

Published in final edited form as:

J Dev Orig Health Dis. 2013 June 1; 4(3): . doi:10.1017/S2040174412000712.

The role of gut microbiota in programming the immune phenotype

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Abstract

The human fetus lives in a germ-free intrauterine environment and enters the outside world containing microorganisms from several sources, resulting in gut colonization. Full-term, vaginally born infants are completely colonized with a diverse array of bacterial families in clusters (Phyla) and species (>1000) by the first year of life. Colonizing bacteria communicating with the gut epithelium and underlying lymphoid tissues ('bacterial-epithelial crosstalk') result in a functional immune phenotype and no expression of disease (immune homeostasis). Appropriate colonization is influenced by the prebiotic effect of breast milk oligosaccharides. Adequate colonization results in an innate and adaptive mucosal immune phenotype via communication between molecular patterns on colonizing bacteria and pattern-recognition receptors (e.g., toll-like receptors) on epithelial and lymphoid cells. This ontogeny affects the immune system's capacity to develop oral tolerance to innocuous bacteria and benign antigens. Inadequate intestinal colonization with premature delivery, delivery by Cesarean section and excessive use of perinatal antibiotics results in the absence of adequate bacterial-epithelial crosstalk and an increased incidence of immune-mediated diseases [e.g., asthma, allergy in general and necrotizing enterocolitis (NEC)]. Fortunately, infants with inadequate intestinal colonization can be restored to a bacterial balance with the intake of probiotics. This has been shown to prevent debilitating diseases such as NEC. Thus, understanding the role of gut microbiota in programming of the immune phenotype may be important in preventing disease expression in later childhood and adulthood.

Keywords

colonization; gut microbiota; 'hygiene hypothesis'; mucosal immune programming

Introduction

If a cross-section of the small intestine in the human fetus *in utero* is examined, an immature, thin epithelium, which turns over very slowly, and a paucity of lymphoid elements can be seen. In contrast, a cross-section of the same portion of the small intestine in the human infant in the extrauterine environment shows a mature, actively turning over epithelium expressing all subclasses of enterocytes and a plethora of lymphoid cells (Fig. 1). The principal difference between these two intestinal sections is the intestinal environment. The human fetus resides in a germ-free environment, whereas the young infant entering the

external environment at term is rapidly exposed to large numbers of diverse microorganisms that colonize the intestine in numbers that exceed the total number of eukaryotic cells in the body by 10-fold. Colonizing bacterial communication with intestinal epithelial and lymphoid elements has a profound effect on the development of intestinal host defense, particularly mucosal immune function. This review will consider the impact of normal colonization of the human gastrointestinal tract on appropriate development of intestinal host defense, particularly regulatory T-cell function, and the disease consequences of inadequate initial intestinal mucosal immune function with disrupted colonization (dysbiosis). Finally, it will review evidence that, under conditions of dysbiosis, the use of probiotics may act as a surrogate colonizer to lessen the severity or even prevent the phenotypic expression of disease.¹

Initial bacterial colonization

Under normal gestational conditions (e.g., full-term gestation and vaginal delivery), the newborn infant leaves a germ-free intrauterine environment to enter a highly contaminated external world. During sequential periods within the first year of life, the infant colonizes its intestine with approximately 10^{14} microbes/ml of intestinal contents containing more than 1000 different species of bacteria. With the development of innate intestinal defenses, most microorganisms reside in the distal small intestine and colon as anaerobes. However, bacteria can also colonize the upper intestine, stomach and esophagus as facultative anaerobes and aerobes. The first and most important phase of normal colonization occurs when the newborn fetus passes through the birth canal and ingests a healthy bolus of maternal vaginal and colonic microorganisms. This bolus further proliferates when oral feedings are introduced. Initial colonization is strongly influenced by the nature of oral feedings (e.g., breast *v.* formula feeding without prebiotics, to be discussed later). At 6 months, weaning to a solid diet leads to complete colonization and the infants have a unique signature of microbiota with them throughout their lifetime. The appropriately colonized infant gut contains a balance of large clusters of bacterial families known as Phyla.² More recently, groups of bacterial families have been classified into ‘enterotypes’ on the basis of their functions, for example, metabolism of dietary components and ability to handle drugs, which should help in our further understanding of the role of enteric microbiota in health and disease.³ In addition to stimulating the immune phenotype, numerous symbiotic bacteria attaching to the gut surface can protect against pathogen penetration and gastroenteritis, or sepsis, by a phenomenon known as ‘colonization resistance’ (Fig. 2), which is very important for the prevention of pathogen-induced gastrointestinal inflammatory disease.

Diet and initial intestinal colonization

Ingestion of food provides a substrate for colonizing bacteria and helps determine enterotypes.³ Bacterial proliferation in many published studies suggests that large clusters of families of gut microbiota (e.g., phyla) are modified by a lifelong diet of processed food *v.* natural high-fiber intake, and this difference may influence the expression of disease (obesity, allergy, etc.).^{4,5} However, diet is most important with the introduction of initial oral feedings. Because colonization has just begun, the nature of oral feeding can have a profound influence on the overall, short-term composition of infant's gut microbiota.⁶ Breast milk has a large percentage of undigestible oligosaccharides (e.g., 8% of total calories), which function as prebiotics, providing substrate for the production of short-chain fatty acids, leading to the proliferation of health-promoting bacteria such as *Bifidobacteria* and *Lactobacillus*.

These health-promoting bacteria show a direct association between the levels of secretory IgA in intestinal secretions and the number of *Bifidobacteria* in the gut at 1 month of age. In

addition, inflammatory cytokine interleukin-6 (IL-6) levels in intestinal secretions are inversely related to the number of *Bacteroides fragilis* organisms in the gut at 1 month,⁷ which is important because excessive inflammation in infancy causes an increased incidence of age-related gastroenteritis. More recently, the report suggests that not only do breast milk oligosaccharides stimulate *Bifidobacteria infantis* proliferation, but they also activate important genes from those organisms that are anti-inflammatory (increased) and pro-inflammatory (decreased) to the host.^{8,9} These observations strongly underscore the protective value of breastfeeding for the newborn infant.⁶ Unfortunately, comparable studies in infants fed infant formula, with the exception of formula with prebiotics added, have not carefully documented their effects on gut microbiota or health-promoting bacteria. With complete colonization of the infant at 1 year, colonizing bacteria exist in a symbiotic relationship with the host, and immunologic homeostasis exists with no expression of disease. We will now discuss in detail studies that have shown the mechanisms by which colonizing bacteria stimulate appropriate programming of mucosal immunity.

