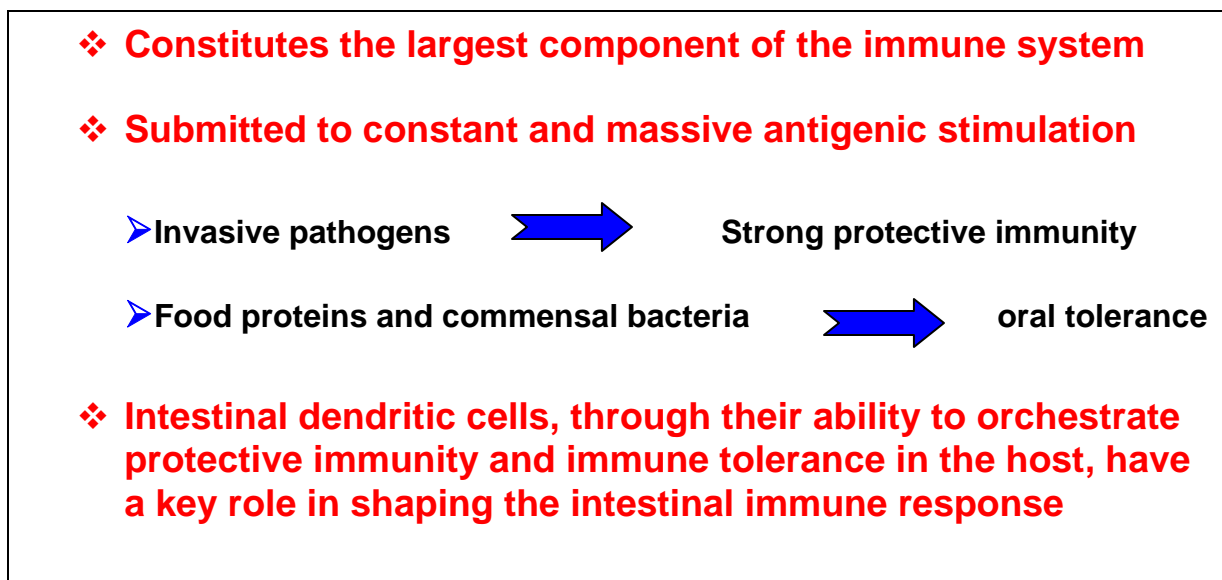


# Early Nutrition, Including the Role of Breast Milk, and Modulation of Tolerogenic and Immunogenic Responses

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The largest component of the immune system is located in the intestine. A key feature of the intestinal immune system is its ability to discriminate between invasive pathogens, for which it generates strong protective immunity against infections, and antigens that are harmless, such as food proteins and commensal bacteria (Fig 1).



**Fig 1. Intestinal immune system.**

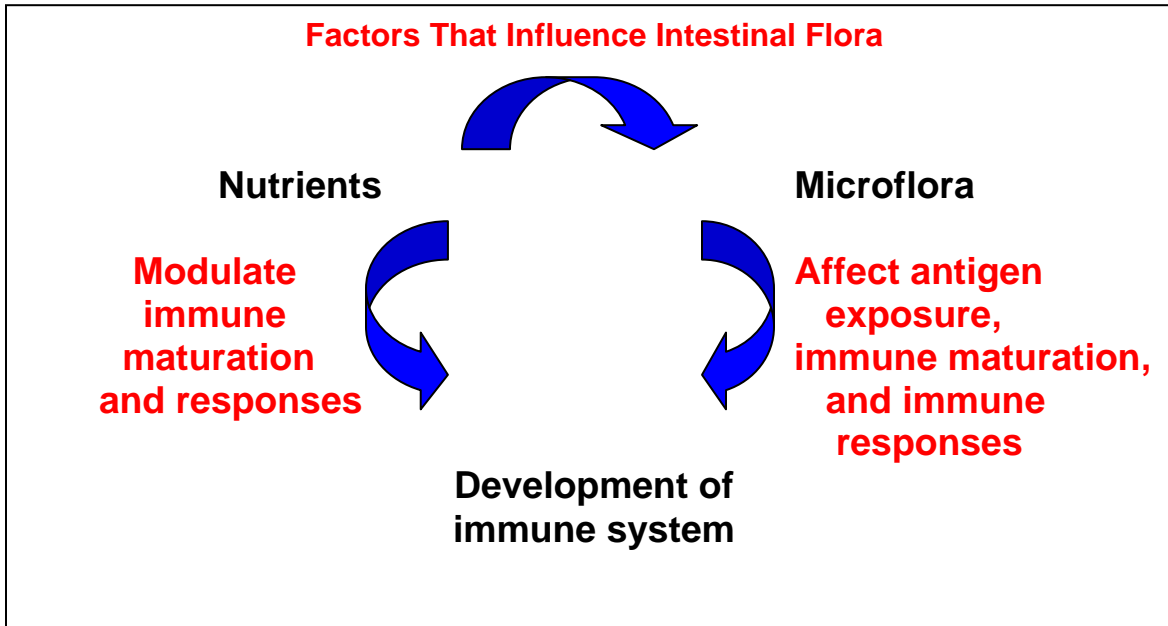
The default response to harmless antigens in the gut is the induction of a state of immunological hyporesponsiveness, known as oral tolerance, and this may be the homeostatic mechanism that prevents intestinal disorders such as celiac disease and Crohn's disease, as well as atopy.<sup>1</sup>

