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Protective Properties of Human Milk

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Human breast milk is an undeniably unique, natural source of nutrition for the human infant. However, in addition to the nutritive value it imparts, breast milk and breast-feeding are well recognized to protect against gastrointestinal infections, diarrheal diseases, and respiratory infections.[1] Such protective effects have been variously ascribed to the presence in milk of maternal leukocytes, immunoglobulins, growth factors, and immune factors. While some of these components interact directly with bacterial cells, others mediate their effects by promoting proliferation, maturation, and activation of neonatal cells and by ensuring homeostasis. The net result is an enhanced protection of the immature intestine against colonization and invasion by pathogenic bacteria.

Much of the immunologic benefit of breast milk previously described in reviews of the subject has been attributed to the passive protection that it provides.[2] It is apparent that although breast milk supplies a combination of protective factors, functional redundancy is not rare. Not only do several factors exhibit the same biological function, a single molecule can have a range of biological activities. This complexity and the change in the concentration of specific components as lactation proceeds are testimony to a dynamic biological fluid, the complete function of which has still to be fully appreciated. Indeed, there is now evidence to suggest that protective effects of breast milk extend beyond the neonatal period and can influence the development of pathologic diseases later in life.[3] This is further support for a more intimate association between the maternally derived milk factors and the neonatal immune system beyond that required to prevent pathogen colonization and infection.

The mechanisms underlying the long-lasting benefits of breast milk are likely to be multifarious. However, at least two different and partially overlapping processes are probably involved. First, breast milk factors, in shaping the composition of the neonatal microbiota, indirectly influence immune system development and response. Second, breast milk directly “educates” the neonatal immune system to react with appropriate innate or adaptive immune responses upon microbial and antigenic challenge. Indeed, there is increasing evidence to suggest that upon exposure to antigen, breast milk educates the neonatal immune system in the decisions to be made.

Breast milk contains a myriad of factors that, qualitatively or quantitatively, may modulate how neonatal cells perceive microbial components or respond to microbes with which they come in contact. Examples are immunoglobulins, glycoproteins, and glycolipids, which interfere with bacterial binding to epithelial cells, and oligosaccharides and antimicrobial peptides, which encourage the preponderance of particular microbial species.

It has also been suggested that the latter is achieved via breast milk–derived bacteria that ultimately colonize the neonatal gut.[4] However, an emerging concept is that breast milk influences the neonatal immune system’s perception of “danger.” To do so, some milk molecules may actually facilitate the intestinal response to specific microbial motifs by activating intracellular signaling pathways such as that of nuclear factor (NF)-κB, a major regulator of the inflammatory response. At first, this might appear to contradict the recognized anti-inflammatory effect of breast milk. However, the present chapter will elaborate further on breast milk factors that modulate the neonate’s perception of danger signals in the absence of exaggerated inflammation. More specifically, it will address those factors that promote a beneficial microbial flora either directly or indirectly via education of neonatal cells. Moreover, it will challenge the theory that milk is strictly anti-inflammatory by proposing novel mechanisms by which the mother already educates her infant in the immediate postnatal period.

THE BASIS OF INNATE AND ADAPTIVE IMMUNE RESPONSES IN THE INTESTINE

The intestine is the largest immune apparatus in the body and, as such, the immunological events taking place in its tissues have a major impact on the overall immune status of the host. The development and effectiveness of this system is influenced by a number of cellular elements and luminal antigens that include but are not limited to epithelial and immune sentinel cells in the mucosal tissue and dietary antigen, immunomodulatory factors, and a substantial, heterogenous inoculum of microbial motifs in the lumen.[5] A prerequisite for a healthy intestine is an immune system that can differentiate these luminal antigens and initiate a protective response to “danger signals” while remaining tolerant to nonthreatening microbes, dietary antigens, and endogenous host cells and molecules.[6,7]

The single layer of epithelial cells overlying the lamina propria and lymphoid follicles of the intestine is in direct contact with luminal contents and functions as an early warning system of impending microbial attack. Any delay in response to real threat could seriously compromise the host; thus, an alert is triggered by all organisms. However, the magnitude and precise nature of the response are determined by the characteristics of the microbe. Broadly speaking, nonpathogens mount limited responses with a transient innate component[8] that might explain the low level of physiological inflammation observed in the intestine. In contrast, pathogens induce rapid and more vigorous responses that are accompanied by some tissue damage.[6] In each case, both innate and adaptive immune processes are engaged. Thus, the host reaction can be considered as a stratified response whose primary tier is triggered by most bacteria and is pro-inflammatory, but whose second level of activation involves an assembly of gene products that are defined by the virulence traits of the organism.[9] However, the nature of the response is also governed by the level of maturation and activation of immune sentinel cells. Upon antigenic challenge, pattern recognition receptors (PRRs), such as the Toll-like receptor (TLR) family of proteins, empower these cells to recognize and interact with a number of microbial components and endogenous host proteins[6,7] and to relate cellular signals needed for appropriate action. Several members of the TLR family, capable of recognizing single or multiple microbial motifs, have been identified (Table 1). Their expression can be inducible or constitutive and is dependent on cell type and intestinal location.[10] Nucleotide-binding oligomerization domain (NOD) 1 and NOD2 are a structurally distinct family of intracellular PRRs that are expressed in intestinal epithelial cells (IECs), Paneth cells,[11] in macrophages, T and B cells, and dendritic cells (DCs).[12] These PRRs have been less studied and, to date, the only known ligands for NOD1 and NOD2 are, respectively, the gram-negative peptidoglycan (PGN) derivative γ-D-glutamyl-*meso*-diamino-pimelic acid and muramyl dipeptide.[10]