Colonization and host defense

With colonizing microbiota, the gut has evolved an elaborate barrier system to defend against the invasion and dissemination of microbes into sub-epithelial intestinal tissues. This system consists of a surface mucus and membrane barrier (Fig. 3). Surface mucus is a layer of viscous fluid rich in mucins, non-specific antimicrobial peptides (AMPs) and secretory immunoglobulin A (sIgA). The intestinal barrier, dependent on bacterial colonization, is a membrane continuity formed by epithelial cells that are attached by tight junctions with dendritic cell (DC) appendages extruding between enterocytes (Fig. 3). The ‘trialogue’ among bacterial inhabitants, intestinal barriers and gut-associated immune components ensures generation and preservation of a stable symbiotic relationship between the intestine and colonizing bacteria. The biological consequences of disruption of this relationship have been the topic of intensive interest and the subject of many excellent reviews, for example.^{10–16} Here we will focus only on recent advances in the role of microbial colonization in programming host defense in the gut.

The role of commensal bacteria in the development of the gut defense system

The innovation of germ-free mice has revolutionized the study of how microbiota orchestrate host defense and vice versa.¹⁷ It is now acknowledged that commensal bacterial colonization modulates many developmental processes of the host, particularly the development of a fully functional host defense system (Fig. 4).¹⁸ Compared with conventionally colonized animals, germ-free mice present numerous indicators of defective development of immunity.¹⁹ There are fewer numbers of CD4⁺ and CD8⁺ T cells and less production of IgA plasma blasts in the gut lamina propria (LP) in germ-free mice relative to conventionally colonized mice.^{20,21} Mono-colonization with *Bacteroides thetaiotaomicron*, *B. fragilis*, *Clostridium*, *Lactobacillus* and *Bifidobacterium* ameliorates various immune deficiencies seen in germ-free mice and augments the expression of genes involved in intestinal development, transport and immune protective functions.^{22–24} Mazmanian *et al.*²⁵ observed that polysaccharide A (PSA), the capsular polysaccharide produced by *B. fragilis*, mediates its effect on directing host immune development. In this case, PSA is processed by CD11c⁺ DCs, which subsequently induce CD4⁺ T-cell expansion and appropriate cytokine production.²² These studies highlight a positive role for commensal bacterial colonization in host defense.

Intestinal microbiota enhance barrier function

Intestinal barriers (mucus and membrane) provide the first line of defense and directly communicate with microbiota in the gut. The barrier (Fig. 3) comprises structural and

secreted products. The mucus barrier includes a thinner inner layer and a thicker extracellular barrier.²⁶ The inner layer, often referred to as the apical glycocalyx, constitutes membrane-bound mucins and glycolipids on intestinal epithelial cells (IECs), including goblet cells.²⁷ The extracellular mucus barrier comprises three components: secreted mucins, AMPs and sIgA.

Secreted mucins serve as an energy source for microbiota and a guardian for the host to prevent pathogen invasion.^{26,28–30} Mucins production can be modulated by colonizing microbes. In the germ-free condition, there is a deficient production and/or function of the mucus barrier, which includes reduction in thickness, compactness, mucin content and a decreased number and function of goblet cells.³⁰

AMPs cause detrimental activities in a broad spectrum of microorganisms including Gram-negative and Gram-positive bacteria, fungi, protozoa and even enveloped viruses. Some AMPs have chemoattractive activities and can neutralize bacterial exotoxins.³¹ The major family of AMPs are defensins, which can be divided into two subfamilies: α - and β -defensin. Paneth cells are the primary source of α -defensins,^{32–34} whereas several subtypes of IECs can secrete β -defensins. All defensins must be processed into an active peptide, which involves matrix metalloproteinase (MMP7) or matrilysin in mice and trypsin in humans.³⁵

Crosstalk between AMPs and gut microbiota plays an important role in sustaining gut homeostasis. Bacterial exposure causes an increase in the production of AMPs.³⁶ Vaishnavi *et al.*³⁷ show that bacterial-triggered secretion of AMPs requires activation of the MyD88 signaling pathway, which is also necessary for restricting the penetration of commensal or pathogenic bacteria into mesenteric lymph node (MLN). On the other hand, AMPs participate in shaping the microbiota community in the gut.³⁸ Salzman *et al.* observed that transgenic expression of DEFA5 (α -defensin) leads to a significant decrease in the percentage of *Phylum Firmicutes*, a significant increase in the percentage of *Bacteroidetes*, and a loss of *Clostridia*, *Bacilli* and *Erysipelotrichi*. Transgenic expression of DEFA5 also causes loss of *segmented filamentous bacteria* (SFB) and IL-17-producing T cells in the intestinal LP. In mice deficient in MMP7, which is essential for generating active defensin, the percentage of *Firmicutes* is significantly increased and the percentage of *Bacteroidetes* is significantly decreased relative to MMP7^{+/+} mice.³⁸

sIgA controls epithelial colonization of microorganisms and prohibits the absorption of potentially dangerous antigens/pathogens. The production of sIgA requires the cooperative action of plasma cells located in intestinal LP and epithelial cells.^{39,40} The involvement of intestinal microbiota in the programming of sIgA can occur in two ways: first, by modulating the production of sIgA;⁴¹ second, by participating, together with other factors including the age of the host and T cells, in modulating the sIgA repertoire.^{42–45}

Microbiota program sIgA excretion by inducing receptor expression or by activating and driving the expansion of plasma cells. Crosstalk between microbiota and epithelial cells is an important mechanism in the regulation of polymeric immunoglobulin receptor (pIgR) expression. Accumulating evidence suggests that microbe-associated molecular patterns (MAMPs) stimulate the expression of pIgR in IECs.^{46,47} Bacteria-induced production of specific sIgA appears to be precisely regulated through a feedback loop. In this case, newly produced sIgA restricts the adhesion of bacteria to the epithelial surface, thereby minimizing bacterial stimulation of host epithelial cells. Hapfelmeier *et al.*⁴³ report that reversible colonization of germ-free animals with a non-dividing mutant of *Escherichia coli* evokes a long-lived, highly specific sIgA response, which can be limited by restimulation with continuous exposure to the commensal bacterium.

