Allergen shedding in human milk: Could it be key for immune system education and allergy prevention?

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In addition to being a source of nutrients for the developing newborn, human milk contains thousands of bioactive compounds, which influence infant health in the short-term as exemplified by its major benefits on infectious disease prevention. Many of the human milk compounds also have the required characteristics to instruct immune development and guide long-term health. Prebiotics, probiotics, and variated antimicrobial molecules all have the potential to shape the composition and function of the establishing gut microbiota, which is known to be a major determinant of immune function. Another and less explored way human milk can instruct long-term immunity is through antigen shedding. Here, we will review the evidence that antigens from maternal environment and more specifically from allergen sources are found in human milk. We will discuss data from rodent models and birth cohorts showing that allergen shedding in breast milk may influence long-term allergy risk. We will uncover the variables that may underlie heterogeneity in oral tolerance induction and allergy prevention in children breast-fed by allergen-exposed mothers. We will focus on the parameters that control antigen transfer to breast milk, on the unique biological characteristics of allergens in breast milk, and on the milk bioactive compounds that were found to influence immune response in offspring. We propose this understanding is fundamental to guide maternal interventions leading to lifelong allergen tolerance. (J Allergy Clin Immunol 2021;148:679-88.)

Abbreviations used
β-LG: β-lactoglobulin
EV: Extracellular vesicle
FcRn: Neonatal Fc receptor
HDM: House dust mite
OVA: Ovalbumin
Treg: Regulatory T

Key words: Allergy, primary prevention, oral tolerance, breast milk, allergen

NEONATAL IMMUNITY, THE FIRST PIECES OF A PUZZLE THAT WILL CONDITION LONG-TERM HEALTH

Children show a high susceptibility to both infectious and allergic disease, revealing deficiencies of the developing immune system in its 2 main arms: immune defenses and regulation (Fig 1). Progressively, the infants’ immune system will acquire the ability to mount tightly regulated responses that are tailored to the target. This ability will be key for a lifelong protection from infectious and noninfectious disease such as allergy, cancer, and metabolic disease. The concept of a “neonatal window of opportunity” has emerged in the last years, suggesting that a critical nonredundant time frame exists in a newborn’s life during which environmental factors drive immune and tissue development and influence the susceptibility to diseases in adult life. Finding the key risk and protective factors in the infant’s environment, and modulating them if necessary, opens new perspectives for promoting long-term health. In this regard, deciphering the influence of the establishing gut microbiota on immune trajectory is a focus of major interest and has been recently reviewed elsewhere. The diet is another key player in immune development and polarization. In this regard, human milk is of particular significance as the physiological food for the developing infant. Its massive impact on infectious disease prevention in childhood is shown repeatedly in meta-analyses concluding that scaling up breast-feeding to a near-universal level could prevent about 1 million annual deaths in children younger than 5 years. Abundant research is investigating the microbiota-shaping properties of breast-feeding because this may represent a way breast-feeding...
could influence long-term immune health in addition to its major impact in the short-term. Whether antigens in human milk play a role in immune imprinting and condition long-term susceptibility to immune-dependent health outcomes is a less explored field. Although systematic reviews and meta-analyses conclude that breast-feeding is not protecting from allergies at the population level, here, we will explore the hypothesis that the presence of allergens in human milk is key to dictate allergic outcomes in breast-fed children (Fig 1). We will further review the knowledge, and its gaps, on the factors directing immune tolerance induction on allergen exposure through breast milk.

**ALLERGENS IN HUMAN MILK: WHAT IS THE EVIDENCE FOR AN IMPACT ON ALLERGY RISK IN OFFSPRING?**

Allergy, including eczema, asthma, and food allergies, is a growing burden among children in both high- and low-income countries. After the era of allergen avoidance, new guidelines recommend early oral allergen exposure aiming at inducing regulatory immune responses and generate tolerance to allergens. This will prevent induction of type 2 inflammatory immune responses that are responsible for allergic disease. There is accumulating evidence that early oral introduction of some dietary-derived allergens such as egg and peanut is beneficial for food allergy prevention. However, recent studies also demonstrate that a significant proportion of infants already have egg sensitization and clinical reactivity (including anaphylaxis) at weaning age and before the first introduction of egg in their solid foods diet. Furthermore, currently, there is no consistent evidence that early introduction of other major allergens in the diet such as milk, wheat, or fish is decreasing allergy risk. Demonstrating the potential of early oral tolerance induction through exposure to allergens in human milk will highlight an attractive strategy to overcome early allergic sensitization. We will review the evidence on the presence of allergens in human milk and data from experimental models and birth cohorts on their contribution to antigen-specific immune education and allergy prevention in offspring.