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Table 1 Recognition of Bacterial and Viral Motifs by Human Toll-Like Receptors

Microbial Components: Bacterial (b) and Viral (v)	Host TLR Molecules	Main Cellular Compartment*
Triacyl lipopeptides (b)	TLR1 / TLR2	Plasma membrane
Proteoglycan, porins, lipomamnan, lipoarabinomannan (b)	TLR2	Plasma membrane
Hemagglutinin protein (v)	TLR2	Plasma membrane and intracellular
dsRNA (v)	TLR3	Intracellular
Lipopolysaccharide (b)	TLR4	Plasma membrane
Envelope proteins (v)	TLR4	Plasma membrane
Flagellin (b)	TLR5	Plasma membrane
Diacyl lipopeptides, lipotheichoic acid (b)	TLR6 / TLR2	Plasma membrane
ssRNA (v)	TLR7 and TLR8	Intracellular
CpG-DNA (b)	TLR9	Intracellular
DNA (v)	TLR9	Intracellular

*Epithelial and immune cells.

NEONATAL INTESTINAL IMMUNITY AND THE DEVELOPMENT OF THE MICROBIOTA

The neonatal intestine is sterile at birth but is immediately exposed, not only to potential pathogens but to a multitude of nonpathogenic organisms, some of which ultimately form the commensal microbiota. This potentially hazardous process is nevertheless vital for the development and maturation of the intestinal immune system.[13] Indeed, the hygiene hypothesis, proposed to explain the rise in atopic diseases, suggests that early in life, microbial antigens interacting with the innate immune system tip adaptive immune responses toward a T helper (Th)-1, and away from a Th2, response. A recent adaptation of this hypothesis proposes that instead of priming immune activation, such microbial stimulation primes immunoregulation via T regulatory (T_{reg}) cells and regulatory antigen presenting cells.[14]

During the first days of life, the intestinal microbiota undergoes major compositional changes until a steady state is reached around weaning.[15] The mode of delivery, the gestational age, hospitalization and length of hospital stay, the use of antibiotics, as well as the type of nutrition all have some bearing on the composition of the neonatal microbiota.[16] Bifidobacteria, lactobacilli, streptococci, staphylococci, *Bacteroides* species, and *Escherichia coli* are present in the newborn fecal matter[17] but while bifidobacteria has a net predominance in the stools of breast-fed infants, enterobacteria, coliforrms, and clostridia reach high levels almost equivalent to those of bifidobacteria in the stools of the formula fed.[18] The number and type of bacteria competing for an intestinal habitat, as well as the high density of microbial antigens and molecular motifs, may be contributory factors in a variety of immune and inflammatory neonatal conditions such as necrotizing enterocolitis (NEC) and gut-derived sepsis. It has been postulated that in human newborns, particularly preterm infants, an inappropriate inflammatory reaction to intestinal microorganisms is a causative factor for NEC.[19] Furthermore, overgrowth of abnormal bacterial flora can cause septicemia in human newborns receiving enteral nutrition.[20] Importantly, breast milk protects against bacterial translocation in neonatal animals and against septicemia and NEC in premature human infants.[21,22]

For successful colonization, healthy immune status and a beneficial relationship with the microbiota throughout life, the most elementary immunoregulatory mechanisms must already exist in the neonatal intestine at birth. Certainly, some immune instruction already begins prenatally and depends on the thymus for the generation of self-tolerant effector and regulatory T cells.[23] Immature T cells interact with thymic epithelial cells in a vital process that ensures the maturation, differentiation, and selection processes of the mature T cell pool.[24] These prenatal events explain the central features of adaptive immunity already present at birth. However, while it is important to restrict antigen exposure in utero for fetal survival, it leads to deficits in adaptive immunity in the newborn period.