According to Mowat,<sup>1</sup> the way in which antigen is presented to the immune system is the critical factor that controls whether tolerance or immunity results, and most evidence indicates that dendritic cells (DCs) are likely to be the antigen-presenting cells involved (Fig 1). In the

presence of inflammation or pathogenic organisms, DCs are activated to express a full range of co-stimulatory molecules and cytokines, ensuring the efficient stimulation and differentiation of effector T cells. However, DCs are also central to the induction of tolerance to both self and foreign antigens, as in the absence of inflammation, DCs can present antigen to T cells but lack the complete range of co-stimulatory molecules necessary for full T-cell activation. DCs of this kind have been referred to as mature but "quiescent," and T cells stimulated in this way become unresponsive (anergic) and/or differentiate into regulatory T cells.

Recent work has begun to identify factors responsible for intestinal conditioning of DC function and the subsequent decision between tolerance and immunity in the intestine.<sup>2</sup> These studies have identified how DCs resident in the intestine, depending on how they are conditioned by epithelial cell-derived factors or pathogenic virulence factors, drive the differentiation of different type of T-cell responses, such as forkhead box P3 (FoxP3)<sup>+</sup> regulatory T (Treg) cells or T helper 1 (Th1)- or Th17-type responses. Furthermore, recent studies addressing the mechanism of oral tolerance also show that mucosal tissues are replete with a unique subset of DCs that secrete factors such as TGF- $\beta$ 1 and retinoic acid that induce FoxP3<sup>+</sup> regulatory T cells. According to these studies mucosal unresponsiveness might be related to the availability of a factor in the food stream, such as vitamin A.<sup>3</sup>

Nutrition may be the source of antigens to which the immune system must become tolerant; provide factors, including nutrients, that themselves might modulate immune maturation and responses; and provide factors that influence intestinal flora, which in turn will affect antigen exposure, immune maturation, and immune responses.<sup>4</sup> Consequently the interaction between nutrients, microflora, and the intestinal immune system highly influences how these processes take place (Fig 2).



**Fig 2. Interaction between nutrients, microflora, and the intestinal immune system.**

This is in agreement with the hygiene hypothesis of atopic disease that suggests that environmental changes in the industrialized world have led to reduced microbial contact at an early age and have thus resulted in the growing epidemic of atopic eczema, allergic rhinoconjunctivitis, and asthma. In addition to protection against atopy, protection against infectious, inflammatory, and autoimmune diseases also may depend on healthy host-microbe interactions implicated in the hygiene hypothesis.<sup>5</sup>

Breast milk, which is generally accepted as the optimal source to feed infants, constitutes a good model to study the interaction between nutrients, microflora, and the intestinal immune system. On the one hand, it is currently accepted that human milk is a source of beneficial bacteria for infant gut development and maturation,<sup>6,7</sup> and on the other hand, it also contains a complex mixture of bioactive compounds that have been demonstrated to have a beneficial role on immune function in infancy (Table).<sup>8</sup>