**Dietary and airborne allergens are found in human milk**

The first investigations on the presence of allergens in human milk were in the early 1980s and focused primarily on the excretion of cow’s milk and egg proteins. The advancement in sensitive techniques revealed the presence of all the major food allergens such as allergens from egg, milk, peanut, and wheat in human milk as detailed in Table I. Food allergens are found in human milk in the range of picogram per milliliter to nanogram per milliliter in about 1 in 2 women under normal and unrestricted diet or after a challenge with the food allergen (Table I). Besides allergens derived from maternal diet, airborne allergens are shed into human milk in similar concentration as food allergens (Table I). Allergens from house dust mite (HDM) Dermatophagoides pteronyssinus and Blomia tropica have been detected in about half of the human milk samples that were collected from various regions of the world.

**FIG 1.** Allergen shedding in human milk as a way to educate immune system and modulate allergy risk in offspring. Both regulatory and effector immune responses are compromised in the neonate and will evolve to become finely regulated and tuned immune responses. Human milk brings multiple molecules including pathogen-specific antibodies that will provide passive help and protect the newborn from infectious disease. We propose that human milk also actively influences offspring’s immune ontogeny through antigen shedding. The breast-fed infant is exposed to a wide variety of maternal diet–derived and environmental allergens. The nature of the allergen, the way antigen is presented, and milk cofactors will dictate long-term allergy susceptibility. Although there is evidence that egg, peanut, and cow-milk dietary antigens in maternal milk can promote tolerance in offspring, HDM-derived allergen in milk might promote allergy risk. Figure created with Biorender.
TABLE I. Allergens in human milk