Although the innate response is not considered to require previous immunological experience to be activated, the sophisticated control mechanisms operating in the adult intestine are not expected of the naïve neonatal immune system, particularly if one considers that the same host receptors recognize both pathogens and nonpathogens.[25] Indeed, the increased susceptibility of neonates to infection and certain allergens suggests that the capacity to handle antigenic challenge is not inherent but develops over time and/or through education and experience. In the immediate postnatal period, the cellular composition of the microbiota is highly variable as many organisms vie to colonize the mucosa. This physiological process can trigger an inflammatory response not only in the gut but also systemically owing to the high permeability of the neonatal intestine.[26] Remarkably, detrimental inflammatory reactions do not normally occur and immune homeostasis prevails. Possible explanations are a low expression of TLR2 and TLR4 on IECs and lamina propria cells[25] and/or limited cellular signaling following engagement with nonpathgens.[27] However, the greater incidence of infections, inflammation, and allergy in formula-fed infants compared to breastfed infants suggests that breast milk is a source of protective regulatory factors.

MILK FACTORS DEFINING THE INTESTINAL MICROBIOTA AND THE ORGANISMS THAT INTERACTS WITH THE NEONATAL IMMUNE SYSTEM

Closer scrutiny of the interplay among specific milk molecules, microbes, and the mucosal immune system is beginning to unravel some of the intricacies of breast milk protection. It is becoming apparent that exogenous factors in milk play a regulatory, or even an educational, role in the underlying immune processes. Simplistically, soluble factors in breast milk can prevent microbial antigens, pathogenic or not, from reaching the cells in host tissues (Table 2). Through their bacteriostatic or bactericidal action, some of these factors define the nature of microbial antigens that succeed in interacting with the intestinal immune system. The most recent research now suggests that breast milk permits, and indeed

Table 2 Human Milk Factors That Define the Intestinal Microbiota

Oligosaccharides and glycoconjugates	Receptor analogues that inhibit binding of pathogens and their toxins to epithelial cells Bifidogenic effect
Mucin 1	Inhibits binding of *S*-fimbriated *E. coli* and rotavirus to epithelial cells
Lactadherin	Inhibits binding of rotavirus to epithelial cells
Defensins and cathelicidins	Bacterial killing via membrane disruption
Lactoferrin	Bacterial killing by lactoferricin peptide Bacteriostatic activity by binding ferric iron Bifidogenic effect
Lysozyme	Bacterial killing via membrane disruption
Secretory IgA	Inhibits binding of pathogens to the intestinal mucosa Facilitates biofilm formation by the normal flora Bifidogenic effect of secretory component
α-Lactalbumin	Antibacterial peptides
Fatty acids	Antibacterial activity

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encourages, interaction between particular microbial motifs and this immune system but curbs any energetic innate reaction. In so doing, breast milk cultivates a healthy relationship between the neonatal immune system and the various bacterial communities that constitute the permanent microbiota of the gut.

Antimicrobial Human Milk Oligosaccharides and Glycoconjugates

Human milk oligosaccharides (HMOs) are synthesized by specific mammary gland glycosyltransferases, which sequentially add fucose, galactose, *N*-acetylglucosamine, and sialic acid to lactose. The large number of oligosaccharides present in human milk decrease in concentration with increased lactation[28] length and are key elements in preventing bacterial adherence to, and colonization of, the neonatal stomach and small bowel.[29] This is achieved by molecular mimicry. Different pathogens, such as *Streptococcus pneumoniae*, *E. coli*, *Campylobacter jejuni*, *Listeria monocytogenes*, *Shigella*, *Salmonella*, and *Vibrio cholera*, initiate the infectious process by adhering, via carbohydrate-binding proteins, to oligosaccharides expressed on epithelial cell membranes.[30] In vitro, epidemio logical and clinical studies[31] suggest that HMOs protect against infection by serving as soluble ligands for the virulence factors of adhesion, the specificity of the effects being determined by the chemical nature of the HMO. However, HMOs may also mediate their protective effects by modifying the composition of the glycocalyx or the expression of glycoproteins on the epithelial surface.[32] Together, these mechanisms suppress colonization by harmful microoganisms. The impact in shaping the commensal microbiota has not been addressed but is implied by the observation that breast-fed infants harbor less P-fimbriated *E. coli* than type 1 fimbriated *E. coli*, and are thereby less likely to suffer from urinary infections.[33]

The milk fat globule membrane glycoproteins, mucin (MUC)-1 and lactadherin, inhibit rotavirus replication and prevent experimental gastroenteritis through their sialic acid residues.[34] Milk MUC1 has also been shown to inhibit the adhesion of S-fimbriated *E. coli* to epithelial cells in vitro.[35] Several other glycoconjugates of human milk are now known and include the ganglioside GM1, which binds to cholera toxin and labile toxins of *E. coli* and *C. jejuni*; the neutral glycolipid globotriosyl ceramide, which binds to toxins of *Shigella* and *E. coli*; a mannosylated glycoprotein that inhibits binding of some strains of *E. coli* to human intestinal epithelium; and finally, glycosaminoglycans, which inhibit binding of the outer membrane protein of the AIDS virus to CD4 T cells.[36]

Antimicrobial Proteins and Peptides

Antimicrobial proteins and peptides (AMPs) are key effectors of the innate immune response. They are expressed by circulating cells and epithelial cells and mediate their effect by disrupting microbial membranes. They have broad-spectrum antibiotic activity against bacteria, viruses, yeast, and fungi and thereby modulate the composition of the intestinal microbiota and confer protection against environmental and pathogenic organisms. Numerous peptides and proteins with antimicrobial activity have been described in humans, but they differ in their tissue localization, regulation, and additional biological activities.[37] Important classes are the cationic α- and β-defensins and the cathelicidins, which protect against bacterial colonization of gut, lung, and skin epithelia.

