# Innate Immunity and Human Milk[1,2]

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ABSTRACT Human neonates are born with an immature and naïve acquired immune system, and many of the innate components of mucosal immunity are not fully developed. Thus, the innate immune system of human milk is an important complement to the mucosal barrier of the developing gut. The nursing mother provides her infant many protective agents through milk, a growing number of which have been identified as isolates of milk in laboratory models of infection. The number, the potency, and the importance of these protective agents are probably greater than previously thought. For example, many potent protective agents are not found in milk until digestion releases antimicrobial agents such as fatty acids and peptides. An alternate conformer of α-lactalbumin forms from milk in the stomach and inhibits cancer cells. Many of the protective constituents of human milk inhibit different aspects of a pathogenic process, creating a synergy, where much lower concentrations of each component become protective. Some components have a temporal and a spatial specificity that would cause their protective role to go unrecognized by most laboratory models of infection. Some protective components had remained underappreciated because of technical challenges in their isolation and testing. Recent reports suggest that human milk contains a highly potent mixture of protective agents that constitute an innate immune system, whereby the mother protects her infant from enteric and other diseases. These human-milk components may represent a rich source of novel classes of therapeutic agents against human pathogens. J. Nutr. 135: 1308–1312, 2005.

KEY WORDS: • *innate immunity* • *disease* • *human milk* • *neonates* • *protection*

The association between breast-feeding and healthy babies had been noted periodically over the past few thousand years. One of the earliest systematic studies of this phenomenon was published by Grulee et al. (1) in 1934. Using techniques that are relatively unsophisticated by today's standards, over 20,000 mother/infant dyads were evaluated for the relation between breast-feeding and incidence of disease in the infant. Relative to breast-fed infants, artificially fed infants had 3.1-fold higher morbidity and 7.1-fold higher mortality because of gastrointestinal disease, 1.4-fold higher morbidity and 1.9-fold higher mortality from respiratory disease, and 2.5-fold higher morbidity and 4.3-fold higher mortality from other diseases, including otitis media. The discovery soon thereafter of secretory antibodies in human milk seemed to provide a facile explanation for this phenomenon, at least for gastrointestinal disease: the acquired immune system of the mother provided antibodies against pathogens through milk to the infant gut. We now know that this is only one of many types of protection that human milk can provide to the infant, which includes elements of both an acquired immune system and an innate immune system.

The burgeoning list of protective components in milk is impressive but not totally unexpected. The infant is born with an immature and a naïve acquired immune system, a gut devoid of microflora, a stomach whose function is less able to exclude pathogens, a mode of locomotion that results in intimate contact with the dirtiest and most contaminated part of our environment, and a propensity to explore the environment orally. Based on these considerations alone, it was reasonable to predict that infants need exogenous protection and that human milk might contain protective agents. These and other considerations have lead to research over the last few decades that have resulted in an impressive array of factors in milk that are bioactive, with most of the activity being antipathogenic or immunomodulatory.

With the growth of this list of bioactive components in human milk that have the potential to protect infants, some complexities have emerged. Some of these milk factors inhibit specific pathogens, some inhibit families of pathogens, and some inhibit a very broad array of pathogens. Some function

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with a direct mechanism, such as inhibiting the binding of a pathogen to its receptor, and some function indirectly, such as by modifying the resident microflora of the gut. More recent data, such as those represented in the accompanying articles of this symposium, suggest that the mechanisms of inhibition may be further confounded by additional considerations. For example, the matrix in which the inhibitor is present may contain synergistic inhibitors, such that inhibitors thought to be weakly active may become highly effective inhibitors, such as with fatty acids. The location at which an inhibitor is produced and the location at which it is effective may result in a spatial specificity that would cause an inhibitor to act entirely differently in vitro from its role in vivo, such as with biocidal peptides. Differences in resident microflora among individuals may influence the efficacy of a probiotic. Different states of inflammation may determine if specific immunomodulators are beneficial under a specific set of circumstances. Individual differences in glycan expression determine to which pathogens an individual is susceptible and which human-milk oligosaccharides would be protective; expression of glycans in gut also differs at different stages of development. Also, different strains of pathogens may have different binding specificities, again, determining which glycans would be effective inhibitors of these strains. Finally, all of these factors may interact. Thus, spatial and temporal specificity, synergy, individual differences and stages of development, and immunological states and context all must be taken into account when defining the protective agents in human milk that participate in the mucosal immunity of the infant.