**Table. Immunological Components in Human Milk**

<ul style="list-style-type: none"><li>• Constituents that promote tolerance/priming of the infant immune system<ul style="list-style-type: none"><li>– Cytokines: TGF-<math>\beta</math>, IL-10</li><li>– N-6, n-3 fatty acids</li><li>– Antigens</li></ul></li><li>• Antimicrobial components<ul style="list-style-type: none"><li>– Immunoglobulins</li><li>– Lysozymes/other enzymes</li><li>– Lactoferrin, mucins, other proteins/peptides</li></ul></li><li>• Factors that promote immune development<ul style="list-style-type: none"><li>– Cytokines: TGF-<math>\beta</math>, IL-6, IL-10</li><li>– Nucleotides, gangliosides</li></ul></li><li>• Anti-inflammatory components<ul style="list-style-type: none"><li>– Cytokines: TGF-<math>\beta</math>, IL-10</li><li>– Antioxidants</li></ul></li></ul>	<ul style="list-style-type: none"><li>– Oligosaccharides</li><li>– Fats/fatty acids</li><li>– Hormones, bioactive peptides</li><li>– Long-chain polyunsaturated fatty acids</li></ul>
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The role of breast milk in regulating immunological tolerance to allergen exposure even outside the intestine was recently demonstrated through an elegant animal study. This study showed that breast milk-mediated transfer of an airborne antigen to the neonate resulted in oral tolerance induction, leading to antigen-specific protection from allergic airway disease. That tolerance induction did not require the transfer of immunoglobulins, relied on the presence of transforming growth factor beta (TGF- $\beta$ ) during lactation, was mediated by regulatory CD4<sup>+</sup> T lymphocytes, and depended on TGF- $\beta$  signaling in T cells.<sup>9</sup>

The inclusion of probiotics, as ingredients in nutritional products, has been one of the tools used to try to influence the process of oral tolerance and response upon infection at the intestinal level. The immunomodulatory effects of probiotics in the intestinal tract were recently reviewed, suggesting that many probiotic organisms are able to influence DCs to induce a nonresponse state, more particularly by encouraging the development of T cells with immunoregulatory properties.<sup>10</sup>

The influence of probiotics on the process of oral tolerance, and consequently on the prevention of atopy, has been demonstrated by several studies, the most representative one showing that *Lactobacillus* GG was effective in prevention of early atopic disease in children at high risk.<sup>11,12</sup> However, another group recently showed, through a similar experimental design, that supplementation with *Lactobacillus* GG during pregnancy and early infancy did not reduce the incidence of atopic dermatitis or alter the severity of atopic dermatitis in affected children but was associated with an increased rate of recurrent episodes of wheezing bronchitis.<sup>13</sup> According to this last study, the effect of *Lactobacillus* GG on the prevention of atopic dermatitis remains controversial. Nonetheless, another recent study has shown that supplementation with another *Lactobacillus* strain (*Lactobacillus rhamnosus*) during pregnancy and early in life substantially reduced the cumulative prevalence of eczema<sup>14</sup> and had the potential to influence several fetal immune parameters as well as immunomodulatory factors in breast milk.<sup>15</sup>

On the other hand, protection against viral or bacterial infections is one of the most frequent claims made for probiotic consumption. Different mechanisms have been suggested to explain this antimicrobial activity, including production of anti-microbial compounds, increase in mucine production, reduction of intestinal permeability, and competition with enterotoxigenic bacteria for nutrients and epithelial intestinal cell-receptor binding sites.<sup>16</sup>