While α-defensins are constitutively expressed in human neutrophils and Paneth cells of the small intestinal crypts, β-defensins are expressed predominantly in the epithelial cells of the gastrointestinal tract, and cathelicidin (LL-37/hCAP18) is expressed by neutrophils and mast cells, as well as by differentiated epithelial cells in the colon and stomach and in Brunner's glands of the duodenum.[37] Paneth cells are considered particularly important in antibacterial defense since they secrete other antimicrobial peptides such as lysozyme and secretory phospholipase A2. Both the numbers of Paneth cells and the expression of antimicrobial peptides are developmentally regulated and can already be detected in human fetal intestine at 24 weeks' gestation.[38] However, the human newborn has fewer numbers of Paneth cells and less antimicrobial peptides than adults.

The importance of Paneth cells and their expression of antimicrobial peptides in neonatal defence are suggested by animal studies demonstrating increased susceptibility to infection in animals following ablation of the Paneth cell population.[39] Furthermore, the absence of lysozyme in the Paneth cells of preterm and term infants with NEC supports a causative link between Paneth cell secretion of antimicrobial peptides and NEC.[40] The presence of an exogenous source of antimicrobial peptides in human breast milk may therefore compensate for the deficit in Paneth cells and antimicrobial peptides observed in the immature neonatal intestine. Certainly, lysozyme, defensins, and cathelicidin are produced by mammary gland epithelial cells and milk cells, are present in milk in significant concentrations throughout lactation, and have demonstrable antimicrobial activity against a range of organisms.[41–43]

Lactoferrin is a major protein of breast milk that is present throughout lactation and has multiple biological effects. By virtue of its iron-binding properties, lactoferrin has been proposed to play a role in iron uptake by the intestinal mucosa and to act as a bacteriostatic agent by withholding iron from iron-requiring bacteria.[44] An antimicrobial effect of lactoferrin, independent of the degree of iron saturation, has also been described[45] and may be due to the generation of the bactericidal peptide, lactoferricin, during digestion.[46] However, lactoferrin is relatively stable in the neonatal intestine and can be found intact or partially hydrolysed in the feces of suckling but not formula-fed infants.[47] In vitro studies have also demonstrated antifungal[48] and antiviral effects of lactoferrin.[49]

Growth Factors for the Commensal Microbiota

Breast milk may directly influence the composition of the neonatal microbiota other than by antimicrobial activity. For example, it also contains a variety of factors that promote the growth of beneficial bacteria. The majority of studies examining such factors have focused on HMO. These are resistant to gastrointestinal digestion and arrive in the colon in an intact form where they serve as nutrients for colonic bacteria and preferentially support the growth of bifidobacteria.[50] However, lactoferrin has also been shown to promote growth of *Bifidobacterium* and *Lactobacillus* species.[51] Moreover, peptides generated from lactoferrin and from the secretory component of sIgA have also been shown to be bifidogenic.[52]

Immunoglobulins

Although intestinal lymphoid structures are present at birth, immunoglobulin-producing B cells are not fully operational for several weeks.[53] This delay in B cell activation causes a deficit in secretory IgA (sIgA) production and reduced protection of the neonatal intestinal mucosa.[54] However, microbial stimulation leads to an active B cell population by 1 month postpartum. These B cells become plasma cells that produce polymeric IgM and, most frequently, dimeric IgA, which are transported through the epithelium by polymeric immunoglobulin receptors.[55]

In the early newborn period, breast milk–derived antibodies compensate for the deficit in neonatal production. These milk antibodies are produced in the mammary gland by B lymphoblasts that have trafficked from gastrointestinal and bronchial lymphoid tissues in the mother.[56] The dominant immunoglobulin in milk is sIgA, which protects neonatal mucosal surfaces by agglutinating and immobilizing different pathogens.[57] The repertoire of milk antibody specificity is governed by the infectious agents to which the mother has been exposed[58] and can be directed against both enteric and respiratory pathogens, including viruses.[59] Concentrations of sIgA are higher in the feces of breast-fed infants than in those of formula-fed infants[60] and may reflect the capacity of milk sIgA to resist gastrointestinal digestion. By a mechanism known as "immune exclusion," sIgA binds and agglutinates pathogens and thereby, prevents their adhesion to the intestinal epithelium and facilitates their elimination by peristalsis.[61] However, although many bacteria in the intestinal microbiota are also coated with sIgA, they are not permanently removed from the intestine.[62] The reasons for this apparent inconsistency are not known but could be related to the type and specificity of the antibody in each case. In addition, sIgA may also facilitate biofilm formation by the

