REVIEW ARTICLE

The Role of Microbes in Developmental Immunologic Programming

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ABSTRACT: The role of microorganisms in the gastrointestinal tract has undergone significant modification in the past few decades with new observations from clinical, epidemiologic, and basic science research. We now know that the perception of these gut microbes as pathogens or even as commensals is somewhat outdated. It is becoming increasingly clear that the gut microbiome plays an important role in a host of activities including digestion, protection from potentially pathogenic organisms, and the regulation and development of the host immune system. The complex interactions between microbes and host combined with recent clinical observations and epidemiologic trends may point to the convergence of two well-supported (though imperfect) hypotheses: the “hygiene hypothesis” and the “fetal programming hypothesis.” We are beginning to understand that exposure to microbes before conception, during gestation, and in the neonatal period have profound effects on the developing immune system. Recent observations from a variety of fields help support the expansion of the “fetal programming hypothesis” to a host-microbe corollary that microbe-host interactions at critical windows influence the future immune phenotype, the maintenance of immune health, and the development of immune-mediated disease. (*Pediatr Res* 69: 465–472, 2011)

The “hygiene hypothesis” was initially formed as an explanation for the observed rise in the incidence of acute appendicitis coinciding with improvements in sanitation during the industrialization of Western societies in the early 20th Century (1–3). Observations supporting the theory’s relevance to atopic diseases were noted as early as the mid-1970s by Gerrard *et al.* (4), but the expansion of the hypothesis to its current and more popularized form is credited to Strachan (5), who noted that an increasing number of siblings in a household and specifically the number of older siblings, both markers of high microbial exposure, were protective against the development of hay fever. The hypothesis presently states that the recent increase in T-helper cell (Th) type 2-mediated diseases over the past half-century can be explained, at least in part, by decreased exposure to microbial antigens early in life through improved sanitation and the relative sterility of the modern world. Decreased antigenic exposure has adverse

effects on the budding immune system and increases the likelihood of developing atopic disease (6).

The resident gut microbial flora and its constant and active communication with the gastrointestinal immune compartment seem to play an important role in immunologic programming and when abnormal or inappropriate can lead to the development of allergic diseases. It is also becoming more clear that intestinal microflora influence the development of both Th1- and Th2-mediated diseases through their effect on regulatory cells and pathways that, when intact, help prevent disease-associated aberrant immune responses (7). Gastrointestinal tract microbes also play an integral role in the development of Th1 and Th2 balance. Examples include mice that are maintained in germ-free conditions tend to have Th2 dominant immune responses. Sellon *et al.* (8) showed that IL-10 deficient mice raised in germ-free conditions do not develop the expected spontaneous Th1-mediated colitis while the same mice reared in specific pathogen-free bacteria do. Although the hygiene hypothesis by itself is an imperfect theory and cannot explain all of the recent increases in certain diseases, there is support for one of its primary predictions that host-microbe interactions that occur early in life have long-term effects on the development of disease across populations.

Barker *et al.* are credited with the “developmental origins of adult disease” or “fetal programming” hypothesis (9), which grew from epidemiologic observations linking LBW and prenatal under-nutrition to adult diseases such as coronary artery disease (10), type 2 diabetes (11), hypertension (12), and stroke. Interpreted more generally, the hypothesis suggests that events and exposures occurring *in utero*, at birth, and in early childhood can have profound and long-term effects on the adult phenotype. The roots of the hypothesis lie in developmental plasticity during gestation and early childhood, a process through which one genotype can lead to various phenotypes in response to environmental signals during critical periods of development (9). Although the original hypothesis specifically relates to fetal nutrition and growth, the concept of environmental conditions at critical windows programming the development of disease can and has been

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Abbreviations: LGG, *Lactobacillus rhamnosus* strain GG; NEC, necrotizing enterocolitis; Th, T-helper cell; TLR, Toll-like receptor

Figure 1. Factors that may influence immune development and related health/disease outcome in the offspring.

expanded well beyond the original observations into a more general “Barker hypothesis.” Maternal dietary factors (13–15) and maternal smoking (16) can modify neonatal immune responses and influence the development of immune-mediated disease. Figure 1 illustrates a range of maternal and environmental factors that may influence fetal immune development and are implicated in short- and long-term immune health. Considering the recent rise in certain immune-mediated disorders, of particular interest is the exposure of the fetus and neonate to microbes and the resultant effects, both deleterious and beneficial, on developmental immune programming. The mechanisms underlying the microbial influences on immune development are beginning to be elucidated with many studies focused on the intrauterine milieu and on epigenetic inheritance. Epigenetic dysregulation, in particular, has been shown to be an important factor in the development of many human diseases, and the role of gene-environment interactions in immune function and immune-mediated disease is just beginning to be established (17).