### *The acquired immune system of human milk*

One of the earliest classes of protective components of human milk to be recognized is the immunoglobulins, consisting of 90% secretory IgA (sIgA). Because this was the only major protective component recognized for some time, whose discovery coincided with the increasing recognition of the central role of antibodies in human defense, and the lower incidence of disease in breast-fed infants, the sIgA in milk was widely thought to account for the protection afforded to the nursing infant against pathogens. The mechanism for this protection is shown in **Figure 1**. When the mother–infant nursing dyad is exposed to a novel enteric pathogen, the Peyer's patch in the maternal intestinal mucosa acquires the pathogen in a sample of luminal contents. The M cell presents the antigens of the pathogen to circulating B cells, priming the cell for antibody production. When the B cell is in the proximity to the basolateral side of the mammary epithelial cell, the IgA that is produced is transported into the acinar cell on the basolateral side, and, as the IgA is transported to the apical side of the cell, the IgA acquires its carbohydrate chain to become sIgA that is excreted from the apical membrane into the milk. The sIgA in milk enters the alimentary canal of the infant where it binds to the enteric pathogen, inhibiting disease. After the mother is exposed to a pathogen, many days elapse before the induction of protective antibody and its secretion into the milk and the gut of the infant. Assuming that both the mother and her infant are exposed to the pathogen simultaneously, this would leave the infant vulnerable to the pathogen were it not for other protective mechanisms. Other forms of infant mucosal protection were the topic of this series of reports and include protective components that are intrinsic constituents of human milk, which we define as components of an innate immune system of human milk, and probiotics, which are food-borne bacteria that confer some positive activity toward protecting the infant.

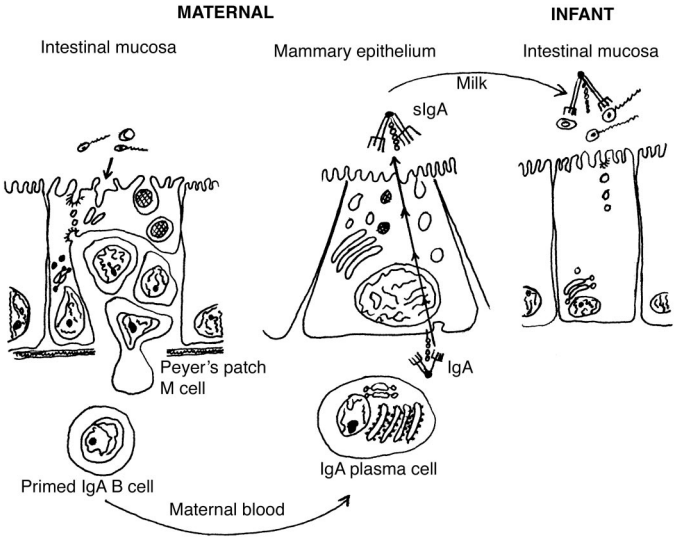


**FIGURE 1** The acquired immune system of human milk. When the nursing dyad is exposed to an enteric pathogen, the Peyer's patch of the mother acquires the pathogen from the lumen of the gut, whereupon the M cell presents its antigen on the serosal side to the B cell, which migrates to the serosal side of the mammary epithelial cell and secretes IgA. As the IgA moves from the serosal to the luminal side of the mammary epithelial cell, it is glycosylated to form secretory IgA, which is secreted into the milk. When the infant consumes the milk, the sIgA, which is resistant to digestion, binds to the pathogen, inhibiting its ability to infect the infant.