An antigen-triggered specific IgA response originates from the germinal centers (GCs) in Peyer's patches (PPs) and isolated lymphoid follicles (ILFs).⁴⁸ There are at least three lines of evidence that suggest that the development of ILFs requires bacterial colonization. First, germ-free mice lack ILFs.⁴⁹ Second, ILFs develop immediately after bacterial colonization of the intestine.^{50,51} Finally, the size and cellular composition of ILFs depend on the load of bacteria colonizing the intestine.⁵²

Microbiota colonization modulates IECs and sub-epithelial gut-associated lymphoid tissue (GALT)

IECs not only provide a physical barrier but also cooperate with components of sub-epithelial GALT in maintaining intestinal immune homeostasis. Both commensal bacteria and pathogenic bacteria express MAMPs that are recognized by pattern-recognition receptors (PRRs) present on the cell surface of IECs and DCs. The most studied PRRs are toll-like receptors (TLRs). IECs also express intracellular nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) that can recognize microbial components.⁵² The signaling cascades downstream of PRRs are to a large extent mediated by MyD88, a cytosolic adaptor protein that directly binds TLRs through its own Toll/IL-1 receptor (TIR) domain. Once engaging TLRs, MyD88 recruits a cohort of signaling molecules, activates MAP kinases and drives nuclear translocation of NF- κ B. Unlike pathogens that use TLR pathways to trigger inflammation, commensal bacteria exploit the TLR pathway to maintain intestinal homeostasis and actively suppress immune reactions probably by inducing production of cytoprotective factors.^{22,53,54} For example, Rakoff-Nahoum *et al.*⁵⁴ found that treatment of mice deficient for MyD88, TLR2 and TLR4 with an epithelial irritant, Dextran Sulfate Sodium (DSS), led to severe ulceration and denudation of the epithelial cell layer, which preceded infiltration of inflammatory cells and resulted in a decreased production of cytoprotective factors such as IL-6 and keratinocyte-derived chemokine (KC-1) by epithelial cells. A similar phenomenon was observed in normal mice treated with a cocktail of multiple antibiotics to deplete microbiota in the gut. Together, these findings suggest that microbiota act to condition signal cascades mediated by TLRs on IECs and maintain gut homeostasis.

There are two prevailing models of how commensal bacteria are sampled in the gut (Fig. 5). Macpherson and Uhr⁵⁵ propose that sampling of commensal bacteria occurs through M cells. In this model, M cells take up by luminal commensal bacteria and/or other intact antigens by endocytosis. Commensal bacteria and/or other intact antigens discharged by M cells are captured by DCs in PPs, which migrate to MLNs and activate B cells in MLNs, eventually resulting in IgA production, which in turn prevents microbiota penetration. Another model posits that DCs directly sample commensal bacteria and/or other intact antigens in the intestinal lumen.⁵⁶ The seminal study in support of this model is the finding that DCs extend dendrites into the gut lumen by penetrating through the epithelial tight junction.^{57,58} Proper sampling and processing of commensal bacteria and/or other intact antigens by penetrating DCs depend on the activation of TLR signaling on IECs.^{59,60} These studies inspired Strober⁶⁰ to hypothesize that crosstalk among IECs, DCs and gut microbiota is essential for establishing and sustaining a healthy stable relationship between microbiota and host defense.

Commensal bacteria via MAMPs bind to TLRs on IECs and DCs. Activation of TLR signaling induces IECs to secrete cytoprotective factors. Upon activation of TLR signaling, IECs release thymic stromal lymphopoietin (TSLP) and transforming growth factor beta (TGF β) by which tolerogenic phenotypes of DCs are elicited.⁶¹ TLR-activated DCs secrete IL-10, which results in an immune response dominated by Treg cells. DC activation through TLRs can also induce Th1 immunity and secrete anti-inflammatory cytokines (Fig. 6).^{62,63}

Taken together, these studies suggest that the TLR signaling pathway orchestrates crosstalk among microbiota, IECs, DCs and related lymphocytes and promotes intestinal mucosal homeostasis.

Gut DCs are continuously exposed to microbial antigens. Commensal bacteria can activate and drive tolerogenic DCs mediated by a wide range of PRRs. For example, in addition to TLRs, DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), a C-type lectin receptor expressed on both the macrophage and DCs, recognizes and binds to MAMPs on microbiota.⁶⁴ DC-SIGN was found to bind to the commensal bacteria *Lactobacillus reuteri* and *Lactobacillus casei*, resulting in Treg cell induction.⁶⁵ Furthermore, Konieczna *et al.* showed that feeding *Bifidobacterium infantis* stimulates DCs to induce FoxP3⁺ Treg and IL-10-secreting T cells. However, the mechanisms of FoxP3⁺ Treg induction differ with myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). Induction of FoxP3 Treg by mDCs is TLR2, DC-SIGN and retinoic acid (RA) dependent, whereas induction of FoxP3 Treg by pDCs requires indoleamine 2,3-dioxygenase (IDO).⁶⁶ By contrast, there is evidence that pathogenic bacteria have the ability to directly prime DCs and promote an effector-type response. Hence, each bacterium is differently sensed by DCs according to the type of PRRs it interacts with.⁶⁷

Colonization and immune tolerance

In principle, microbiota–IECs interaction may engender two immune responses: protective immunity against pathogens and immune tolerance from non-pathogens. Germ-free mice exist in a Th2-biased state, and in this condition oral tolerance cannot be efficiently induced. Oral OVA sensitization before a systemic challenge abrogates the Th1-mediated immune response in germ-free mice; however, the Th2-mediated immune response is still preserved. Colonization with the commensal bacterium *Bifidobacterium infantis* constricts the Th2 response and restores oral tolerance in germ-free mice. However, oral tolerance can be induced only in neonatal, but not in older, mice.⁶⁸ Karlsson *et al.*⁶⁹ using a different approach to induce oral tolerance reached the same conclusion that only neonatally colonized animals can develop immune tolerance. These studies indicate that appropriate colonization with microbiota in early life is a key to induction of host immune tolerance. Despite being not fully understood, possible mechanisms are discussed below (Fig. 7).