Maternal exposure to microbes, the sequential colonization of the gastrointestinal tract after birth, the influence on that colonization by birth conditions and exposures in the first few weeks of life, and the influence of prebiotics and probiotics all support the adaptation of the “fetal programming” hypothesis to a host-microbe corollary that specific (though not yet defined) interactions between the microbes and host during critical periods of immune development (including interactions between the fetus and the maternal immune system and microbiome during gestation) may have consequences well into adulthood.

Gestation

Placental mammals are faced with a difficult task during pregnancy. The maternal immune system must not only actively eliminate potentially harmful microbial stimuli but also be tolerant of the fetus and protect it from immune rejection. Maternal health during gestation has significant effects on the health of offspring and nutritional, toxic, genetic, metabolic, and infectious factors all contribute to the eventual newborn phenotype.

There is building evidence that maternal immune status and maternal exposure to microbes during pregnancy affects the development of the fetal immune system. Blümer *et al.* (18) showed in a murine model that maternal exposure to lipopolysaccharide during gestation resulted in less allergic sensitiza-

tion and airway inflammation in their offspring. The most convincing evidence in humans comes from studies showing that offspring born to mothers living in farming environments (a marker for high maternal microbial exposure during gestation) are protected from the development of asthma (19) and have an up-regulation of receptors of the innate immune system (20). Human offspring of mothers exposed to pets during pregnancy have lower cord blood IgE levels at birth (21), which may be protective against the development of allergic disease. The mechanisms behind such protections are being investigated, and maternal Toll-like receptor (TLR) signaling has recently been shown to be an important participant (22). Maternal infections with parasites (23), viruses (24), and exposure to viral antigens (25) during gestation have also been recently shown to modulate the immune response in the offspring in both helpful and harmful ways. These data, along with observations from probiotic intervention studies (to be discussed in detail below), show that maternal microbial exposure during gestation may lead to long-term health consequences for the offspring by influencing fetal immune development.

The Maternal/Fetal Interface and *In Utero* Priming

The role of maternal dietary avoidance in the prevention of atopic diseases in offspring has been well studied and hotly debated over the past few decades as is evidenced by a recent change in recommendations from the American Academy of Pediatrics against routine avoidance of specific dietary antigens (26) largely based on results from a 2006 Cochrane review (27). Some studies have even shown that maternal dietary and allergen avoidance during pregnancy results in an increased risk of allergy (28,29). At the crux of the issue of maternal allergen avoidance is the ongoing debate over whether prenatal allergen exposure leads to *in utero* priming of the fetal immune system or not. Although not directly related to microbes, a review of the current literature on this topic yields important clues to the potential mechanisms behind the influence of microbes, as well as other environmental exposures, on the developing immune system. To date, there is evidence supporting both sides of the discussion.

In utero priming garners backing from studies showing an association between cord blood IgE levels and the development of atopic disease (30) and the presence of allergen-specific IgE in cord blood of neonates (31). However, the clinical relevance of cord blood IgE has been called into question as it does not seem to consistently correlate with or predict the development of disease (32). Others have shown that cord blood allergen-specific IgE is not of fetal origin but rather due to maternal contamination likely at the time of delivery (33), although one recent study claims to have controlled for maternal contamination (34). More support for fetal immune priming comes from studies showing allergen-induced T-cell reactivity in cord blood (35,36), whereas other studies show a lack of a quantitative relationship between maternal allergen exposure and T-cell responses in their newborn offspring (37). The potential mechanism behind *in utero* priming could include transport of small, yet immunogenic

allergen fragments (38) or IgG/allergen complexes across the placenta to prime naïve fetal T cells. Environmental allergens have been documented both in cord blood and amniotic fluid (39). Although it is well known that maternal IgG readily crosses the placenta, IgG/allergen complexes have also been shown to cross (40) and may exert fetal immune reactivity through binding with FcRn (neonatal Fc receptor) known to be distributed on placental cells and on human fetal intestine (41).