<table>
<thead>
<tr>
<th>Allergen source</th>
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<th>Study design</th>
<th>n/total n (% of milk samples with detectable allergen)</th>
<th>Allergen concentration (ng/mL), median (range)</th>
<th>Population</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Kilshaw and Cant, 16 1984</td>
<td>Ovomucoid</td>
<td>Mothers were put under egg-free diet and then challenged with 1 raw egg. Milk collected after 24 h</td>
<td>7/9 (77.7%) 13/22 (59%)</td>
<td>1.29 (0.44-2.88) 2.09 (0.26-6.17)</td>
<td>NA</td>
<td>1 wk-12 mo</td>
</tr>
<tr>
<td>Cant et al, 15 1985</td>
<td>OVA</td>
<td>Mothers were put under egg-free diet and then challenged with 1 raw egg. Milk collected after 24 h</td>
<td>14/19 (73.7%)</td>
<td>NA (0.2-4.0)</td>
<td>England</td>
<td>&lt;6 mo</td>
</tr>
<tr>
<td>Fukushima et al, 17 1997</td>
<td>OVA</td>
<td>Mothers under unrestricted diet</td>
<td>2/24 (8.3%)</td>
<td>NA (0.0-0.7)</td>
<td>Japan</td>
<td>4-10 mo</td>
</tr>
<tr>
<td>Hirose et al, 18 2001</td>
<td>Ovomucoid</td>
<td></td>
<td>12/37 (32%)</td>
<td>NA (0.33-37)</td>
<td>Japan</td>
<td>NA</td>
</tr>
<tr>
<td>Palmer et al, 19 2005</td>
<td>OVA</td>
<td>RCT: Mothers were challenged with a breakfast that contained egg or not. Milk samples collected after 8 h</td>
<td>3/41 (7%) 22/41 (54%) 19/41 (46%) 28/41 (68%)</td>
<td>0.00 (NA) 10.28 (NA) 0.00 (NA) 1.18 (NA)</td>
<td>Australia</td>
<td>&gt;8 wk</td>
</tr>
<tr>
<td>Vance et al, 20 2005</td>
<td>OVA</td>
<td>RCT: Mothers avoided egg or not during pregnancy until the end of lactation</td>
<td>18/60 (30%) 22/65 (33.8%)</td>
<td>1.41 (0.12-170.9) 0.63 (0.14-1258)</td>
<td>United Kingdom</td>
<td>3 mo</td>
</tr>
<tr>
<td>Palmer et al, 21 2008</td>
<td>OVA</td>
<td>RCT: Mothers were challenged with a muffin that contained 1 egg or not for 3 d</td>
<td>1/16 (6%) 10/16 (63%)</td>
<td>1.0 (0.23-0.63) 0.63 (0.01-3.32)</td>
<td>Australia</td>
<td>7.0 mo 7.1 mo</td>
</tr>
<tr>
<td>Macchiaverni et al, 22 2014</td>
<td>OVA</td>
<td>Women under unrestricted diet</td>
<td>51/97 (52.5%) 22/34 (64.7%) 31/70 (44.3%) 64/136 (47%)</td>
<td>0.12 (0-3.9) 0.12 (0-0.7) 0.11 (0-2.0) 0.13 (0-3.7)</td>
<td>France</td>
<td>1-3 d Australia 1-3 d</td>
</tr>
<tr>
<td>Metcalfe et al, 23 2016</td>
<td>OVA</td>
<td>RCT: Mothers were submitted to a dietary intervention for the first 6 wk of lactation. Milk collected 2-6 h after egg ingestion:</td>
<td>- High-egg diet (&gt;4 eggs per week) 19/33 (58%) 18/39 (46%) 10/27 (37%)</td>
<td>0.20 (NA) 0.05 (NA) 0.05 (NA)</td>
<td>Australia</td>
<td>6 wk</td>
</tr>
<tr>
<td>Verhasselt et al, 24 2020</td>
<td>OVA</td>
<td>Atopic mothers under unrestricted diet</td>
<td>60/122 (49%) 46/102 (45%)</td>
<td>0.15 (0-NA) 0.17 (0-NA)</td>
<td>Australia</td>
<td>3 mo 6 mo</td>
</tr>
<tr>
<td>Rekima et al, 25 2020</td>
<td>OVA</td>
<td>Atopic mothers under unrestricted diet</td>
<td>45/100 (45%)</td>
<td>0.17 (0-3.7)</td>
<td>Australia</td>
<td>6 mo</td>
</tr>
<tr>
<td>Kilshaw and Cant, 16 1984</td>
<td>β-LG</td>
<td>Mothers were put under cow milk–free diet and then challenged with 500 mL cow milk. Maternal milk collected after 24 h</td>
<td>10/19 (52%)</td>
<td>0.71 (0.11-6.4)</td>
<td>NA</td>
<td>1wk-12 mo</td>
</tr>
<tr>
<td>Axelsson et al, 26 1986</td>
<td>β-LG</td>
<td>Mothers with cow milk–containing diet (0.2-1.5 L/d)</td>
<td>93/232 (40%)</td>
<td>NA (5-800)</td>
<td>Sweden</td>
<td>1-31 wk</td>
</tr>
<tr>
<td>Host et al, 27 1990</td>
<td>β-LG</td>
<td>Mothers ingested 500 mL of milk. Maternal milk collected in the first 24 h</td>
<td>19/20 (95%)</td>
<td>4.2 (0.9-150.0)</td>
<td>Denmark</td>
<td>7-9 wk</td>
</tr>
<tr>
<td>Sorva et al, 28 1994</td>
<td>β-LG</td>
<td>Mothers were put under milk-free diet during 24 h and then ingested 400 mL of fat-free milk. Maternal milk collected at 0, 1, and 2 h</td>
<td>23/47 (49%)</td>
<td>0.01 (0.0-3.5) 0.12 (0.01-7.84) 0.07 (0.01-2.34)</td>
<td>Finland</td>
<td>2.5-12 mo</td>
</tr>
<tr>
<td>Fukushima et al, 17 1997</td>
<td>β-LG</td>
<td>Mothers ingested 200 mL cow milk per day for 7 d. Maternal milk collected in the first 15 h</td>
<td>15/24 (62.5)</td>
<td>NA (&lt;0.1-16.5)</td>
<td>Japan</td>
<td>4-10 mo</td>
</tr>
</tbody>
</table>

(Continued)
Currently, there are no data on airborne allergens from sources other than mites such as from birch pollen, cat dander, or mold. Breast-fed infants are thus exposed to a multitude of allergens, which originate from maternal diet and environment and have much chance to be found in child’s diet and environment after weaning. In contrast, if not breast-fed, infants would not be exposed to most of these allergens before weaning (Fig 2). We may then formulate the hypothesis that early oral allergen exposure through breast milk is designed to educate the child immune system and get it ready to mount an appropriate immune response for later encounter. The evidence of that hypothesis is discussed in the next 2 sections.