Subsets of immune cells that confer suppressive effects on the immune response include CD4⁺FoxP3⁺ Treg cells, IL-10-producing CD4⁺ T cells (Tr1 cells), TGF- β -producing Th3 cells, tolerogenic DCs, myeloid suppressor cells and IL-10-producing B cells (Breg cells). Among these factors, CD4⁺FoxP3⁺ Treg cells are believed to play a dominant role in maintaining immune tolerance and in suppressing an excessive immune response¹⁵ (Fig. 7). The finding that FoxP3⁺ Treg cells are more abundant in the intestinal LP than in peripheral lymph nodes or in the spleen supports this notion.⁷⁰ Treg cells can also be isolated from PPs and MLN in the gut.⁷¹ Recent evidence suggests that FoxP3⁺ Treg cells in the intestine differ from those in secondary lymphoid organs.^{72,73} Treg cells in intestinal LP express CCR9, CD103, IL-10, IL-35, granzyme B and killer cell lectin-like receptor G1, many of which are regulated by the signaling molecule STAT3.

Generation of FoxP3⁺ Treg and concomitant establishment of immune tolerance involve two steps according to Hadis *et al.*⁷⁴ First, activation of antigen-specific naïve T cells in gut-draining MLNs produces a founder pool of FoxP3⁺ Tregs, by which a latent tolerance is established. The second step involves homing of activated Treg cells to intestinal LP. This is driven by the intestinal macrophage. In the LP, homing FoxP3⁺ Treg cells propagate, and immune tolerance is irreversibly installed. Evidence suggests that gut microbiota program Treg cells in the intestine. In a gnotobiotic mouse model of cow's milk allergy, Rodriguez *et*

*al.*⁷⁵ found that the FoxP3 gene was highly expressed in the ileum of gnotobiotic and conventional infant mice when compared with germ-free infant mice. In addition, resident commensal bacterial GroEL, a typical member of bacterial heat shock protein (HSP) family, but not mouse-derived HSP60, causes naïve T cells to differentiate into CD4⁺CD25⁺FoxP3⁺ Treg cells, indicating that the production of Tregs in the gut depends on commensal bacterial components.⁷⁶

Yet, neither the efficiency of different bacterial species nor their bacterial component(s) in Treg cell induction are fully understood, as there are so many different strains of commensal bacteria in the gut. *Clostridium cluster XIVa* and *Clostridium cluster IV*, which account for 10–40% of the total bacterial number of gut microbiota, have been shown to affect the number and function of FoxP3⁺CD4⁺ Treg cells.^{77,78} Furthermore, probiotics including *Lactobacillus rhamnosus GG* and/or *Bifidobacterium infantis* were seen to promote the expansion of CD4⁺CD25⁺FoxP3⁺ Treg in the gut in a food allergy murine model.^{79–81} A recent study by Round *et al.* demonstrates that PSA from *B. fragilis* activates TLR2 on Treg cells and increases the suppressive capacity of Tregs, for example, production of IL-10. Thus, PSA from *B. fragilis* provides two arms to modulate the host immune response: one is to expand CD4⁺ T cells and increase Th1 frequency and the other is to activate Treg cells.^{82–84}

As both commensal bacteria and pathogens have MAMPs, an important question is why do pathogenic bacteria cause gut inflammation, but commensal bacteria do not. Recent studies suggest that commensal bacteria inhibit the NF-κB pathway. *In vitro* studies found that commensal bacteria such as *Lactobacillus spp.*, *Bacteroides spp.* and *E. coli* and the attenuated pathogenic strain *Salmonella spp.* can inhibit polyubiquitylation and the subsequent degradation of IκBα required for efficient nuclear translocation of NF-κB and activation of this signaling pathway.^{85–87} Furthermore, commensal bacteria *B. thetaiotaomicron* inhibit the NF-κB pathway by relocalization of peroxisome proliferation-activated receptor-γ (PPARγ)-binding into a complex with RelA in the nucleus,⁸⁸ thus inhibiting NFκB transcription.

Inadequate colonization

There are circumstances in which newborn infants have inadequate or abnormal colonization resulting in either delayed or inappropriate programming of intestinal defenses and in an increased incidence of disease. Inadequate colonization occurs most commonly with: (1) premature delivery, (2) Cesarean section or (3) the excessive use of antibiotics in the perinatal period. In each, the first and most important phase of colonization is inadequate, and, despite the stimulus of oral feeding and weaning, complete colonization is delayed to between 4 and 6 years during which time they become more susceptible to both intestinal and systemic infections. In addition, if inadequate colonization persists, they show an increase in immune-mediated diseases, for example, the so-called 'hygiene hypothesis'. In addition, 'colonization resistance' is not operational, leading to a tendency to increased mucosal penetration by pathogens resulting in more diseases (Fig. 2). If we compare the microbiome of full-term infants born naturally by vaginal delivery with those born by Cesarean section, we find that vaginally born infants have more colonizing bacteria and greater species diversity as well as microbiota that resemble the mother's intestinal contents compared with C-section babies in whom the microbiome contains fewer microorganisms with less diversity and resembles bacteria on the mother's skin rather than in her intestine. The end result of this colonization is an inadequate symbiotic relationship between colonizing bacteria and the underlying intestinal epithelium and lymphoid tissues, with a greater potential for pathogen overgrowth and more disease expression.

Clinical consequences

in this review, we discuss three common examples of clinical conditions that are directly related to inadequate colonization and aberrant programming of the immune phenotype. These include the expression of asthma with excessive perinatal antibiotic use, an increased incidence of atopic disease in general after C-section and the expression of necrotizing enterocolitis (NEC) in very premature babies.