It is also now known that the role of maternal IgG itself is no longer limited to the passive protection of the fetus against harmful environmental exposures. These antibodies also provide the developing fetus with a wealth of maternal immunologic memory and experience. Maternal antibodies influence the selection of T- and B-Cell repertoires in the offspring that are conserved well into adulthood. They also aid in the suppression of IgE antibody responsiveness, which seems to be long lasting (42). The maternal-fetal unit is infinitely complex, and more study is needed, *e.g.* the impact of the cytokine milieu, other soluble immune factors, and microbes at the maternal/fetal interface. However, the framework for the mechanisms underlying the influence of microbes and other stimuli on the developing immune system exists.

Gastrointestinal Immunity

The intestine is now recognized as the largest and perhaps most influential immune organ in the body. It is charged with actively responding to potentially harmful pathogens and antigens, while creating and maintaining tolerance (systemic unresponsiveness) to other antigens and to potentially beneficial commensal and symbiotic bacteria.

The gastrointestinal mucosal barrier must be selective in what molecules and signals are allowed to transfer across it. However, the role of the intestinal epithelium goes far beyond just that of a physical barrier. Mucosal barrier components such as transmembrane TLRs and cytoplasmic Nucleotide-binding oligomerization domains (NOD) receptors are members of the innate immune system that act as microbial pattern recognition receptors (43). These families of receptors bind novel ligands encountered in the gut lumen and they have an important role in the interaction of luminal microbes with host-immune defense, immune cell recruitment, and mucosal inflammation (Fig. 2). The importance of these families of receptors in inflammation and tissue homeostasis is most famously exemplified by the association of certain *NOD2* gene mutations with Crohn's Disease, a chronic inflammatory disorder (44), and by the development of more severe experimental colitis in various TLR knockout mice (45). *NOD1* recognition of intestinal microbiota-derived peptidoglycan has been shown to enhance systemic innate immunity (46), illustrating the influence of intestinal microflora far beyond the gut. In addition, TLR recognition of specific commensal bacteria has been shown to be required for the maintenance of intestinal homeostasis (47), lending support to the symbiotic relationship between certain microbes and the host.

The interaction of the microbes and immune cells of the gastrointestinal tract also effects the development and maturation of both the innate and adaptive immune system.

Figure 2. Microbe-driven T-cell differentiation: mucosal dendritic cells (DCs) sample bacterial antigens from the intestinal lumen *via* Toll-like receptors (TLRs) and are activated. Activated/mature DCs produce cytokines that activates naïve T cells (Th0) to mature into a balanced T-helper cell response [Th1, Th2, and regulatory T cell (Treg)]. MHC, major histocompatibility complex. Reprinted from Walker WA Functional Food Reviews 1:13–19. Copyright © 2009, BC Decker Inc, with permission.

Mazmanian *et al.* (48) showed that a bacterial polysaccharide (PSA) from *Bacteroides fragilis*, a specific colonizer of the gastrointestinal tract, has direct immunomodulatory activities including correction of systemic T-cell deficiencies and Th1/Th2 imbalance and has a role in immune maturation by directing lymphoid organogenesis. Hooper *et al.* (49) showed that angiogenin 4 (Ang4), a product of the commensal bacterium *Bacteroides thetaiotaomicron*, acts as a mediator of host defense in the intestine. Finally, it is becoming accepted that commensal microflora can induce an anti-inflammatory immune response through induction of regulatory T cells that help guide Th1 and Th2 balance (50). This is supported by the demonstration of impaired oral tolerance induction in germ-free mice which later regain their ability to be tolerized after reconstitution of their microflora with *Bifidobacteria* during infancy (51). Commensal gut microflora DNA (gfDNA) has also been shown to limit regulatory T-cell conversion through their interaction with TLR9 and thus play a role in intestinal homeostasis (52). These examples highlight the importance of reciprocal interactions between microbes and the host and the significance of this symbiosis with regard to human immune health.