### Table 1 (Continued)

<table>
<thead>
<tr>
<th>Allergen source</th>
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<th>n/total n (%) of milk samples with detectable allergen</th>
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<th>Population</th>
<th>Time at milk collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matangkasombut et al,39 2017</td>
<td>α-LG</td>
<td>Mothers avoided cow milk products for 7 d and were then challenged once with 240 mL of pasteurized cow milk. Maternal milk was collected over a 7-d period. - Baseline - After ingestion</td>
<td>15/15 (100%) 15/15 (100%)</td>
<td>0.58 (NA) 1.23 (0.41-3.80)</td>
<td>Bangkok</td>
<td>0-12 mo</td>
</tr>
<tr>
<td>Peanut</td>
<td>Vadas et al,30 2001</td>
<td>Ara h 1 and Ara h 2</td>
<td>Mothers ingested 50 g of dry roasted peanut. Milk collected over a 12-h period</td>
<td>11/23 (48%)</td>
<td>200 (120-430)</td>
<td>Canada</td>
</tr>
<tr>
<td>Bernard et al,31 2014</td>
<td>Ara h 6</td>
<td>Mothers ingested 30 g of dry roasted peanuts on an empty stomach or after breakfast. Milk collected before and over a period of 26 h after ingestion</td>
<td>2/2 (100%)</td>
<td>NA (&gt;1-3340)</td>
<td>United Kingdom</td>
<td>12 wk</td>
</tr>
<tr>
<td>Schocker et al,32 2017</td>
<td>Ara h 2</td>
<td>Mothers ingested 100 g of dry roasted peanut. Milk collected after 1 h</td>
<td>14/40 (35%) 9/40 (22%)</td>
<td>NA (2.3-184) NA (1.1-79)</td>
<td>Germany</td>
<td>NA</td>
</tr>
<tr>
<td>Wheat</td>
<td>Troncone et al,33 1987</td>
<td>Gliadin</td>
<td>Mothers were put on a gluten-free diet for 12 h and then ingested 20 g of gluten. Milk collected after 1 h</td>
<td>54/80 (67.5%)</td>
<td>NA (5-95)</td>
<td>Italy</td>
</tr>
<tr>
<td>Chirdo et al,34 1998</td>
<td>Gliadin</td>
<td>Mothers under unrestricted diet</td>
<td>13/14 (93%) 49/49 (100%)</td>
<td>883 (28-9000) 178 (5-1200)</td>
<td>Argentina</td>
<td>1-3 d 2 wk</td>
</tr>
<tr>
<td>HDM</td>
<td>Macchiaverni et al,22 2014</td>
<td>Der p 1</td>
<td>Mothers selected from general population</td>
<td>44/76 (58%) 66/97 (70.1%) 25/32 (78.1%) 37/71 (52.1%) 89/161 (55.2%)</td>
<td>0.10 (0.0-1.12) 0.07 (0.0-1.18) 0.11 (0.0-1.29) 0.05 (0.0-0.65) 0.05 (0.0-0.83)</td>
<td>Brazil</td>
</tr>
<tr>
<td>Macchiaverni et al,35 2015</td>
<td>Der p 1</td>
<td>Mothers selected from general population</td>
<td>44/75 (58.6%) 31/75 (41.3%)</td>
<td>0.10 (0.0-1.12) 2.00 (0.0-28.8)</td>
<td>Brazil</td>
<td>1-3 d</td>
</tr>
<tr>
<td>Baiz et al,36 2017</td>
<td>Blot 5</td>
<td>Mothers selected from general population</td>
<td>62/84 (73.8%)</td>
<td>0.067 (0.0-1.37)</td>
<td>France</td>
<td>3-7 d</td>
</tr>
<tr>
<td>Rekima et al,37 2020</td>
<td>Der p 1</td>
<td>Mothers selected from general population</td>
<td>51/100 (51%)</td>
<td>0.064 (0.0-0.83)</td>
<td>Australia</td>
<td>6 mo</td>
</tr>
<tr>
<td>Macchiaverni et al,38 2020</td>
<td>Der p 1</td>
<td>Mothers selected from general population</td>
<td>79/218 (36%)</td>
<td>0.17 (0.0-1.23)</td>
<td>The Netherlands</td>
<td>2-35 wk</td>
</tr>
</tbody>
</table>

Ara h, Arachis hypogaea; Blot 1, Blomia tropicalis; Der p, Dermatophagoides pteronyssinus; NA, not applicable/available; RCT, randomized controlled trial.

### Proof-of-concept studies in rodents showing that allergens in breast milk influence later risk of allergy in offspring

Exposing lactating mice to egg antigen ovalbumin (OVA) by oral or intranasal route has been demonstrated to result in antigen transfer into the milk,38,39 and this proved to be an efficient way to induce oral tolerance in offspring.38-45 OVA-exposed lactating mice that are lacking adaptive immunity were still able to induce oral tolerance in offspring,38 strongly arguing that it is the presence of the antigen itself in breast milk that is leading to oral tolerance. The plasticity of immune responses induced in offspring by antigen shedding in breast milk was exemplified with HDM allergens (Fig 1). In contrast to OVA, the transfer of HDM allergens to offspring through breast milk resulted in immune priming. Mice nursed by mothers inhaling HDM during breast-feeding showed an increased susceptibility to respiratory allergic response on re-exposure to HDM in young adulthood.22 Furthermore, they could not be protected from egg allergy by oral tolerance induction, revealing the immune imbalance caused by neonatal exposure to HDM through breast milk.25