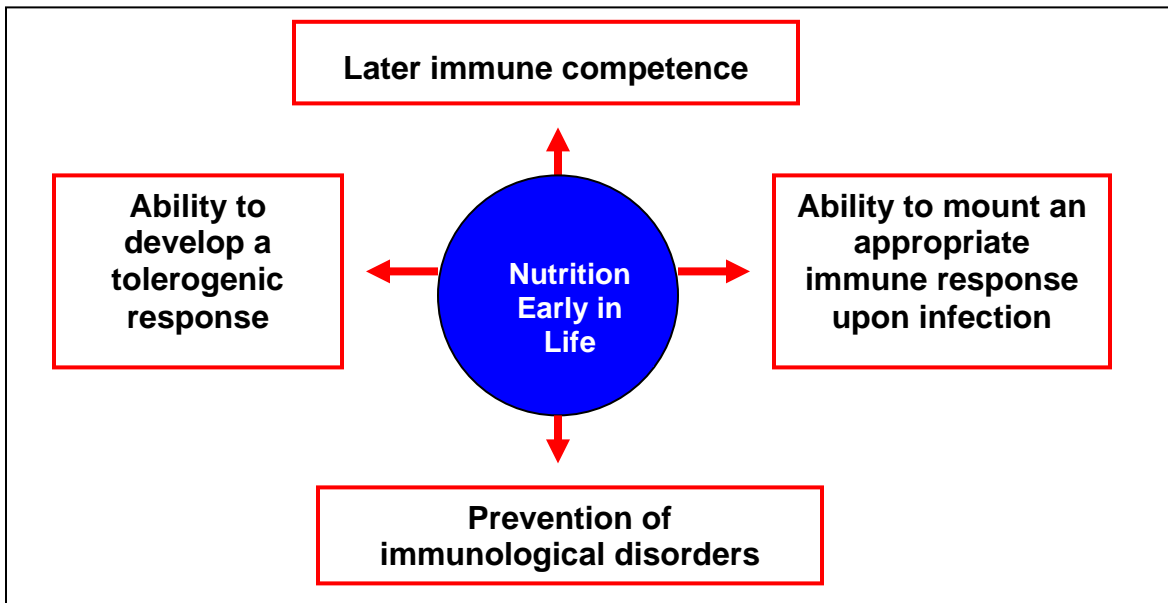
Human milk also contains a wide range of oligosaccharides, which are considered as the first prebiotics in humans, thus constituting also the first soluble fibers to which humans are exposed.<sup>17</sup> Although several biological functions have been proposed for human-milk oligosaccharides,<sup>18</sup> one indisputable role is as primordial soluble fibers and selective prebiotics. In addition, human-milk oligosaccharides act as decoys for pathogens and toxins that adhere to oligosaccharides in the surfaces of target cells. Several studies support this particular role for

human-milk oligosaccharides,<sup>19</sup> which is also shown for other glycoconjugates, such as gangliosides. Gangliosides are also present in human milk, and they may function as “unintended” target receptors for bacterial adhesion to the intestine.

Ganglioside-supplemented infant formula has been reported to modify the intestinal ecology of preterm newborns, increasing the *Bifidobacteria* content and lowering that of *Escherichia coli*.<sup>20</sup> After oral administration, gangliosides can be putative decoys that interfere with pathogenic binding in the intestine, this being the main mechanism by which these compounds can prevent infection. Recently, the influence of milk gangliosides on DC maturation and effector functionalities also has been reported, suggesting a role, especially for GD3, in modulating the process of oral tolerance during first stages of life.<sup>21-24</sup>

On the other hand, gangliosides also may stimulate the ability to mount an appropriate immune response upon infection. In fact, the influence of dietary gangliosides on several parameters related to the development of the intestinal immune system, such as cytokine and intestinal IgA production, also has been described in animal models.<sup>20</sup> This is also the case for other ingredients, such as nucleotides, which are shown in animal studies to have a role modulating IgA production and lymphocyte populations at the intestinal level.<sup>25</sup> Several clinical studies also have shown the role of nucleotides influencing both humoral and cellular responses, and reducing diarrhea incidence and duration when they are incorporated into infant feeding early in life.<sup>26</sup> The effect of nucleotides reducing diarrhea incidence might be influenced, at least partially, by also influencing the composition of intestinal microflora. Although this topic has remained controversial because of studies reporting contradictory results,<sup>27,28</sup> a recent study using more robust molecular techniques to assess fecal microbiology has demonstrated that nucleotide supplementation improves the composition of the gut microbiota in formula-fed infants.<sup>29</sup>

In summary, nutrition early in life might affect later immune competence, the ability to develop a tolerogenic response to “self” and to benign environmental antigens, and the ability to mount an appropriate immune response upon infection; consequently, it also might prevent the development of immunologic disorders (Fig 3).