Colonization in the Peripartum Period

The adult human intestinal tract contains 10^{14} bacteria with a density of between 10^{11} and 10^{12} microbes/mL of luminal content (53). Each microbiome is unique and each is also fairly stable over time (54). By 1 year of age, the infant microbiome appears to be fairly similar to that of adults. However, during that first year, the infant intestinal microbiota is far more impressionable and far less stable. Initial intestinal colonization provides an enormous microbial stimulus to the host, which leads to profound changes in intestinal development and defense (Fig. 3). At the time of birth, the neonate passes from the relatively sterile conditions within the amnion into an environment filled with microbes. Exposure of the

Figure 3. Cross section of a noncolonized human fetal small intestine (left) and the same cross section of a fully colonized infant (right). Note the contrast in active proliferation, epithelial maturity, and abundance of lymphoid elements. Reprinted from Walker WA Functional Food Reviews 1:13–19. Copyright © 2009, BC Decker Inc, with permission.

infant's mucosal surfaces to the mother's vaginal and fecal flora, in addition to other environmental exposures, is thought to begin the complex process of intestinal colonization. There is wide variability in colonization across individuals and over time, and initial colonization seems to be dependent on the bacterial population to which the infant is first exposed. Bacteria begin to appear in feces within a few hours of life. With vaginal delivery, facultative anaerobes such as *Escherichia coli* and *Streptococcus* dominate in the first few days of life, taking advantage of the abundance of oxygen (55). As oxygen begins to be deprived, the facultative bacteria are replaced by anaerobic bacteria such as *Bacteroides*, *Clostridium*, and *Bifidobacterium*, typically by 1–2 wk of age (56).

Neonatal intestinal colonization is influenced by numerous perinatal factors including mode of delivery, composition of early feeding (breast milk *versus* formula), and antibiotic use. More importantly, the resulting differences in early microflora composition may have lasting effects on immune function. For example, infants born *via* cesarean section are more frequently colonized with *Klebsiella*, *Enterobacter*, and *Clostridium* than those born vaginally (57) and less frequently colonized by *Bifidobacterium* and *Bacteroides* (58). These differences in colonization may help account for the increased risk of asthma (59), atopy, and allergic disease (60) in children born by cesarean section.

The next major event that impacts the pattern of microbial colonization and immune status is the initiation of feeding. Although results are somewhat less consistent, it does appear that breast-fed infants are colonized with less *Escherichia coli*, *Clostridium difficile*, and *Bacteroides* than formula-fed infants. Breastfeeding provides many immune-related benefits for the infant including short-term protection from gastrointestinal illness (61), respiratory illness and infections (62), and otitis media (63). These advantages have been attributed to a range of different components of human milk including immunoglobulins, antimicrobial peptides, growth factors, nutrients, lysozyme, lactoferrin, and complement (64). These effects may also be due, at least in part, to the observed differences in microbial colonization in the neonatal period.

Intestinal colonization is also influenced by the use of antibiotics early in life. In premature infants, the duration of antibiotic treatment in the first month of life correlates with decreased bacterial diversity (65), and certain antibiotic regimens have been shown to decrease anaerobic bacteria and result in an overgrowth of specific organisms such as *Klebsiella* (66) and *Staphylococci* (67). Term infants given antibiotics in the first month of life have decreased colonization with *Bifidobacteria* and *Bacteroides fragilis* (58). These differences in microbiota might contribute to the somewhat disputed clinical observation, supported by one meta-analysis (68), that antibiotic use in the newborn period may increase the risk of atopic disease later in life. Once again, although the mechanism is not clear and more study is needed, these examples show that microbe-host interactions during critical periods of development may have long-lasting effects on the future immune phenotype.

Bifidobacteria are one of the better studied intestinal colonizers. As previously mentioned, they are less abundant colonizers of neonates exposed to antibiotics and those born *via* cesarean section. Colonization with *Bifidobacteria* seems to play an important role in humoral immune development exemplified by its role in the maturation of the mucosal salivary secretory IgA system (69) and its association with more circulating IgA and IgM secreting cells (70). In addition, *Bifidobacteria* are generally found in lower numbers in the feces of infants who go on to develop atopy (71) and thus may have long-term effects on an immune phenotype. This effect is likely species-specific as allergic infants have been reported to be colonized with different *Bifidobacterium* species than their healthy counterparts (72).