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normal flora in the large bowel, a process that may aid in the development of the normal flora and attenuate growth of pathogenic microorganisms.[63]

IMMUNOMODULATORY ACTIVITIES OF BREAST MILK DURING NEONATAL INTESTINAL COLONIZATION

Protection and education of offspring are integral parts of evolutionary success, and breast milk composition has adapted to meet these requirements. However, while the passive protection afforded by breast milk is well recognized, the manner by which it educates the neonatal immune system is less obvious. Through their immunomodulatory activity, milk growth factors and cytokines may affect neonatal immune development, but arguably, the finest education is achieved through experience. To this end, milk limits but does not completely veto bacterial interaction with the intestinal immune system. Indeed, such interactions are important for postnatal maturation of intestinal immunity,[13] and any delay to this maturation, such as that caused by reduced microbial stimulation, may increase susceptibility to allergic disease.[14] The following sections discuss how milk may modulate microbial interactions with receptors on host cells thereby encouraging cellular activation to particular microbial motifs but preventing crude, exaggerated inflammatory responses that may occur more readily in immature cells.

PRR–Bacteria Interactions in the Presence of Breast Milk

Lipopolysaccharide of gram-negative bacteria is a potent stimulator of the innate immune response, but it requires several host humoral and cell-surface proteins to mediate its effects. These include the acute-phase lipopolysaccharide (LPS)-binding protein (LBP), and the glycoproteins CD14, TLR4, and myeloid differentiation protein–2 (MD-2).[64] Cellular activation begins when LPS binds to LBP, which subsequently accelerates LPS binding to CD14 on the cell surface[65] However, LPS is then transferred from the CD14–LBP complex to a cell-surface TLR4–MD-2 complex, which transduces intracellular signaling.[66] In contrast, cellular activation by the lipoteichoic acid (LTA) of gram-positive bacteria involves LBP-, CD14-, and TLR2-mediated signals.[67]

TLRs belong to a superfamily called the TLR/IL-1R whose members have a Toll/IL-1 receptor (TIR) domain.[68] Upon ligand binding to TLRs, this cytoplasmic domain activates a cascade of adapter signaling molecules that include myeloid differentiation marker (MyD88), IL-1 receptor–activated kinase (IRAK), and tumor necrosis factor receptor–associated factor (TRAF)-6. Their sequential recruitment leads to activation of NF-κB and mitogen-activated protein kinases[69] and induction of an inflammatory response.

Human breast milk contains high levels of soluble forms of CD14 (sCD14)[70,71] and TLR2,[72] which with lactoferrin may modulate TLR signaling (Table 3). Depending on its concentration and environment, sCD14 can activate or inhibit responses to LPS by respectively transferring LPS to[73] or diverting LPS from[74] membrane CD14 (mCD14). Furthermore, sCD14 can transfer LPS directly to the TLR4–MD2 receptor complex in cells that lack mCD14, such as endothelial and epithelial cells.[75] It is feasible therefore that the ambivalent nature of sCD14 allows physiological activation of cells for homeostasis but prevents exaggerated inflammation during infection or sepsis. Further assurance may be provided by additional milk proteins that bind sCD14 and lessen sCD14–LPS or sCD14-bacterial lipoprotein (BLP)-mediated cell activation. An example of the former is the antimicrobial protein lactoferrin that binds specifically and with high affinity to the lipid A region of LPS[76] as well as to sCD14 and the sCD14–LPS complex.[77] The more recently discovered sTLR2 also decreases in concentration over the lactation period and, by binding to sCD14 in milk, has been postulated to moderate responses to BLP.[72]

Thus, some receptors in cell-surface TLR complexes exist as soluble forms in milk and act as decoy receptors for microbial motifs (Table 3). Still other proteins found in milk, such as IL-10 and transforming growth factor (TGF)-β, at constant levels throughout the first months of lactation,[59] may modify TLR-mediated responses by regulating the expression of the various proteins in the TLR receptor complex. In this respect, IL-10 increases the expression of CD14 on monocytes[78] but does not alter their expression of TLR4.[79] In contrast, TGF-β downregulates the expression of CD14 on macrophages.[80] Importantly, TGF-β null mice have increased TLR4 expression[81] and inflammatory lesions in multiple tissues.[82]