**Fig 3. Influence of early nutrition on immunity.**

Breast milk contains a complex mixture of bioactive compounds that greatly contribute to regulation of those abilities. The incorporation of ingredients such as nucleotides, probiotics, and prebiotics into the composition of infant formulas constitutes a good example of the effort to not only provide the nutritional requirements of the neonate, but also to emulate the immunological development observed in breastfed infants both early and later in life.

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## Q & A

Q: In one study, Dr Rueda, you described inhibition of dendritic cells by several species of gangliosides. In another study, you observed an increase of IgA production in your subjects. Will you speculate about the possible underlying mechanisms in these differing observations?

Dr Rueda: We have not studied dendritic cells with gangliosides, so we could not interpret what the mechanism might be. Regarding IgA, although we described it for another milk compound (it was for nucleotides and not for gangliosides), we could see that nucleotides were able to modify the expression of some markers, especially those of B1a cells, which are precursors of plasma cells producing IgA at the intestinal level [Aggett P et al: *Nutrition* 2003;19:375-384.] We also were able to demonstrate that both nucleotides and gangliosides were able to stimulate the production of some cytokines that are involved in the maturation and differentiation from B cells to plasma cells producing IgA. That is the case, for example, with IL-2 and IL-6. We could see that process for both nucleotides and gangliosides, so that might be one mechanism explaining the increase of IgA production promoted by both milk compounds. There might be others, however, that we cannot interpret.

Q: With respect to the profile of oligosaccharides, there is a difference among species. What about in rodent species? What are the oligosaccharide levels and distribution?

Dr Rueda: We have not studied that. As far as I know, there is a varied population of oligosaccharides in sheep and goats, but I am not aware of the specific role or concentration of oligosaccharides in rodents.

Q: Can you comment on the proposed mechanism or hypothesis as to why nucleotides might affect the microbiota? Are they food for the microbiome?

Dr Rueda: I do not have a clear answer. It has been debated whether nucleotides are prebiotics or not. We do not know whether they reach the colon, because they are absorbed earlier in the small intestine. They might be a specific nutrient, but because they do not reach the colon, I do not know how they are able to modify the microbiota.

Q: Is it possible to give a rough gauge of the relative importance of oligosaccharides, nucleotides, and gangliosides on the direct effect on commensal bacteria, which, in turn, affect the host? What about the relative importance of breast-milk components on direct effects on the host and on commensal bacteria in the host?

Dr Rueda: It is difficult to isolate one from the other, because they probably are related. On the other hand, we cannot forget that it also is difficult to isolate one specific ingredient present in breast milk from the others. There might be complex interactions between them. The only way to do it is in animal studies in which we isolate a specific ingredient and have a control group that does not get this ingredient, and then interpret the potential mechanism. We always have several mechanisms that might affect the intended outcome, however, so such research is difficult.

Q: Yes, it is with conventional animals and humans. Has anyone done this on germ-free animals to illustrate whether there is a direct or indirect effect on commensal bacteria?

Dr Rueda: Dr McCoy previously described experiments using that wonderful technology—germ-free animals. We have not done any experiments in which we fed germ-free mice with a specific compound and studied intestinal microflora because we did not have that focus. We focused mainly on the immune activity regulated by these ingredients and how they modify some immunological parameters at the intestinal level.

Q: Here is a controversial issue: What sort of advice would you give to mothers who are severely affected by allergic asthma, with high levels of IL-4 and perhaps even T-cell specificity for allergens in the breast milk? Should they breastfeed their babies?

Dr Rueda: We could talk about that question for an hour. Although some studies indicate that probiotics taken by the mother and/or the infant might have a role in preventing allergies, results do not clearly indicate which probiotic produces this effect. For a mother of a child who is probably at risk to develop atopy, I would advise controlling the feeding of the infant in the first days of life. At this moment, the tools we have are infant formulas with hydrolyzed protein that help prevent atopy. However, it is not clear whether partially hydrolyzed or extensively hydrolyzed formula should be the first option. This is controversial, but at this moment, international pediatric committees recommend feeding infants at risk for atopy a formula with an extensively hydrolyzed protein.