### Evidence from birth cohorts of an impact of allergen shedding in human milk on allergy risk in offspring

Recent meta-analyses and systematic reviews conclude that maternal allergen consumption during lactation does not affect allergy risk in offspring.8,7 However, a major limitation for the
interpretation of these studies is the absence of measurement of allergens in maternal milk. Because there is a poor relation between maternal diet and allergen content in breast milk (see below), maternal allergen consumption cannot be used as a proxy of allergen content in breast milk. Up to date, only 1 birth cohort study did analyze the association between allergen levels in human milk itself and allergy risk.24 Although limited in size, the study showed that the presence of OVA in breast milk was associated with a reduced risk of egg allergy in children at age 2.5 years as compared with breast milk with undetectable OVA.24 Another study found a positive association between allergic sensitization and respiratory allergies in children and the presence of *D. pteronyssinus* 1 levels in breast milk.36 Furthermore, and in agreement with mice studies, there is preliminary evidence of a higher risk of egg allergy in children exposed to HDM allergen through human milk.25

Data reviewed in this section demonstrate that the necessary condition to induce oral tolerance to allergens via breast milk is met as allergens are found in human breast milk (Table I). Rodent experiments further demonstrate a causal relation between allergen exposure through breast milk and allergy risk in offspring.38-45 Preliminary evidence in observational birth cohorts where allergens were measured in breast milk supports the concept of an impact of allergen in human milk on offspring’s allergy risk.24,25,36 However, the lack of consistency in birth cohorts on the relationship between maternal allergen intake during breast-feeding and allergic outcome in offspring6,9,46 highlights 2 major gaps in knowledge: (1) what parameters control allergen shedding in human milk and (2) which allergen characteristics and/or milk properties are required for oral tolerance induction to allergen in human milk. The next sections will review existing data on these key points to move toward efficient intervention in lactating mother for allergy prevention.

**WHAT IS CONTROLLING ANTIGEN SHEDDING IN BREAST MILK?**

To translate observations made in rodent experimental models to the human setting, and promote allergy prevention by allergen exposure through breast milk, there is a need to know which maternal intervention will be the most successful for allergen shedding in breast milk. The existing, and still scarce, knowledge is reviewed here.

**Maternal exposure**

A correlation between the amount of egg ingested and OVA concentration in breast milk was demonstrated in 2 randomized controlled trials.19,21,23 However, about half the lactating mothers did not secrete allergen in breast milk, even after ingestion of a well-controlled amount of egg, peanut, milk, or wheat (Table I). What is even more surprising is that women consuming an egg-exclusion diet were as likely to have detectable egg allergens (OVA and ovomucoid) in breast milk as women consuming an unmodified healthy diet.18,20,23 This may be explained by accidental consumption of egg-containing foods or exposure to egg in the environment of the household. A great variability is also found in the kinetic of allergen shedding after its ingestion. In most cases, the peak concentration in breast milk is detected in the first 1 to 4 hours after allergen consumption17,27,30,31,33 and is then rapidly cleared. However, in some cases, the allergen was detected in milk only within 12 to 24 hours17,27,32 and persisted up to 1 week after ingestion.37 When mothers ingested peanut on an empty stomach, allergen in human milk was detected as early as 10 minutes after consumption.31 To date, there is no randomized controlled trial measuring HDM allergen concentration in human milk after controlled maternal exposure. A recent study revealed that *D. pteronyssinus* 1 levels in human milk were not associated with allergen’s levels in mother’s mattress.37 These
data are supported by another study of 255 mothers showing that *D. pteronyssinus* 1 levels in human milk are not related to common indoor sources of HDM exposure, such as the presence of a carpet at home and cleaning habits. No data are available on other respiratory allergens in breast milk.

**Gut-breast axis**

An enteromammary pathway has been described in rodents for gut plasma cells and microbiota that traffic from the maternal gut mucosa toward the mammary gland. This suggests that such a pathway could also happen for dietary antigen as well as for airborne antigens, which are mostly ingested. Although the correlation between maternal exposure and antigen level in human milk is poor, a study showed a correlation between cow’s milk β-lactoglobulin (β-LG) antigen levels in blood and milk. We therefore propose that factors affecting antigen digestion and absorption across the gut barrier are key in controlling the allergen concentration in human milk.

**Mammary gland permeability**

Mechanisms of antigen transfer across the mammary gland are poorly identified too. Protected transfer of proteins across the gut epithelium was shown for IgG-antigen immune complexes passage via the neonatal Fc receptor (FcRn). FcRn is also expressed in the human mammary gland epithelium, and antigen-IgG immune complexes may facilitate the transfer of circulating antigen-immune complexes from serum to the mammary gland. Sodium/potassium (Na/K) ratio is considered as a marker of mammary epithelial paracellular permeability, and the only study that correlated Na/K ratio in human milk to the levels of food antigens failed to find any association. Other factors such as local infection, that is, mastitis, may also influence mammary gland permeability and antigen transfer to milk, but this has not been addressed yet.