Asthma

A recent clinical study has shown a direct correlation between antibiotic use during the first year of life and the incidence of asthma in childhood.⁸⁹ The authors determined the number of treatment exposures to antibiotics during the first year of life and the type of antibiotic used to treat (e.g., broad v. narrow spectrum) and the likelihood of asthma expression occurring during childhood. With each exposure to antibiotics, asthma expression increased markedly, and broad spectrum antibiotic exposure produced more asthmatic phenotypes compared with narrow spectrum exposure. The study illustrates that disruption of the normal colonization process during the critical neonatal and infancy periods contributed to an aberrant reaction to ingested and inhaled allergens, causing an allergic immunologic response localized to the lungs rather than of tolerance to these antigens.

Generalized atopic disease

A study from Australia published in the *Journal of Allergy and Clinical Immunology* underscores the effect of C-section delivery on the development of atopic disease.⁹⁰ In this study, infants born by vaginal delivery with no family history of allergy were ascribed an odds ratio risk of one compared with vaginally born infants with a family history of allergy in whom the odds ratio of risk increased to two and a half. However, compared with infants born by C-section with a family history of allergy, the odds ratio increased to 8, suggesting that birth by C-section is an important risk factor for atopic disease. A recent study has compared the mode of delivery (vaginal v. C-section) with the incidence of autoimmune diseases and has shown an increase in the expression of celiac disease⁹¹, suggesting that inadequate initial colonization affects the mucosal immune system's ability to regulate immune exposure to autoantigens.

NEC

The incidence of premature deliveries in the United States over the last several decades has remained unchanged for a variety of reasons including multiple births in older mothers with infertility problems. As a result, the incidence of NEC in very low birth weight infants (<1500 g) has remained at about 7%, and therefore represents a major medical problem for neonatologists. Inadequate colonization occurs in premature infants because of their rapid passage through the birth canal, preventing them from ingesting maternal vaginal and colonic microbiota (the principal basis for phase 1 of colonization). As a result, the premature infant has an imbalanced bacterial phyla and less diversity of individual species. A combination of inadequate colonization and an immaturity in intestinal development results in abnormal intestinal responses to colonizing bacteria and predisposes the infant to an increased inflammatory necrosis of the distal small intestine and colon (e.g., NEC). We have developed intestinal models of human fetuses, which include a small intestinal crypt cell line (H4 cells),⁹² use of organ cultures⁹³ and small intestinal/colonic xenograft transplants into the subcutaneous capsule of immune-deficient [severe combined immunodeficiency (SCID)] mice.⁹⁴ Using these fetal human intestinal models, developmental immaturities in innate immunity in these enterocytes have been identified (described below). In addition to a rapid passage through the birth canal for premature delivery, another basis for inadequate colonization is the excessive use of empiric antibiotics

at birth to prevent sepsis. A large multi-center trial in 19 intensive care newborn nurseries (NICUs) throughout the United States showed a direct association between the number of days of empiric antibiotic treatment and the incidence of NEC.⁹⁵

We know from other studies that trophic factors in amniotic fluid bathing the fetal intestine during the third trimester of pregnancy are needed to produce a profound maturational effect on the intestine's ability to appropriately respond to colonizing bacteria.⁹⁶ For example, mature enterocytes interacting with colonizing bacteria can distinguish between commensal bacteria and potential pathogens. The enterocyte responds to pathogen interaction with a self-limited inflammatory response to prevent penetration, leading to gastroenteritis or sepsis. In contrast, the mature enterocyte does not respond to commensal bacteria by a variety of mature reactions.^{85,88} Using our fetal cell line (H4 cells) compared with a mature cell line (T84 cells), we showed that fetal enterocytes produce a substantial IL-8 inflammatory response to a commensal organism, compared with adult cells.⁹⁷ We further showed that the enhanced response to commensal organisms was partly because of the underexpression of I κ B, the molecule that binds NF κ B and prevents its passage as a transcription factor into the nucleus.

We have also shown that fetal intestine compared with child intestine responds to an exogenous lipopolysaccharid (LPS) and endogenous (IL-1 β) inflammatory stimulus with an excessive IL-8 inflammatory response.⁹⁸ More recently, we have examined the genes that mediate the innate immune inflammatory response and have shown that toll receptors (TLR4, TLR2), signaling molecules (MyD88 and IRAK) and transcription factor (NF κ B) genes are upregulated in fetal *v.* child intestine, and negative regulators that mediate a self-limited innate immune inflammatory response (e.g., IRAK-M, A-20, Tollip, etc.) are underexpressed developmentally.⁹⁹ We believe that these developmental differences in epithelial gene expression that fail to distinguish between commensal bacteria and pathogens as well as the excessive inflammatory responses to stimulation account in part for the pathogenesis of NEC in premature infants.

Probiotics and NEC

We know from clinical studies that feeding the premature infant its mother's own breast milk can either prevent or lessen the severity of NEC. As previously mentioned, studies have been conducted to show that breast milk oligosaccharides can preferentially stimulate an increase in *Bifidobacteria infantis* and activate genes from this organism that stimulate anti-inflammation in the IEC.⁸ Other studies show that probiotics can prevent or lessen the expression of NEC. Unfortunately, these studies involve small numbers of patients and use different probiotics and dosages. Despite a meta-analysis of these studies strongly favoring NEC protection,¹⁰⁰ a single protocol, multi-center trial using one probiotic at a fixed dosage would be necessary before this approach can be routinely recommended to neonatologists.

Our laboratory has taken a different approach to the possible treatment of NEC. Using two probiotics, *Bifidobacteria infantis* and *Lactobacillus acidophilus*, a group of neonatologists in Taiwan showed a striking reduction in morbidity and mortality in infants weighing <1500 gm.¹⁰¹ This study was extended to include eight nurseries in Taiwan with similar results.¹⁰² We have shown that these two probiotics secrete anti-inflammatory factors into their culture media. These isolated secreted products within a 10–15 KD fraction were incubated with either fetal human organ cultures or infused into fetal intestinal xenograft loops. This resulted in an excessive IL-8 response to inflammatory stimuli being strikingly reduced.¹⁰³ More recently, we have separated the two probiotics into individual cultures and have shown that *Bifidobacteria infantis* secretions have a greater anti-inflammatory effect.¹⁰³ Furthermore, these secretions, when incubated for a prolonged period with fetal intestine,

can stimulate a mature pattern for innate immune inflammatory response genes, which we believe represent the probiotic mechanism for the reduction of excessive inflammation in the premature intestine (Fig. 8). Why do we use probiotic secretions and not just probiotics, as was done in Taiwan? The rationale is based on the fact that the Food and Drug Administration (FDA) will not allow live organisms to be given routinely to an immune-compromised host (e.g., the premature infant). Accordingly, we suggest that the secreted products of known probiotics can effectively prevent NEC. Accordingly, we suggest that these secretions mixed with expressed breast milk may be the most effective way to treat all very low birth weight infants. Our future clinical studies will involve a single protocol using secretions at a fixed dosage to determine their efficacy in the NICU environment.