Intestinal Colonization and Specific Disease States

Recent advances in culture-independent techniques to better identify gut microflora have led to a wealth of investigations into patterns of colonization in specific disease states and the potential role for gut microbiota in the etiology and course of immune-mediated diseases.

Necrotizing enterocolitis (NEC). NEC is an inflammatory disease of the bowel that remains a devastating cause of neonatal morbidity and mortality. Although the etiology is likely multifactorial, the end result is a marked inflammatory response that often ends in intestinal tissue necrosis. Microbial colonization seems to be required for NEC pathogenesis. Recent studies have shown differences in gut microbiota between preterm infants with and without NEC both at the time of diagnosis (73) and before disease development (74), providing some evidence for the role of microbes in disease development. Both pathogenic and commensal bacteria (75) and inflammatory stimuli (76) induce exaggerated cytokine responses from fetal intestinal epithelial cells (IECs) compared with adult IECs, which may help explain the susceptibility of premature infants to NEC. The success of some probiotics in preventing NEC in preterm infants underscores the importance of microbe-host interactions in an immune-mediated disease (77).

Atopic diseases. Perhaps nowhere has the role of gut microbes in immune-mediated disease been more studied than in regard to atopic diseases. These studies also provide the best evidence about the critical timing of host-microbiome interactions. We know that, in mice, intestinal bacteria are required for the development of oral tolerance and that these bacteria must be introduced to the gut during the neonatal period or the effect is lost (51). This again indicates that microbe-host interactions at critical windows of time can alter the future immune phenotype. There is good evidence supporting reduced bacterial diversity (78) and differences in colonization patterns in infants with atopic diseases compared with controls (79). Overall trends point most consistently to a protective effect of *Bifidobacteria* and an increased risk of atopic disease in those colonized with more *Enterobacteriaceae*, *Clostridium*, *Bacteroides*, and *Staphylococcus* (79). There may be different mechanisms underlying the associations because colonization with some microbes increases the risk of atopic eczema only while others seem to increase the risk of all atopic diseases (80). With differences in microbiota fairly well established between these groups, investigators have gone on to determine whether disease outcome can be affected by manipulating the microbiota with probiotics. Although probiotics seem to have little role in the treatment of established atopic dermatitis, they may help prevent the condition. Some early studies showed an impressive reduction in the development of atopic eczema at the age of 2 y in children receiving *Lactobacillus rhamnosus* strain GG (LGG) during the final 4 wk of gestation (*via* maternal consumption) and the first 6 mo of life (81). This prevention of eczema was confirmed at ages 4 and 7 within the same cohorts (82,83). However, other studies with LGG with slightly different experimental designs have failed to show a protective effect (84). The mechanism of maternal LGG administration in reducing atopic dermatitis in offspring is not entirely clear. Probiotics alter maternal microbiota, affecting the bacteria that the newborn infant is exposed to and thus, potentially, the neonate's initial intestinal colonization patterns. There is some data to support that probiotics may affect the developing immune system *in utero* (85). However, other studies have shown that fetal immune responses are not affected by LGG given during pregnancy (86), suggesting the effect may be due to postnatal exposure to LGG through breast milk or *via* direct administration to the developing infant. Numerous other probiotics, prebiotics, and pro- and prebiotics mixtures have been evaluated as well (87). A recent meta-analysis did support a preventative effect of certain probiotics on atopic eczema (88). Probiotics have not been shown to be effective in the prevention of asthma and allergic rhinitis in humans to date. Other observations may support the importance of the colonization of the gastrointestinal tract in allergic disease including differences in the bacterial composition of intestinal flora before the onset of atopy (80), the promotion of immunologic tolerance in the neonatal period by bacterial colonization (89), and the requirement of intestinal microbial flora in the neonatal period for the induction of oral tolerance (51).