Q: So would you recommend that a highly allergic asthmatic mother avoid breastfeeding?

Dr Rueda: No, no, no!

Q: No? But some people do that when mothers have high levels of IL-4 and T cells.

Dr Rueda: That is not what the international committees are saying. However, I have read that there might be a risk—that if the mother feeds human milk to the infant, it might be more

difficult to control that process later after child has developed allergy [Zeiger RS: *Pediatrics* 2003;111:1662-1671].

Q: In the mouse study you talked about, you referred to the transfer of antigen into the breast milk. How do you think the ovalbumin moved from the respiratory tract into the milk?

Dr Rueda: I do not know. The only explanation I can give is the same one that Dr Versalovic suggested previously for probiotics. Apparently, there is an increase in susceptibility among pregnant women, especially during the last part of gestation, to having some antigens circulating for some time. The antigens can migrate to, for example, the mammary gland. That is the only mechanism suggested, but we do not have evidence for it. In a study in Spain using probiotics, but not ovalbumin, researchers fed mothers a specific strain and then tried to isolate that strain from human milk. As far as I know, their results are not available or published.

Q: We tend to swallow inhalant allergens, as well, so we get a lot of inhalants in the gut. Might that be involved?

Dr Rueda: Yes.

Q: I would like to follow up on a previous question. The American Academy of Pediatrics and, I believe, the European pediatric groups recommend feeding practices for high-risk infants, but they are not clear. These groups seem silent on what to do with a mother who is profoundly allergic and expressing the symptoms of allergy. Should she breastfeed? That is an important question. Dr Bienenstock, what do you think about that?

Dr Bienenstock: Pat Holt in Western Australia is conducting a study of oral immunization of high-risk infants with large doses of antigen. The infants have been followed since birth and so far have not had any problems or adverse effects in this important and as yet unpublished study. The obvious alternative view we may wish to take is that it makes no difference whether a

mother breastfeeds or not, and that one should encourage the normal process, which is breastfeeding, in this situation. The evidence is highly controversial, and I do not accept the position that one should forbid breastfeeding in a high-risk mother. I think we have to entertain the possibility that what is needed is high exposure levels in these infants to promote tolerance to the antigen.

Dr Brandtzaeg: I am not referring to the genetic risk of the child. I am referring to the drive for a Th2 response, perhaps depending on high levels of IL-4 in breast milk and, possibly, T cells with specificity for allergens that could be transferred to the infant and start an allergic response. The large immunological studies from Arizona show that highly asthmatic women could actually transfer with breast milk this drive toward the Th2 response when atopic children grow up (around 6 years of age). I know this is a controversial issue, despite being supported by mouse studies [Wright AL et al: *Thorax* 2001;56:192-197; Leme AS et al: *J Immunol* 2006;176:762-769].

Dr Bienenstock: This is just a proposal, Dr Brandtzaeg. We are translating the evidence of IL-4 in the breast milk and so on to what we think is going to happen next. I do not believe that the evidence is there.

Dr Brandtzaeg: No, it is controversial and not easy, but an audience like this perhaps could give a clear-cut opinion on it. I take the silence as evidence that there is no answer yet.

Dr Larry Williams (Abbott Faculty): I will not give you an answer, but I will give you the practical pediatric allergy response. We are talking about risk reduction, not prevention. For an individual mother, it is a question of risk reduction, and none of the techniques that have been suggested for allergen avoidance over the last 20 years is associated with more than a 30% reduction in risk. That means that if the physician takes the authoritative stance “you must do

this,” he or she is setting up a 70% chance that those mothers will fail. That is unacceptable. So the science is such that we do not know what to do. My view is that physicians should not be prescribing extensive dietary manipulation of small infants, but that standard practice should reign until we have better science.

Q: Is there any evidence that the presence of IL-4 in breast milk is maintained in the infant? It has to go through a lot of systemic paths. Most of it must be destroyed. Would it even be active?

Dr Rueda: Not as far as we know.