### *Probiotics*

One of the early documented differences between breast-fed and artificially fed infants was the microflora of their gut. Breast-fed infants have a higher percentage of lactobacilli, especially *Lactobacillus bifidus* (now *Bifidobacterium bifidum*), whereas artificially fed infant microflora has a composition that more closely resembles that of the adult gut. A human-milk component, a glycan, was found to stimulate the growth of, and colonization by, *L. bifidus*, and such probiotics (indigestible glycans that promote beneficial microflora) are an active area of research on modifying gut colonization. Another more direct mechanism for altering intestinal microflora is to feed beneficial bacteria, probiotics, in the diet to benefit from their presence in the gut. From the use of lactobacilli commonly found in traditional yogurts, the selection of probiotics that protect the recipient from diseases has advanced in the past several years to the highly popular and more effective *Lactobacillus* GG, an isolate with origins in the human gut. Much work on *Lactobacillus* GG, and the continued search for other probiotics, was and is still being performed in Finland; Salminen and colleagues (2) describe the basis for the multiple criteria being used for the selection of new probiotics. Each probiotic has a specific target based on its specificity of binding to host cell receptors, which are usually glycans expressed on the cell surface of intestinal mucosa. Likewise, specific probiotics are able to protect against specific pathogens. For a probiotic to be able to colonize the host, thereby extending its useful duration, the interdependence of microbiota must be considered. Administration of probiotics early in development has a greater chance of coinciding with a critical period of gut colonization and thus of producing lasting consequences. Ultimately, we would like to be able to choose probiotics with long-term sequelae for prophylaxis against chronic conditions; for example, evidence is presented that specific probiotics early

in life may attenuate or prevent the development of manifestations of allergy in later life. Thus, the search for effective probiotics has moved from looking for one or a few probiotics for treating acute infections by any intestinal pathogen to looking for complex combinations of probiotics that would balance the indigenous microflora in a way that has lifelong benefits to the recipient.

### *The innate immune system of human milk*

Several milk components have been found that are important nutrients but that have, or whose partial digestion products have, antipathogenic activity. These can be classified as multifunctional agents, and, because these are intrinsic components of milk, we will consider them as part of the innate immune system of human milk. Two of the most widely recognized have been the FFAs, and especially the monoglycerides, that are released as human-milk triglycerides are digested in the infant stomach, and lactoferrin, a major protein of human milk that has been reported to have several inhibitory mechanisms, including the sequestering of iron, which may be bacteriostatic, a direct antibacterial effect by the whole protein, and the release of peptides during its digestion. These peptides inhibit many distinct pathogenic bacteria, most commonly through local disruption of the membrane, causing it to become leaky. Recent research is not only adding more recognized components to this category of multifunctional protective agents in milk but is also defining the conditions under which they are most active. The examples in the accompanying articles are the FFAs and monoglycerides, the antibacterial peptides, and a protein structural conformer.

***Fatty acids and monoglycerides.*** In the adult, triglyceride is digested slowly, but, in nursing infants, as milk is entering the stomach, it is already releasing FFAs from the digestion of the milk triglycerides by lingual and gastric lipases. Thus, gastric contents of nursing infants already have appreciable fatty acids and monoglycerides, and these have been shown to be antiviral, antibacterial, and antiprotozoal. This activity may augment the ability of the stomach to act as a barrier against ingested pathogens. An illustration of the ability of linoleate, at concentrations typical of the nursling's stomach, to destroy vesicular stomatitis virus is found in **Figure 2** (3). In earlier studies, the Isaacs laboratory had defined oleic and linoleic acid as having very high activity at killing enveloped viruses and found that monoglycerides had the highest activity. Of these, oleic acid is released from milk in the highest concentration, making it a primary source of protection to the breastfed infant. However, when fatty acids are tested in combination, even those that show no inhibitory activity at the concentrations found in stomach aspirates may display potent activity when tested in combination. Furthermore, these lipid activities may be further enhanced when tested in combination with inhibitory peptides (4). This implies that there may be many more bioactive milk components than those whose activity is apparent when they are tested separately. More globally, this is just one example of how dependent we are on seemingly irrelevant conditions of assays for finding biological activities relevant to human protection and on how the synergies of these components might produce powerful protection from simple, common compounds. Another point is the temporal and spatial specificity as compounds are released and then destroyed during specific phases of digestion at specific regions of the gut. This latter may be best illustrated by the release of bioactive peptides, described below.