In conclusion, evidence is pointing out that maternal exposure is not the key parameter in determining allergen levels in human milk. This means that maternal exposure cannot be used as a proxy of antigen levels in milk. Importantly, it implies that more research in humans and animal models is needed to understand the mechanisms that govern antigen shedding into human milk to efficiently influence offspring immune trajectory. In particular, (multiple) data on allergen levels in breast milk should be gathered in randomized controlled trials that address maternal intake of allergens during breast-feeding. Importantly, interventions that may influence maternal allergen digestion and absorption may have a high impact on the impact of such intervention on allergen shedding in breast milk.

**WHICH PARAMETERS CONDITION IMMUNE TOLERANCE INDUCTION TO ALLERGEN SHED IN BREAST MILK?**

The limited efficacy of food allergy prevention by early introduction of allergens in infants’ diet highlights that ingesting an antigen is necessary but not sufficient to induce oral tolerance in early life. Work from 4 decades ago showed that feeding OVA antigen to neonatal rodents was not efficient in inducing immune tolerance and may even result in immune priming in contrast to findings on oral tolerance induction in adults. The efficacy of inducing oral tolerance to OVA via breast milk (as reviewed here above) suggests that antigen in breast milk may be more suitable to activate the newborn’s immune system than its direct oral administration. This section will uncover the current knowledge on specificities of antigen in breast milk and the accompanying bioactive compounds that will guide early-life immune system trajectories toward tolerance or immune priming.

**Nature of the allergen**

Data from rodent experiments indicated that transfer of deggregated human gamma-globulin, BSA, or OVA induced oral tolerance in offspring on their transfer through breast milk of naive mothers (and even if mothers are devoid of adaptive immunity). In contrast, peanut and cow milk require prior maternal immunity to these allergens to elicit protection in offspring on their transfer through breast milk. (The mechanisms for increased protection by maternal immunity are discussed here below.) The understanding of offspring immune priming by only a few picogram of HDM allergens in breast milk uncovered the key role of HDM protease. Protease activity in breast milk of mothers inhaling HDM was increased, which was responsible for gut epithelium barrier damage, IL-33 secretion leading to type 2 innate lymphoid cell activation, Th2 cells’ expansion, and defective oral tolerance induction. Preliminary human data support that the nature of the allergen that is shed in breast milk will dictate immune outcomes because exposure to OVA is associated with protection whereas exposure to HDM is associated with increased allergy risk.

Altogether, these data stress that if the presence of the antigen in breast milk is necessary to induce tolerance, it is not sufficient. Uncovering parameters that promote tolerance to allergens in breast milk, whatever their nature, is fundamental for developing strategies of allergy prevention through breast-feeding–induced tolerance. We will review here existing knowledge, and areas in need of research, on parameters that we expect to influence immune outcomes to allergens that are shed in breast milk.

**Minute dose of allergens in human milk**

A striking observation is the very low amounts of diet-derived antigens in human breast milk (picogram per milliliter to nanogram per milliliter), which results in a daily dose of maximum 1 μg for the breast-fed child (Table I). In contrast, solid food ingestion results in exposure to dietary antigen at levels at least 1 million times higher. For instance, β-LG is in the range of milligram per milliliter in cow’s milk, which makes a daily dose of 1 g/d for a child consuming 1 L of cow milk (Fig 2). The same applies for 1 egg, which contains approximately 2 g of OVA. One may hypothesize that the very low amount of external antigen in human milk may fit specific requirements of activation of the developing immune system. Accordingly, elegant studies in mice demonstrated that neonates are fully able to mount cytotoxic immune response toward a viral infection when exposed to a dose that is 10,000 times lower than in the adult, whereas an adult dose does not elicit protection. The induction of regulatory immune response in mice on transfer of only a few nanogram of allergen through breast milk further supports that the induction of regulatory immune responses in early life would also require only
very low amounts of antigens. The efficacy of food allergy prevention by early introduction of food allergens in the PETIT trial\(^6\) in which infants ingested 10 times less egg allergen as compared with other trials\(^8\) supports the concept of the need of very low amount of antigen for activation of mucosal immunity in early life.

These data highlight that the physiological dose to induce oral tolerance in early life may be extremely low. This may be particularly true when infants are exposed to antigens via breast milk where antigens have unique characteristics and are surrounded by multiple bioactive compounds as we will describe in the next paragraphs (Fig 2).