Prebiotics and Probiotics

Probiotics are live microorganisms that are beneficial to the host beyond their nutritional value. They have been shown to have favorable immunologic effects that influence both systemic and gut-associated immune responses. Many aspects of the immune system have been suggested to be effected by probiotics, including alteration of tolerance induction (90), anti-inflammatory effects *via* TLRs (91), Th1 skewing, augmentation of Regulatory T-cell function (92), modulation of dendritic cell function (93), and increased mucosal IgA production. Not all probiotics are alike, and it is becoming clearer that probiotic combinations are likely to be more effective than monotherapy. Some probiotics have been used successfully in the prevention and/or treatment of childhood infectious gastroenteritis (94), antibiotic associated diarrhea (95), and inflammatory bowel disease (96). Probiotics may exert their greatest effects when given during the perinatal period and during early childhood. This is supported by some of the strongest data showing prevention of immune-mediated diseases such as atopic dermatitis (97) and NEC (98) in high-risk neonates. Mechanisms of action have been proposed (99,100), but more studies are needed to determine precise pathways and safety of use. These studies, along with research into stable, probiotic-secreted products may lead to more widespread use in appropriate clinical situations in the near future as the concept of giving live bacteria to high-risk infants is still quite controversial.

Prebiotics are indigestible food ingredients, normally complex carbohydrates that benefit the host by stimulating the growth and/or activity of certain intestinal bacteria like *Bifidobacterium* (101). Although well-controlled trials are limited at this time, there is some evidence that prebiotics alone or in combination with probiotics may be effective in preventing atopic dermatitis in high-risk populations (102,103). More trials are clearly needed, but the promising early results support the importance of microbe-host interactions at critical periods on the development and potentially prevention of human disease.

Conclusions

Microbes, among other environmental exposures, play an important role in developmental immune programming. Although the majority of this review focused on gastrointestinal tract microbiota, postnatal immune development can also be affected by exposure to nonenteric microbes, microbial products, and nonmicrobial antigens at the interface of multiple other immune compartments (*e.g.* respiratory and skin epithelium), which may prove to be equally important. These different mucosal surfaces all have continuous contact with the outside world and thus all contribute to the development of an immune response and/or immune tolerance. The gastrointestinal tract seems to play a particularly significant role in the development of the immune system and the mechanisms underlying its role are being explored. For example, the development of innate immune tolerance in the neonatal intestine has recently been shown to be dependent on microRNA-146a-mediated repression and degradation of the

IL-1 receptor-associated kinase (IRAK1), a TLR signaling molecule (104). Continued detailed investigation of the development of intestinal immune homeostasis and the microbiome-host relationship during the neonatal period will provide invaluable information that may better illuminate and define the windows for therapeutic intervention in the future.

The timing of these "critical periods" is inherently important. Animal studies showing that preconception maternal immunization protects the offspring from allergic sensitization support the importance of the prenatal period (105). Human probiotic prevention studies would point to the last month of gestation and first few months of life as the critical periods of immune development (81). Differences in microflora and the risk for atopic disease between infants born vaginally and those born by caesarean section highlight the importance of the immediate postnatal period (60) as do studies implicating breast milk components in the induction of oral tolerance and prevention of asthma (106). There may indeed be multiple windows of opportunity, and more work is clearly needed to better define them to help guide future therapeutic interventions.

More research is also needed to further elucidate how the microbiome is maintained, the associations of specific microbial species and/or components with specific clinical diseases, and the complex mechanisms of immune tolerance. New avenues of research include microbial influences on epigenetic interactions *in utero* and their potential effect on developmental immune programming through alterations in fetal DNA through altered histone methylation, histone acetylation, and chromatin structure (107). For example, bacterial infections have already been shown to promote DNA hypermethylation (108). It will also be important to better define communications not only between the microbe and host but also between microbes themselves. Novel research has led to new therapeutic strategies that are actively being trialed. These include polymicrobial probiotic cocktails, prebiotics, and other immunostimulatory molecules such as DNA and helminthes. New technology will allow us to address the interactions of entire microbial communities and their role in immune development and function spanning from gestation through adulthood. Ultimately, these efforts will generate new models and interventions with the goal of promoting human health and preventing human disease.

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