***Antimicrobial peptides.*** The article by the Mietzner group (5) notes that, although human milk contains nutrients in

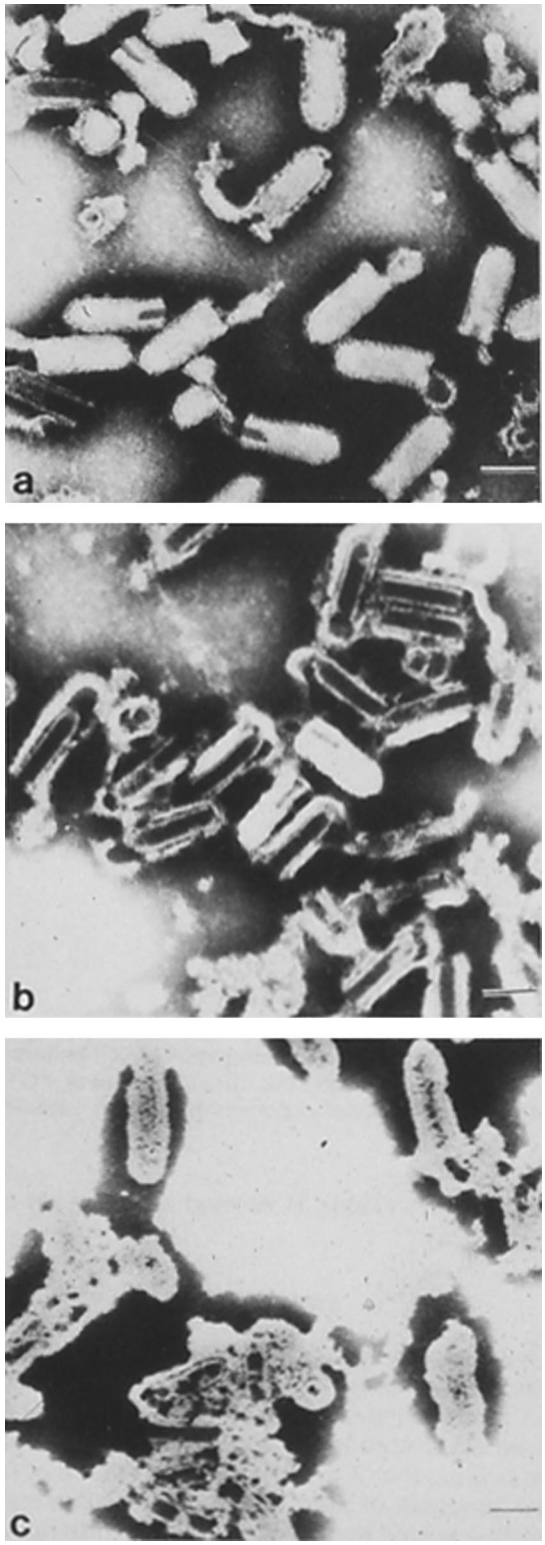


**FIGURE 2** Destruction of enveloped virus by FFAs. Vesicular stomatitis virus (*a*) is incubated (*b*) for 30 min with linoleic acid at half the concentration found in gastric aspirate of nursing infants (0.5 g/L) or (*c*) at the full concentration (1 g/L). The linoleic acid released from human-milk triglycerides makes the envelope of the viruses become leaky, ultimately destroying the virus under conditions that mimic those found in the stomach of nursing infants. (Reprinted from Antimicrob. Agents Chemother, vol. 31 (1), Thormar et al., Inactivation of enveloped viruses and killing of cells by fatty acids and monoglycerides, pp. 27–31, Copyright 1987, with permission from the American Society for Microbiology.)

proportions that should support profuse bacterial growth, it is highly resistant to bacterial overgrowth. This is consistent with their conclusion that antimicrobial peptides are abundant in human milk, both pre- and postdigestion. They ask why there are so many, and whether they share common modes of action, and they investigate whether a "superpeptide" can be engineered with optimum pathogen inhibition. Model peptides were constructed with cationic amphipathic motifs, which are common to antibacterial peptides, and some of these structures had optimum activity in bacteria-killing assays. But the same structure was not optimum under all conditions. For example, the ability of a peptide to kill one pathogen was eliminated by the presence of salt at physiologic concentration, whereas the ability to kill another was not. The efficacy of 2 peptides was compared in different environments: one was more potent than the other when the isolated peptides were tested, but the other was more potent when administered in human milk. These findings in model peptides may help in understanding how human-milk peptides may function in mucosal immunity. The release of various peptides from milk proteins and their subsequent degradation occur in specific stages of digestion of milk, usually in specific locations. The multiplicity of peptides released from milk may be necessary because of the different conditions under which they must function; each may vary in its ability to inhibit in a specific location with a unique environment and may be active against a specific infection. Furthermore, many peptides must be secreted in their inactive forms, to become active only in particular locations and specific conditions. Some peptides may be optimized to inhibit pathogens in the absence of inflammation, whereas others inhibit best in an inflamed environment. A given peptide may have different functions at different local concentrations, with low concentrations being immunomodulatory, and higher concentrations being lethal to pathogens. Moreover, low concentrations may induce mild membrane perturbation that is not lethal per se but may become lethal when in combination with another inhibitor. Another example of a milk component that becomes biologically active only under a specific set of conditions is the conversion of $\alpha$-lactalbumin to an alternate conformer, named HAMLET.