Summary and conclusions

In this review of the role of gut microbiota in programming the immune phenotype, we provide evidence that initial appropriate newborn colonization is necessary to stimulate innate and adaptive immunity development and for the prevention of infant intestinal inflammatory and immune-mediated diseases (allergy and autoimmunity) in later life. We provide evidence that oligosaccharides in human milk given preferentially during the first few postnatal months stimulate health-promoting (probiotic-like) organisms, such as *Bifidobacteria infantis*, and evoke gene transcriptions in this microorganism, which promotes anti-inflammation. We have reviewed the birth circumstances of inadequate colonization (prematurity, birth by C-section and excessive use of perinatal antibiotics) and its short-term and longer-term clinical consequences (NEC, allergy and asthma). Adequate colonization must occur in the immediate *post-partum* period. Alternatively, probiotics can be used as a surrogate colonizer during the period of inadequate colonization to prevent the expression of these diseases.

Acknowledgments

These studies were supported by grants from the National Institutes of Health (R01 HD012437, R01 HD059126, P01DK 033506, P30DK 040561) to Dr Walker.

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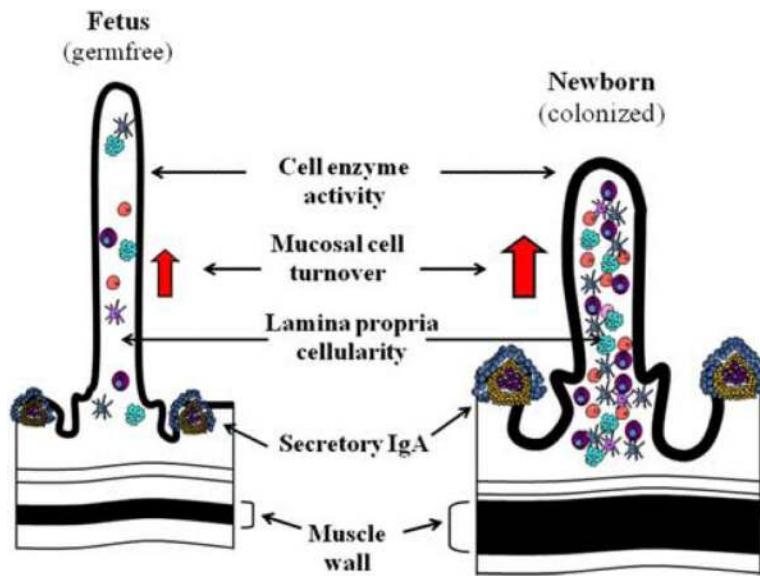


Fig. 1.

A schematic representation of a cross section of small intestine of human fetus *in utero* v. the newborn human infant. Fetal intestine appears thin and exhibits a slow epithelial proliferation rate with a paucity of gut-associated lymphoid tissue (GALT), whereas infant intestine manifests a robust, diverse epithelium with a fast turnover rate and abundant GALT elements. Reprinted with permission from Walker.¹

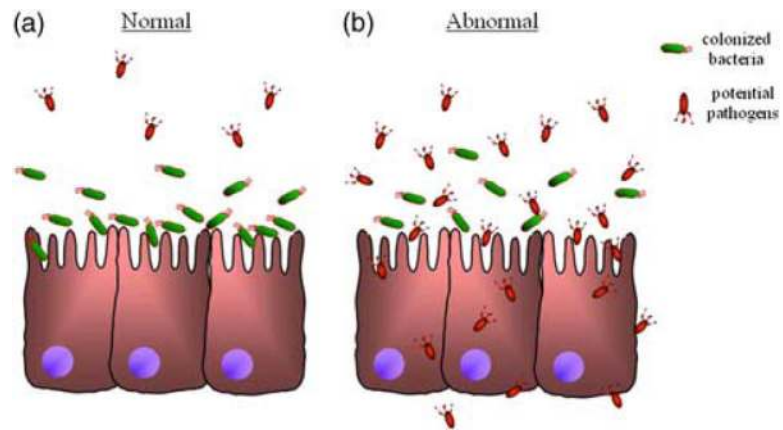


Fig. 2. Diagram of 'colonization resistance' in extraterine intestine. Upon shift from a liquid to a solid diet, a larger number of anaerobic symbiotic microbiota attach to the luminal surface of the intestinal epithelium that prevents potential penetration by pathogenic (mostly aerobic) flora (a), which does occur with abnormal colonization (b) lacking 'colonization resistance'.