**Maternal digestion of allergens in human milk**

There is evidence from preclinical and clinical studies that gut protein digestion influences the generation of tolerogenic products. Peng et al\(^6\)\(^,\)\(^6\)\(^,\) showed decades ago in mice that intestinal generation of a tolerogenic form of OVA was time-dependent. The recent clinical association of antacid treatment and increased risk of allergic sensitization also points toward the role of gut digestion in the control of oral tolerance induction.\(^6\) In this regard, data showing that human milk is predigested within the mammary gland itself\(^6\)\(^,\)\(^6\) strongly suggest that this process might be key for the generation of tolerogenic peptides. Predigestion of allergens in breast milk may be especially important to compensate for the weak digestive ability of newborns.\(^6\)\(^,\)\(^6\) The balance of proteases and antiproteases in human milk may guide protein-specific digestion of proteins within the mammary gland.\(^6\)\(^,\)\(^6\) As a matter of fact, most of the \(\beta\)-LG in human milk was found as peptides\(^6\)\(^,\)\(^6\) and unique protein fragments of casein were detected.\(^6\) This contrasts with antigen exposure in formula-fed infant where most of cow’s milk antigens are intact unless hydrolyzed formula is used (Fig 2). Interestingly, the presence of antiprotease in human milk may also be critical for allergic outcomes in offspring because it may dampen the allergenic properties of HDM protease allergens.\(^6\)\(^,\)\(^6\)

The data summarized here suggest that the variability in allergen digestion by lactating mothers may be an important factor in the generation of tolerogenic versus immunogenic peptides. Future studies will need to address whether maternal intake of drugs that influence protein digestion such as antacid drug influences allergy outcome in offspring.

**Allergens in the presence of breast milk immune modulators**

Breast milk is a source of thousands of bioactive compounds that have the potential to influence infant immune development and immune reactivity to antigens transferred through breast milk.\(^8\)\(^,\)\(^1\)\(^,\)\(^2\) (Fig 2). These include molecules that will shape offspring’s microbiota and thereby shift immune reactivity to be more or less prone to tolerance as recently reviewed.\(^7\) A recent and elegant work in mice further demonstrated the key role of maternal specific IgA in breast milk in setting up gut ROR-\(\gamma\)+ Treg cells’ frequency across generations.\(^8\) Although no data are available in humans, studies in mice have identified a few factors to be especially important in instructing the immune response in offspring to antigens that are shed through breast milk:

- **Growth factors.** Studies have strengthened how breast milk complements the immune system of the neonate and provides immune modulators that are physiologically low in early life but required for tolerance induction. As an example, TGF-\(\beta\) is found in low amount in the gut of neonates\(^6\)\(^,\)\(^6\)\(^,\)\(^6\) and breast milk–derived TGF-\(\beta\) was essential to induce oral tolerance to egg antigen transferred to mice offspring through breast milk.\(^8\) Surprisingly, although TGF-\(\beta\) is known to promote the generation and maintenance of FoxP3 Treg cells, \(T_{H}1\) cells were induced and responsible for egg allergy prevention by OVA transfer through breast milk.\(^8\) A recent study in mice further suggested the importance of milk-derived insulin-like growth factor (IGF)-1 for oral tolerance induction to OVA in breast milk.\(^8\) The data on the role of TGF-\(\beta\) in human milk in allergy prevention are conflictual.\(^8\) However, no study so far

**Allergen peptides displayed on antigen presenting cells and extracellular vesicles in human milk**

**Cells.** Human milk contains about \(10^{5}\) to \(10^{6}\) cells/mL composed of epithelial cells, leucocytes, and stem cells.\(^6\)\(^,\)\(^6\)\(^,\)\(^6\) The proportion of leucocytes is especially high in colostrum where they reach up to 70% of milk cells.\(^6\) At later lactation stages, they decrease to a few percent and are replaced by epithelial cells that represent 98% of cells. Maternal milk memory lymphocytes were found to reach the offspring’s blood and tissues and to contribute to immune defense of the offspring against pathogens.\(^6\)\(^,\)\(^6\) In addition, multiple cells in milk express MHCII molecules and are endowed with antigen presentation capabilities such as dendritic cells, macrophages, and innate lymphoid cells that might play a significant role in inducing antigen-specific immune responses in the breast-fed child. Human milk–derived macrophages were shown to easily differentiate into dendritic cells and to efficiently activate T lymphocytes.\(^7\)