***α-Lactalbumin, in the form of HAMLET, inhibits tumors.*** Lactalbumin in the mammary acinar cell is an essential part of the enzyme complex that synthesizes lactose, and it is the principal protein found in human milk. A novel type of protein modification is the conversion of $\alpha$-lactalbumin to an alternate conformation under the conditions that exist in the stomach of the nursing infant. This alternate conformation had been discovered by the Svanborg laboratory in an acid precipitate of human-milk protein as a factor that resulted in the death of tumor cells and was thus named "human $\alpha$-lactalbumin made lethal to tumor cells," with the acronym HAMLET (6). The HAMLET complex consists of $\alpha$-lactalbumin and oleic acid, and is formed under acidic conditions in the presence of free oleic acid, of which, as mentioned earlier, large amounts are released from milk in the stomach. The oleic acid seems to "freeze" the conformation of the protein into the more open (partially unfolded) form that occurs under acidic conditions. The specificity of this complex is noteworthy: many isomers of C18 will complex with $\alpha$-lactalbumin and induce the open conformation but only the C18:1(9)*cis* isomer, which is the isomer released from human-milk triglyceride, will form a complex that is biologically active. The Svanborg group reports that this protein complex, which is a unique product of human-milk consumption, also seems to have a unique mechanism of action. It will bind to both malignant and normal cells and be transported to the cytoplasm of both, but more efficiently in malignant cells, and only in malignant cells will it be transported to the nucleus. HAMLET binds to histones such as H3, inhibits histone binding to DNA, and results in condensation of the chromatin in the nucleus, ultimately leading to apoptosis. In animal models, HAMLET inhibited a broad array of malignant tumors, and in humans topical treatment with HAMLET reduced the volume of >95% of skin papillomas compared with 20% of papillomas reduced by placebo.

These exciting, unique findings raise the question of whether normal immature cells are also inhibited by HAMLET, and, if so, what the role of HAMLET might be in normal development, particularly that of the intestinal mucosa. The formation of HAMLET in the stomach of the nursing infant is perhaps the most startling example of the utilization of common nutrients to form transient but extremely potent bioactive molecules as part of an innate immune system of human milk. Non-nutritive components, whose effects would be expected to be less transient, are also found in human milk and are best exemplified by the human-milk glycans, especially oligosaccharides.

***Human-milk glycans protect against diarrhea.*** Glycans are complex carbohydrate structures usually found as glycoproteins, glycolipids, mucins, and glycosaminoglycans; unique to milk are the free oligosaccharides that terminate in lactose, which are found in exceptionally high amounts in human milk. At ~10 g/L (1%) of human milk, the oligosaccharides represent the third largest constituent, yet they are indigestible. However, the nonreducing terminus of these molecules contains moieties that resemble the human intestinal receptors that many pathogens use to find, bind, and infect, resulting in diarrhea. The glycans protect by inhibiting the ability of the pathogen to bind to its host, as illustrated in **Figure 3**. This led the "Human-Milk Project" to investigate the role of human-milk glycans in protecting nursing infants from enteric pathogens. The milk components that protect against many of