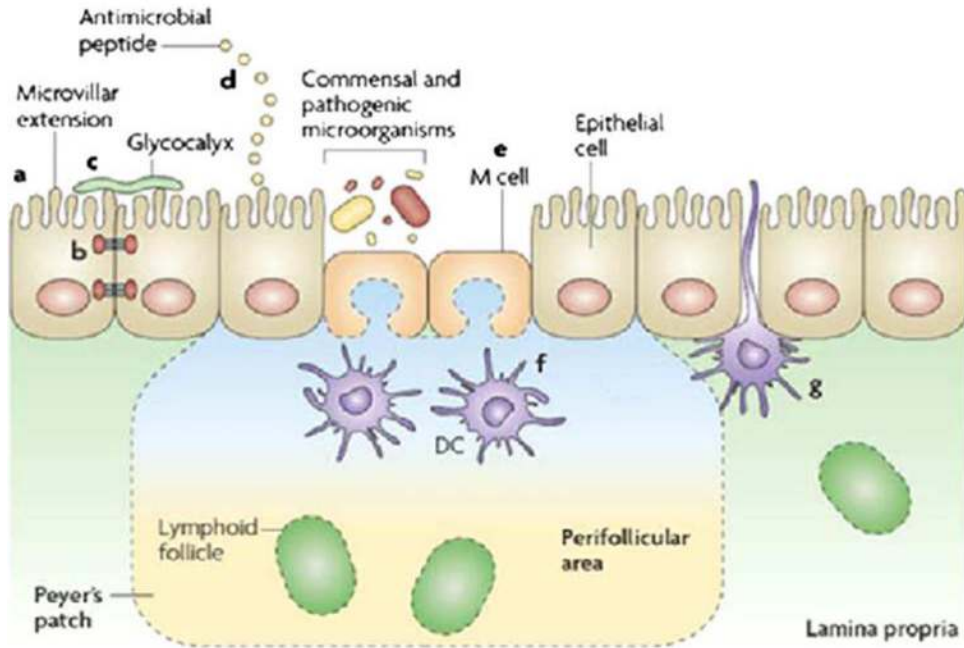


Fig. 3. This figure shows the importance of bacterial colonization, intestinal mucus and epithelial barriers that work in concert with components of submucosal gut-associated lymphoid tissue to maintain immune homeostasis in the intestine. Shown are key elements including (a) microvilli, (b) epithelial cell tight junctions, (c) apical glycocalyx, (d) antimicrobial peptides, (e) microfold cells (M cells), (f) dendritic cells (DCs) in Peyer's patch and (g) specialized DCs extending dendrites into the gut lumen for sampling lumina intact antigens and/or microbiota. Reprinted with permission from Chichlowski *et al.*⁸

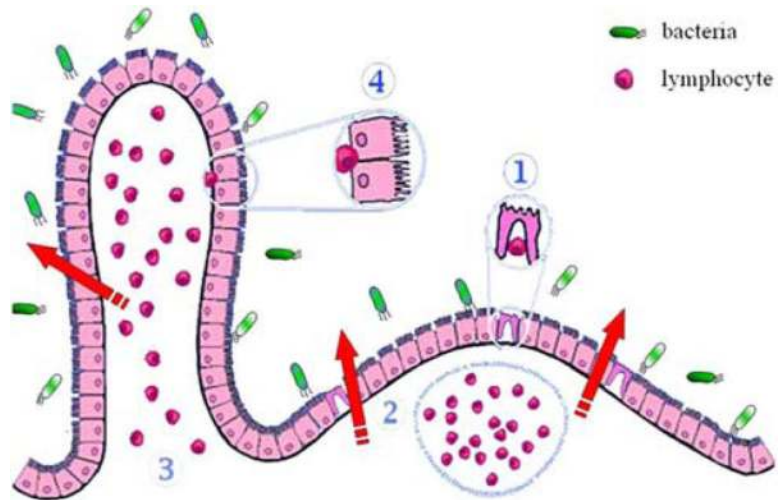


Fig. 4.

A diagram of human neonatal intestine mucosal immunologic development. At birth, these components including (1) M cells, (2) Peyer's patches rich in lymphoid elements, (3) interstitial lymphocytes and (4) intraepithelial lymphocytes. The proper development of these defense components requires the stimulation from colonizing bacteria. Reprinted with permission from Walker.¹

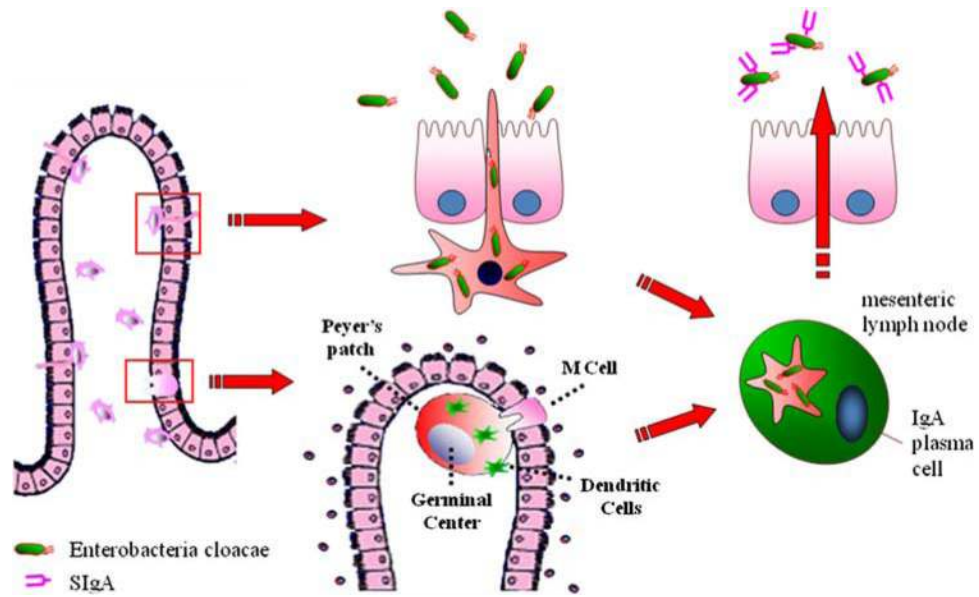


Fig. 5.

A diagram that shows two ways by which symbiotic bacteria can condition dendritic cells to promote immunoglobulin A (IgA) production. One is by way of M cells, which transfer commensal bacteria to dendritic cells in Peyer's patches; the other is that dendritic cells (DCs) directly sample commensal bacteria by extending dendrites into the gut lumen. Bacterial-laden DCs home to mesenteric lymph nodes, where an efficient anti-commensal microbiota IgA response is elicited from plasma cells. sIgA, secretory immunoglobulin A.