Cellular Reactivity to Bacteria in the Presence of Breast Milk

The predominant production of Th2 cytokines in fetal and neonatal life is a key element in depressing innate immune responses in the newborn[83] This deficiency results in impaired LPS-induced production of the proinflammatory, Th1-polarizing cytokines tumor necrosis factor (TNF)-α, interferon (IFN)-γ,[84] and IL-12[85] by neonatal mononuclear cells. An increased ratio of IL-6/TNF-α further polarizes toward a Th2 response.[86] Deficits in TLR4 and TLR2 expression may explain this.[85]

A similar deficit may exist in intestinal epithelium. It has been postulated that hyporeactivity to the microbiota is due to low expression of TLR2 and TLR4[87,88] and no expression of MD2[88] on IECs. In contrast to neonatal mononuclear cells and adult IECs, human fetal enterocytes express TLR2 and TLR4 and are hyperresponsive to LPS.[89,90] This hyperreactivity may also depend on a lower expression of the inhibitory intracellular molecule I-κBα, which binds NF-κB and retains it in the cytosol.[89]

An animal model suggests that an interaction between luminal microbes and aberrantly overexpressed TLR4 on neonatal IECs underlies the development of NEC.[91] Thus, increased TLR expression on human fetal enterocytes could explain the increased susceptiblity of premature infants to NEC. Although studies in mice suggest that the TLR expression and responsiveness of IECs diminish shortly after birth,[92] this apparent physiological window of hyperreactivity may compromise newborns. Nevertheless, a transient activation of IECs, with IκB-α phosphorylation and subsequent NF-κB nuclear translocation, may be required for subsequent postnatal tolerance to endotoxin.[92] Indeed, continuous activation in the presence of bacterial ligands is then thought to downregulate TLR expression on IECs,[88] perhaps via shedding of the receptors.[72] It is relevant that TLR4 expression gradually decreases on the intestinal epithelium of mother-fed newborns in an animal model of NEC.[91]

Remarkably, the presence of soluble PRRs and their partners in breast milk may participate in both the transient activation of innate immunity and in its subsequent downregulation. Certainly, milk sCD14 mediates the production of proinflammatory cytokines IL-8 and TNF-α and of the chemokine ENA-78 by IECs exposed to LPS[70] and the production of IL-8 in monocytes and DCs exposed to LPS.[70,93] At first glance, it is difficult to reconcile why high levels of sCD14 in breast milk, in the presence of an important bacterial inoculum, do not lead to excessive

Table 3 Human Milk Factors That Modulate Responses to Bacteria

Soluble CD14	Transfers lipopolysaccharide and lipoteichoic acid to Toll-like receptor (TLR) receptor complexes or to lipoproteins Modulates TLR signaling Proinflammatory
Soluble TLR2	Modulates TLR signalling Anti-inflammatory
Lactoferrin	Modulates TLR signalling Anti-inflammatory
Interleukin 10	Increases expression of CD14 Anti-inflammatory
Transforming growth factor β	Decreases expression of CD14 and TLR4 Anti-inflammatory
Osteoprotegerin	Anti-inflammatory
β-Defensin 2	Activates dendritic cells and induces Th1 immune responses via TLR4

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inflammation in the intestine. One possible explanation is that strong activation leads to shedding of the TLR involved in the response.[72] However, it is also possible that other proteins in milk refine the response by interacting with sCD14 or sCD14 complexes as described earlier. An example is lactoferrin, which in addition to downregulating LPS-induced production of proinflammatory cytokines via NF-κB,[94] also inhibits sCD14–LPS-induced expression of IL-8 by human endothelial cells[95] as well as their increased expression of adhesion molecules, E-selectin and intercellular adhesion molecule (ICAM)-1.[77]

It is also noteworthy that the TLR2 ligand LTA does not stimulate proinflammatory cytokine production by IECs in the presence of milk, but rather inhibits sCD14-mediated LPS effects.[96] This observation suggests that gram-positive bacteria, via their LTA, may moderate cellular reactivity to LPS. However, more recent work has shown that sTLR2 in human milk can bind sCD14 and that sTLR2 can inhibit monocyte production of IL-8 and TNF-α in response to synthetic BLP.[72]

It is now known that multiple rounds of NF-κB activation are necessary in a first instance for immune defence and thereafter for resolution of inflammation and tissue repair[97] and that the latter is achieved through IKK-α inhibition of NF-κB.[98] It is becoming apparent that breast milk has evolved to provide molecules that act at several levels of this process. First, it permits the neonate to mount a limited response to the microbial “danger” signal by promoting the production of proinflammatory cytokines. Thereafter, it may activate a second set of genes that may be part of a negative feedback mechanism.[93]

Perhaps as a precautionary measure, breast milk already provides anti-inflammatory factors and antimicrobial peptides whose expression in the neonatal mucosa would otherwise require additional NF-κB activation (Table 3).[99,100] In agreement with this, a certain ambivalency is seen with some milk factors such as TGF-β,[101] sCD14,[73] and the antimicrobial factors lactoferrin and lysozyme,[102] which have the capacity to activate and inhibit NF-κ activation depending on concentration and cellular environment.