**Extracellular vesicles.** Human milk contains extracellular vesicles (EVs) possibly holding critical mediators for offspring immune system education. EVs are submicron-size vehicles released by cells for intercellular communication via selectively incorporated lipids, nucleic acids, and proteins.\(^7\)\(^,\)\(^7\)\(^,\)\(^7\) EVs are in higher abundance in early-stage milk as compared with mature milk.\(^7\) EVs’ origin in human milk is unclear, but proteomic analysis of milk EVs indicates they might originate from mammary epithelial tissue.\(^7\) They most probably also derive from immune cells in breast milk or from cells elsewhere in the body. Human milk EVs were found to express MHC-I and MHC-II molecules and to modulate \textit{in vitro} T-cell activation and promote FoxP3 regulatory T (Treg) cell expansion.\(^8\) The research on the factors that control milk EVs composition in human milk, and thereby their functional properties, is at its start. Nearly 2000 proteins have been detected in human milk EVs,\(^7\) and there is some evidence that maternal lifestyle influences their expression.\(^7\) The cellular origin of milk EVs is also expected to influence the composition of milk EVs. The higher percentage of immune cells in early-stage milk as compared with mature milk probably contributes to a higher expression of MHCII molecules on colostrum-derived EVs.\(^7\)

Although evidence exists on the presence of cells and EVs and their potential role in allergen presentation, currently there is a lack of knowledge on allergen peptides loaded onto milk EVs and cells and their contribution to the induction of allergen-specific immune response in offspring.
has addressed whether the risk of food allergy in children exposed to OVA through human milk varied with the accompanying levels of TGF-β.

**Vitamin A.** Retinol is maintained at a low level in utero for proper organ development, and the human newborn is vitamin A deficient if we compare its values to normal values in adults. Mouse experiments demonstrated that this physiological deficiency interferes with oral tolerance induction because of inefficient barrier function and deficient antigen presentation by dendritic cells. Maternal supplementation with vitamin A early postpartum proved to accelerate gut immune development in mice and to allow for oral tolerance induction from the first days of life. Only 1 clinical trial addressed whether vitamin A supplementation at birth would influence allergy susceptibility, and this study performed in Guinea-Bissau indicated an increased risk for atopy in girls supplemented with vitamin A at birth. Because of the environment and particularly the high prevalence of gastrointestinal infection in the study population, these data should be verified in a context with low gut inflammation. Furthermore, no study has addressed the importance of vitamin A levels in milk in the context of allergen shedding in milk on allergic outcome in offspring.

**Maternal immunity to transferred antigen strongly influences allergic outcome in offspring.** A recent report analyzed in detail the influence of maternal immune status on induction of protection against cow’s milk allergen sensitization on β-LG transfer through breast milk in mice. By using 2 different protocols for maternal immunization, the study showed that the levels of β-LG specific IgG in breast milk positively correlated with the inhibition of allergic sensitization in offspring and no protection was induced by the antigen transfer only. Similarly, maternal exposure to peanut during breast-feeding was able to inhibit the allergic response to peanut in offspring only when dams had been immunized but not if naive to peanut. Mechanistic studies demonstrated that OVA-IgG immune complexes in breast milk of immunized dams resulted in the induction of OVA-specific FoxP3+ Treg cells responsible for prolonged and profound tolerance to OVA in offspring. This appeared to result from intact transport of OVA across the gut barrier and an enhanced presentation by dendritic cells, both depending on the use of FcRn. Importantly, FcRn is expressed in human gut throughout life, and allergens bound to maternal IgG have been described in human milk for ovomucoid, gliadin, and peanut, suggesting a possible contribution of intestinal immune complexes in influencing allergy risk in breast-fed children as well.

**The influence of milk microbiota and associated metabolites on offspring immune trajectories to antigens in breast milk is a field that needs to be explored.**

There is growing evidence that oral exposure to allergens through breast milk dramatically differs from exposure through solid food or through environmental exposure (Fig 2). The amount of allergen, the presence of peptides that are free or associated with cell membranes, the existence of immune complexes, and the accompanying multiple and diverse immune modulators will all condition immune outcomes in offspring. Currently, there is a major gap in knowledge on factors controlling these parameters in human milk.

**CONCLUSIONS**

Every day, more than 350,000 babies are born and at least 80% will receive human milk during their first 2 years of life. However, no clear recommendation exists for a mother to increase the chance of allergy prevention through breast-feeding. The literature reviewed here highlights the importance that allergen shedding in human milk may have on allergy risk in offspring. It reveals the multiple breast milk factors that will influence immune outcomes and the need of research to fill major gaps in knowledge on what is controlling allergen shedding and how to ensure immune tolerance induction. We expect that filling these gaps will lead to the possibility of identifying subgroups of breast-feeding mothers where exposure to allergens will result in tolerance in offspring, or the opposite. Importantly, we anticipate that this research will instruct evidence-based maternal interventions that will contribute to alleviate the worldwide growing burden of allergic disease.

**REFERENCES**


