteobacteria is a potent agonist of TLR-4 and more likely to elicit an immune response because of its hexacylated characteristics.[58,59] The intestinal mucosa has mechanisms of recognition of bacterial products via highly specialized pattern recognition receptors called TLRs, which recognize specific microbial-associated molecular patterns (MAMPs).[60] Each MAMP has its own specific TLR.[61] For example, LPS, which acts as the endotoxin for gram-negative bacteria, is recognized by TLR-4. Flagellin is recognized by TLR-5, whereas peptidoglycan and lipotechoic acid from gram-positive bacteria are detected by TLR-2. TLR expression seems to be regulated by patterns of intestinal colonization, and therefore, abnormal patterns of colonization can trigger inappropriate responses. These MAMPs activate the specific TLR that leads to the activation of nuclear factor κ B (NF-κB) and caspases, which in turn activate transcription genes and induce cytokines such as IL-1, IL-6, IL-8, TNF-α, and interferon (IFN-1) (**Fig. 4**). Commensal healthy bacteria are believed to regulate this intestinal inflammatory response.[60,62] For example, studies have shown that *Lactobacillus* protects against cytokine-mediated cell injury.[63] *Bifidobacterium*, *Bacteroides thetaiotaomicron*, and *Streptococcus thermophilus* are examples of other bacteria that play a role in cytoprotection of the enterocyte and intestinal mucosa.[64] Many of these bacteria have been studied as therapeutic agents in the treatment of NEC, with promising results. Several studies, including a recent meta-analysis of the literature,[65,66] showed that the incidence of NEC was decreased in infants treated with probiotics containing different combination of these beneficial bacteria. Probiotics seems to help maintain a normal intestinal flora in neonates, thereby decreasing the incidence of NEC. Current evidence led to a recommendation level B in 2011 of effectiveness (recommendation is based on positive-controlled studies, but 1 negative study did not support the primary outcome).[67] Others[68,69] have suggested that dead microbes may be as effective as live microbes in modulating excessive inflammatory stimuli (see **Fig. 4**).
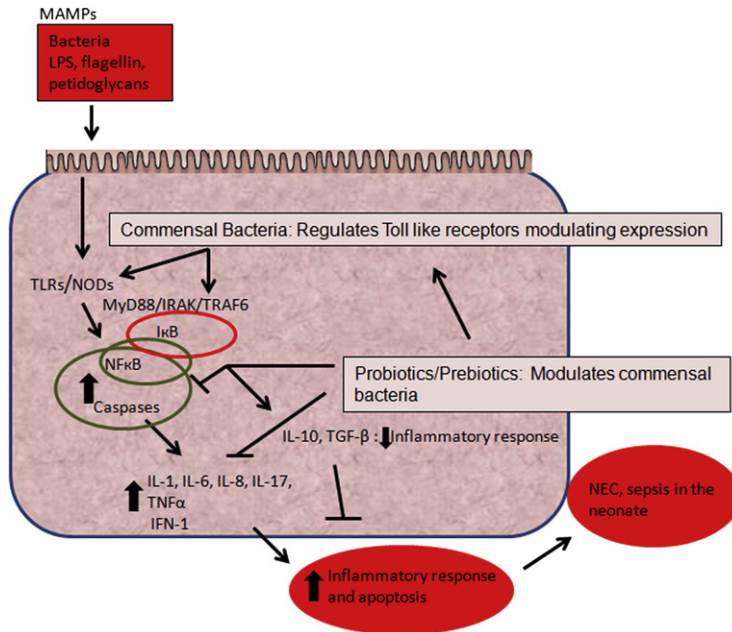
Fig. 4. Simplified TLR signaling leading to NF-κB activation, inflammatory response, and possible NEC. Surface enterocytes can recognize MAMPs via TLRs. Each of these receptors recognizes a specific bacterial product. For example, TLR-2 recognizes products of gram-positive bacteria, TLR-4 recognizes LPS from gram-negative bacteria, and TLR-5 recognizes flagellin. Cell stimulation signals recruitment of MyD88, IRAK, and TRAF6, then triggers activation of IκB. NF-κB activates the transcription of genes, including cytokines and chemokines. IκB: inhibitor of kappa B.

There are many opportunities for the physicians taking care of neonates to provide interventions that improve the adequate colonization of the neonatal gut and to minimize the deleterious but often unavoidable effects of medical treatment.

INTESTINAL MICROBIOTA BACTERIAL TRANSLOCATION AND OTHER RISK FACTORS FOR NEC

Bacterial translocation has been suggested as one of the mechanisms for developing NEC.[70,71] Bacterial translocation is not restricted only to the invasion of intestinal bacteria but can also include bacterial toxins or antigens that damage the intestinal epithelia and enter the circulation, resulting in a systemic inflammatory response.[72] During inflammation, production of nitric oxide alters expression and localization of the tight junction. Disruption of the tight junction zonulin proteins ZO-1, ZO-2, ZO-3, and occludin leads to intestinal permeability and bacterial translocation.[73] Few studies have shown the relationship between bacterial translocation and NEC. In 1 study[74] of bloodstream infections (BSIs) in very-low-birth-weight infants, BSI was more common after diagnosis of NEC in very-low-birth-weight infants who developed intestinal failure (60%) compared with those with surgical NEC without intestinal failure (42%) and those with medical NEC (20%). Coagulase-negative *Staphylococcus* and *Klebsiella* were the most frequently identified microorganisms. Alteration in the intestinal permeability was believed to be one of the mechanisms of BSI. Causation is difficult to

establish. Nonculture-based analyses of intestinal microbiota have given us some indication of the alterations in diversity and intestinal flora composition of those infants who develop NEC.

However, what causes bacterial translocation? Bacterial translocation is believed to be secondary to abnormal intestinal colonization. Risk factors for bacterial translocation are discussed elsewhere in this article. Other factors have been associated with increased risk of NEC probably secondary to bacterial translocation. Histamine 2 (H_2) blockers, which are sometimes used in NICUs, have been shown to increase the risk of sepsis and meningitis.[75] It seems that increasing the gastric pH in preterm infants predisposes the neonate to bacterial translocation and infection.[76] Guillet and colleagues[75] showed in their study that those infants less than 1500 g treated with H_2 blocker were more likely to develop NEC. In a study performed several years ago, Carrion and Egan[77] reported that by maintaining an acidic gastric pH less than 3.0, the incidence of NEC decreased and higher gastric enteric bacterial colony counts were strongly correlated with gastric pH level of more than 4.

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