The TRAF6 signal transducer of the TLR/IL-1R superfamily is also important for signaling of the receptor activator of NF-κB (RANK) and its ligand (RANKL),[103,104] both of which are key regulators of lymph node formation and T cell–DC crosstalk.[105] Osteoprotegerin (OPG), a natural, soluble decoy receptor for RANKL, is expressed by human mammary gland and breast milk cells, is present in human breast milk for at least the first 6 months postpartum and can be detected in the serum of neonatal animals after gavage.[106] OPG is also expressed by IECs and is upregulated in the tissues of inflammatory bowel disease patients.[107] This increased production may be a protective responsive since OPG has been demonstrated in animal models of colitis, to inhibit intestinal inflammation by reducing DC numbers.[108] Interestingly, mice lacking OPG produce lower levels of proinflammatory cytokines after LPS injection.[109]

Maternal milk also inhibits IL-1β–induced NF-κB signaling in human IECs in vitro,[110] perhaps due to the presence of the IL-1 receptor antagonist (IL-1ra) whose levels persist throughout lactation.[59,111] Certainly, both human milk and an infant formula supplemented with human IL-1ra, have anti-inflammatory effects on chemically induced colitis in rats.[112] Soluble TNF-α receptor[111,113] and IL-10[114] in milk may behave in a similar manner.

As well as being a fuel for bacteria, HMO may also keep the reactivity of the neonatal immune response in check. They can interfere in protein–carbohydrate interactions such as those mediated by selectins and can inhibit formation of platelet–neutrophil complexes and the subsequent transmigration of neutrophils through activated endothelium at sites of inflammation.[50]

Linking Innate and Adaptive Immunity

Besides alerting the immune system of danger signals in the lumen, the innate system activates and coordinates adaptive immunity. DCs are at the heart of the decision-making processes and through the expression of TLRs determine the outcome of the primary response.[115] Following TLR engagement, DCs traffic to lymphoid organs upregulates expression of major histocompatibility complex and costimulatory molecules and acquires the capacity to prime naïve T cells, which then develop into Th1, Th2, or T_{reg} effector cells. Th1 cells produce IFN-γ and TNF-β and activate cell-mediated immunity, while Th2 cells favor humoral responses by secreting IL-4, IL-5, and IL-13 and by promoting B cell isotype switching to IgG1, IgA, and IgE or T_{reg} cells.[116] T_{reg} cells, which mediate their effects via secretion of IL-10 or TGF-β, are thought to prevent autoimmune responses by specifically suppressing activation and proliferation of CD4 and CD8 effector T cells.[115]

Neonates are susceptible to microbial infection and mount poor adaptive immune responses. The reasons for this are multifactorial and may include a lack of memory B and T cells and reduced T cell responses,[117] reduced numbers[118] and maturation[119] of DCs, a high proportion of T_{reg} cells but with naïve phenotype, and a bias toward Th2 immune responses.[83] However, specific TLR stimulation can overcome some of these defects[119,120] and may inhibit the development of Th2 responses.[120]

Breast-fed infants are less prone to develop asthma, atopic dermatitis, eczema, and allergy than formula-fed infants.[59] Some studies have reported a correlation between the development of these diseases and the concentration of milk molecules sCD14, TGF-β, and sIgA.[121,122] Mechanistic studies certainly support a regulatory role for milk molecules in adaptive immunity and in correcting imbalances in Th1 and Th2 responses. For example, sCD14 mediates LPS-induced TNF-α, IL-6, IL-8, and IL-12 release by DCs as well as their expression of costimulatory molecules.[123] Furthermore, it acts directly on activated human T cells to inhibit IL-2 production and cell proliferation, as well as the production of another Th1-like cytokine, IFN-γ, and a Th2-like cytokine, IL-4.[124] Direct interaction of sCD14 with B cells, resulting in higher levels of IgG1 and marked inhibition of IgE production, has also been observed.[125] Consistent with these observations, administration of milk sCD14 to neonatal mice enhances antibody secretion.[71]

TGF-β is a multifunctional cytokine that inhibits the development of exaggerated responses to self or nonharmful antigens without compromising immune responses to pathogens.[101] It controls the initiation and resolution of inflammation through the regulation and survival of multiple cell types, including lymphocytes, NK cells, DCs and macrophages, mast cells, and granulocytes[101] and influences IgA production by B cells.[126] Indeed, a correlation has been observed between TGF-β levels in milk and newborn serum levels of IgA.[127] Furthermore, given orally to allergic neonatal animals, TGF-β downregulates the antigen-specific IgG1 and total IgE levels, inhibits mucosal mast cell activation, and increases the expression of the Th1 cytokines, IL-18, IL-12, and IFN-γ .[128]

Several in vitro and in vivo studies have now demonstrated a role for lactoferrin in T cell differentiation (reviewed in reference 129) and the capacity of orally administered lactoferrin to correct the Th1/Th2 cytokine imbalance in both Th1 and Th2 models of disease.