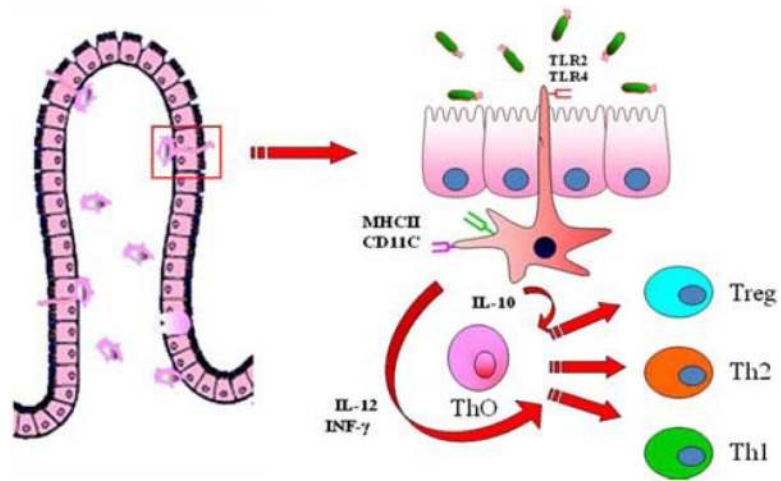


Fig. 6. Commensal bacteria evoke T-cell responses via dendritic cells (DCs) by binding to toll-like receptor 2 (TLR2) and/or TLR4 on the surface of penetrating dendrites. Commensal microbiota induce MHCII⁺ CD11c⁺ DCs to secrete interleukin-10 (IL-10) or IL-12 and IFN γ by which naïve Th0 cells are primed to differentiate into TH1, TH2 or Treg, respectively. Reprinted with permission from Walker.¹

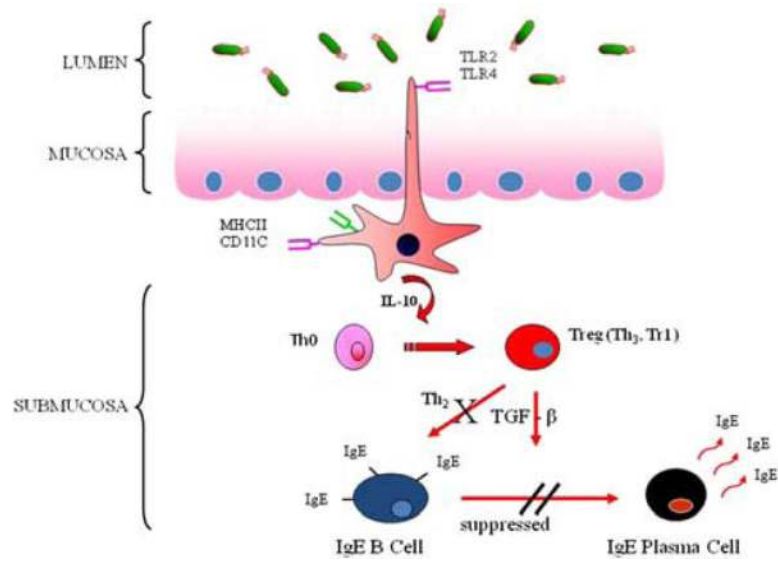


Fig. 7. Schematic representation of oral tolerance induction by gut microbiota. In the intestinal lumen, gut microbiota activate dendritic cells (DCs) via the toll-like receptor 2 (TLR2)/TLR4 signaling pathways. Activated DCs cause maturation of TH0 to subsets (TH3, Tr1) of Treg cells via release of interleukin-10 (IL-10) to stimulate transforming growth factor beta (TGF- β) release and thereby suppress immunoglobulin E (IgE) production.

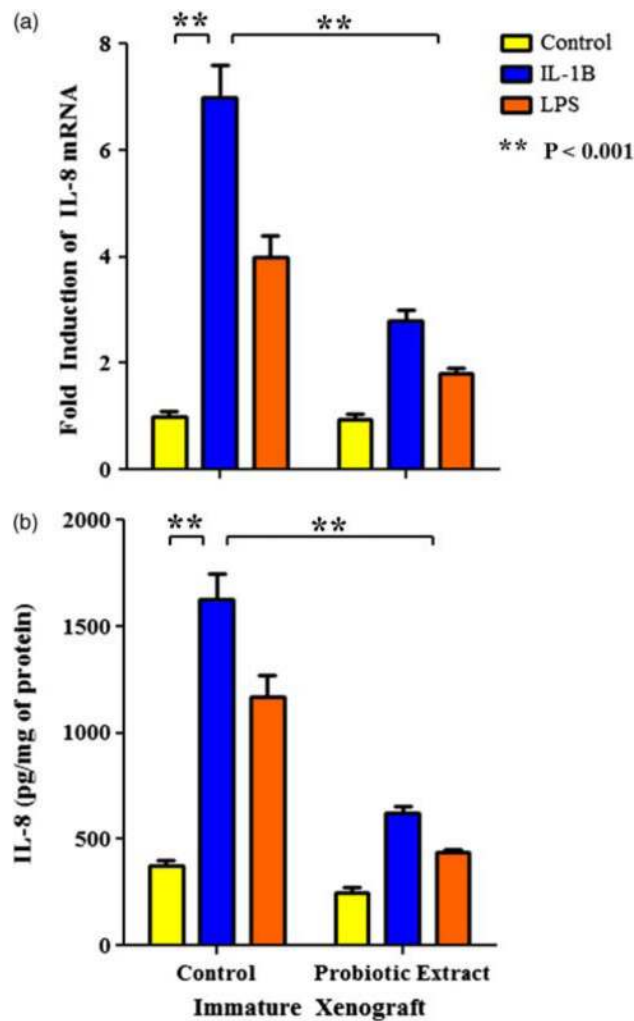


Fig. 8. Probiotic secretions attenuate LPS/interleukin (IL)-1 β -induced excessive IL-8 secretion in the immature human intestinal xenografts. Organ cultures of immature human xenografts were challenged by LPS (50 μ g/ml) or IL-1 β (1 ng/ml) with and without 48 h pre-exposure to probiotic-conditioned media. (a) IL-8 mRNA expression was measured after 12 h and (b) IL-8 (pg/mg of protein) was measured after 16–18 h. A highly significant induction of IL-8 mRNA as well as IL-8 secretion was observed in immature xenografts. However, exposure to probiotic-conditioned media before stimulation resulted in a highly significant decrease in IL-8 mRNA levels ($P < 0.001$, **) and IL-8 secretion ($P < 0.001$, **), as well as in immature xenografts. Reprinted with permission from Ganguli *et al.*¹⁰³