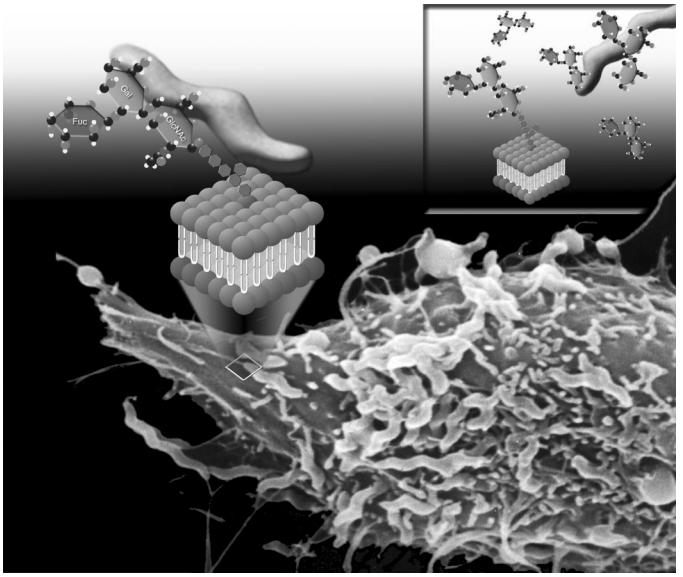


**FIGURE 3** Binding by *Campylobacter jejuni* to the H-2 epitope and its inhibition by milk oligosaccharide. *C. jejuni* binds specifically to the H-2 epitope (a fucosylated glycan) on the cell surface of its target as depicted in the central figure. This binding is inhibited in the presence of the milk oligosaccharide 2′-fucosyllactose by competitive binding, as depicted in the insert on the upper right.

the pathogens under investigation were those that contain a fucose attached by an $\alpha$1,2 linkage to the milk glycan. Finding that mothers of different Lewis blood group types produce milk with significantly different characteristic patterns of fucosylated glycans allowed investigations to be designed to address whether these milk glycans had a role in protecting breast-fed infants against diarrhea. The results, presented in the report by Morrow et al. (7), indicate a strong significant relation between concentrations of specific fucosylated oligosaccharides in milk and the incidence of the specific diseases in the infants consuming the milk. Thus, the oligosaccharides in milk whose moieties are known inhibitors of host cell binding by specific pathogens strongly inhibit these pathogens in the intestine of nursing infants. The conclusion is that the human-milk glycans are powerful components of an innate immune system of human milk whereby the mother protects the infant. Also, mothers differ in their ability to produce specific types of glycans, leading to selective pressures unique to each complement of endemic pathogens for each population, thereby contributing to the diversity of glycan expression seen in humans. This also adds another level of complexity to the interactions of components of the mucosal immune system of human milk.

### *Common principles*

It seems increasingly evident that the benefits conferred by human milk to the nursing infant are attributed to many distinct families of bioactive components, as outlined on **Figure 4**. Emerging research indicates that the protection is more complex than previously assumed. The milk components interact with each other and with elements of the infant gut. Many of the protective components are effective only at specific times, specific locations, and in only some specific types of individuals. This specificity of time, place, and individual variation for many of the components may be offset by the redundancy of the system and the synergistic amplification of protection among components. The protective components of milk described herein seem to comprise an innate immune system of human milk whereby the nursing mother confers powerful protection to her infant. This innate immune system of milk, in concert with the acquired immune system and intestinal microflora, is a robust part of the mucosal immunity that protects breast-fed infants from environmental pathogens.

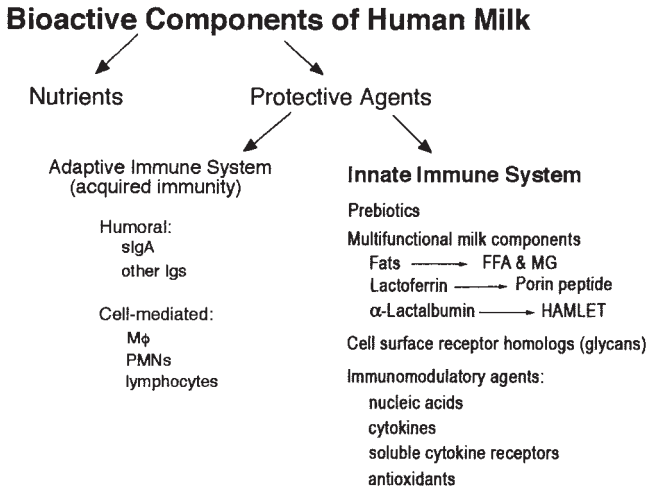


**FIGURE 4** Bioactive components of human milk. Many human-milk components have now been identified as antipathogenic. Many can be classified as part of an innate immune system whereby the mother protects the infant from both endemic and emerging pathogens. In addition to the increasing numbers of known components, increased complexity arises from the interactions among these components and with the infant gut and its microflora.

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