Studies in OPG null mice have shown that OPG regulates B cell maturation and the development of efficient antibody responses.[130] Furthermore, OPG is a survival factor for DCs but can reduce DC numbers in animal models of colitis.[131] It is also noteworthy that Peyer’s patch DCs express RANK and that treatment with antagonists of RANKL enhances the induction of tolerogenic immune responses.[132] It is feasible therefore that the high levels of OPG in milk may mediate similar effects.

HMO, specifically the Lewis X component, can interact with DCs via the cell-surface molecule dendritic cell–specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN).[133] DC-SIGN is implicated in various functions, mainly related to pathogen recognition and antigen processing and presentation to $CD4^+$ lymphocytes,[133] and can function both as an adhesion receptor and as a phagocytic pathogen-recognition receptor, similar to the Toll-like receptors.[134] Although likely, more studies are required to determine whether HMO interference with the interaction of DC-$CD4^+$ cell interactions renders the newborn host tolerant to components of the microbiota.

Role of Milk Leukocytes in Immune Education

Many viable cells are present in human milk but their concentration declines during lactation.[135] The cell populations present include granulocytes, macrophages, and lymphocytes, which are predominantly T cells. A significant proportion of $CD8^+$ T cells with an effector memory phenotype are constantly present in human milk and may be important in the control of viral passage from mother to infant .[136] Recent work demonstrating that CD14$^+$ milk mononuclear cells in milk express HLA-DR, CD86, CD83, and DC-specific ICAM-3–grabbing nonintegrin suggests that these cells are partially differentiated DCs.[137] They are a source of some of the soluble factors found in milk such as sCD14,[70,71] OPG,[106] as well as several cytokines and chemokines.[59] There is some evidence that the neutrophils and macrophages in milk are phagocytic[138,139] and that upon ingestion they induce a respiratory burst.[140]

The milk leukocytes seem capable of surviving gastrointestinal transport, penetrating the neonatal intestinal barrier and migrating to the mesenteric lymph nodes.[141] In addition to maternal eukaryotic cells, there is accumulating evidence that bacteria of maternal origin are transmitted to the infant via the colostrum and milk.[4,142] Most of these organisms arise from the mother's skin but certain milk species may colonize the neonatal intestine. Interestingly, bacteria considered intrinsic to human milk are associated with mononuclear cells and perhaps cells with an immature DC phenotype.[143] It has previously been shown that milk leukocytes are cells that have migrated from the gut- and bronchial-associated lymphoid tissue to the lactating mammary gland via the lymphatics and the circulation.[56,59,144] It now appears that such migrating cells also transport bacterial and their genetic material.[143] The role of these cells in the neonate is not known but they may represent an inoculum for the development of the microbiota and/or be a way to educate the neonatal immune system.

Other Protective Factors in Milk

Additional immunomodulatory factors in milk may regulate innate and/or adaptive immune responses. These include the antimicrobial β-defensin 2,[145] which induces cytokines and chemokines, is chemotactic for DCs, and uses TLR4 to activate DCs and induce Th1 immune responses (reviewed in reference 146). Numerous cytokines and chemokines, such as IL-1β, IL-6, and TNF-α, monocyte colony stimulating factor, monocyte chemotactic protein 1, IL-8, and RANTES,[59] as well as fatty acids[147] have also been described.

α-Lactalbumin is a major milk protein in all mammals. Its digestion provides a source of biologically important amino acids, antimicrobial peptides, and an immunomodulatory peptide that enhances macrophage phagocytic activity.[148] In addition, a unique form of α-lactalbumin in human milk can induce apoptosis.[149]

Milk is also a rich source of hormones and growth factors that may aid in nutrient transport and the growth and differentiation of neonatal tissues.[59,150]

CONCLUSION

Breast milk provides a well-recognized passive protection against pathogens in the neonatal period. However, recently discovered functions suggest that protection extends beyond the newborn period. The cocktail of biologically active factors that milk provides targets neonatal innate and adaptive immune responses at multiple levels. Milk's primordial task is to alert the innate arm of potential danger. To do so, it supplies molecules that actively promote a proinflammatory primary response upon exposure to antigens or microbial components. However, as a pivotal link between innate and adaptive immunity, it ultimately instructs the neonatal immune system to remain tolerant to nonthreatening antigens and components of the commensal microbiota but to eliminate potential pathogens. To achieve this, it already provides a variety of anti-inflammatory factors that would otherwise imply further activation of neonatal cells. These additional factors fine-tune the type and level of reactivity. Differences between breast-fed and formula-fed infants suggest that in some infant formulas, certain elements needed for the control of these processes are lacking and the infant is more susceptible to antigenic challenge.

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