

ORIGINAL ARTICLE

Relationship between breast milk sCD14, TGF- β 1 and total IgA in the first month and development of eczema during infancy

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Keywords

breast milk; eczema; immunomodulatory factors; infant; sCD14; sensitization; TGF- β 1; total IgA

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Abstract

Introduction: The overall beneficial effects of breastfeeding for infants have been well documented, but its role in allergy prevention is controversial.

Objective: We investigated the relationship between breast milk immunomodulatory factors and subsequent development of eczema and atopic sensitization in the first year of life.

Methods: Day 7 and 28 breast milk samples were collected from mothers carrying infants at high risk of allergic disease. Aqueous-phase breast milk samples were assayed for TGF- β 1, sCD14 and total IgA. Infants were assessed for the presence of eczema and atopic sensitization at 12 months of age. The levels of breast milk TGF- β 1, sCD14 and total IgA were compared in infants who subsequently developed eczema and sensitization in the first year and those who did not.

Results: The levels of breast milk sCD14, total IgA, and TGF- β 1 at either day 7 or 28 were not associated with subsequent development of eczema or atopic sensitization during the first year of life.

Conclusion: Levels of breast milk immune parameters were not associated with eczema outcomes or sensitization in infants at 12 months. This suggests that apparent immunological effects on breast milk immunomodulatory factors may not necessarily lead to clinical benefits, and these immune markers may not be critical determinants of allergic disease in infancy.

The first few months of life represent a critical period in the development of the infant functional immune system. Exogenous sources of immunomodulatory substances such as from breast milk can support the maintenance of homeostatic balance between pathogen defence and tolerance induction at a time when the infant immune system is still immature. Breast milk contains numerous immunological components such as immunoglobulins, cytokines, chemokines, oligosaccharides and polyunsaturated fatty acids that can both promote protection against microbial infections and modulate the development and maturation of the infant mucosal immune system (1).

With the increasing rates of allergic disease particularly in developed countries, there is a growing interest regarding the

potential effects of breast milk in modulating immune responses. There are two plausible mechanistic pathways by which breast milk may protect against allergic disease, including direct immunomodulation by complex and possibly interrelated biological factors within breast milk, or indirectly via effects on bacterial colonization of the intestinal tract or through the introduction of potentially allergenic dietary antigens (2). The bioactive factors present in breast milk include the regulatory cytokine transforming growth factor- β (TGF- β), secretory immunoglobulin A (IgA) antibodies and the innate immune receptor soluble CD14 (sCD14). Breast milk TGF- β has been shown to initiate IgA production in newborns (3) as well as to induce mucosal tolerance in the presence of allergens (2). The potent immunoregulatory

properties of TGF- β in breast milk have been demonstrated in animal studies. TGF- β 1 knockout mice develop lethal inflammatory disease after weaning (4), and only survive for the first few weeks of life while breastfeeding (5), suggesting the importance of external TGF- β through maternal milk. Moreover, recent animal studies suggest that the copresentation of food or inhaled allergen together with immunomodulatory compounds within breast milk is sufficient to induce tolerance to that allergen (2). Secretory IgA in breast milk provides passive antimicrobial protection to infants, and allergen-specific IgA antibodies prevent allergen absorption from the gut and the activation of IgE production (6). Together with TLR4, soluble CD14 activates cells that lack CD14, such as epithelial cells and dendritic cells, to interact with lipopolysaccharide (LPS) (7). Thus, high levels of sCD14 in human breast milk may play an important role in facilitating LPS-induced activation as well as monitoring bacterial load of neonatal intestinal epithelial cells and therefore promoting Th1 responses and averting IgE production (8).

Breastfeeding has an established role in reducing the risk of infectious disease, but its precise role in the prevention of allergic disease remains controversial. Several large prospective birth cohort studies have shown that exclusive breastfeeding for at least 4 months protects against development of asthma during the first 4–8 yrs of life (9,10). In contrast, more recent prospective studies reported that breastfeeding does not confer a protective effect against development of atopic dermatitis and may instead be a risk factor for the disease (11, 12). Furthermore, recent meta-analyses did not find a protective effect for breastfeeding against development of atopic dermatitis after adjusting for known confounders (13). Similarly, high levels of IgA (14, 15) and TGF- β (16, 17) in breast milk were shown to confer protection against development of allergic disease in some studies, while others reported no significant association between breast milk TGF- β and the risk of atopic disease in childhood (18). The substantial variations in breast milk composition between individuals and genetic factors may influence the effects of breast milk on development of allergic disease and in turn contribute to these conflicting results. Nonetheless, some studies have suggested that breastfed infants of mothers receiving probiotic supplementation during pregnancy and breastfeeding have the greatest benefit from the treatment (19,20,21), and the beneficial effects in these breastfed infants were suggested to be related to increased breast milk TGF- β 2 levels in breastfeeding mothers treated with probiotics (21).

We have previously demonstrated that prenatal LGG administration to pregnant mothers during the last 4 wks of pregnancy modulated a number of immunomodulatory factors in breast milk, namely sCD14 and total IgA (22). In this study, we aimed to further explore the relationship of breast milk immune markers, sCD14, TGF- β 1 and total IgA, to subsequent development of eczema and sensitization in infants during the first 12 months of life. A secondary aim was to investigate whether maternal allergic status influences levels of sCD14, TGF- β 1 and total IgA in the breast milk.

Methods

Subjects and study design

This study was conducted as part of a randomised double-blind, placebo-controlled trial, evaluating the potential effects of prenatal probiotic *Lactobacillus rhamnosus* GG (LGG) supplementation for the prevention of infant eczema (Probiotic Eczema Prevention Study registered with Cochrane Skin Group www.nottingham.ac.uk/ongoingskintrials/, trial no. 36), as described in detail elsewhere (22). Briefly, 250 pregnant women carrying infants at high risk of developing allergic disease were recruited between February 2006 and February 2008 at antenatal clinics of the Mercy Hospital for Women and through community advertising in metropolitan Melbourne, Australia. Women were eligible for the study if they, their partner or a previous child had a history of doctor-diagnosed asthma, allergic rhinitis, food allergy or atopic eczema. Women were excluded from the study if they had immune deficiency, planned to move from the area before delivery, delivered before 37 wks gestation, or had been taking probiotics during the current pregnancy. Subjects were also excluded if infants had significant congenital anomalies or immune deficiency. Pregnant women were randomised to receive either capsules of 1.8×10^{10} colony-forming units (CFU) of probiotic LGG (Dicofarm SpA, Rome, Italy) or maltodextrin placebo once daily from 36 wks gestation until delivery. Maternal allergic disease was determined during an interview at enrolment, which assessed former and present symptoms of allergic disease such as eczema, allergic rhinitis, asthma and food allergy. The study was approved by the Human Research Ethics Committees of the Royal Children's Hospital and Mercy Hospital for Women. Written informed consent was obtained from all participants. Seventy-nine of the 250 mother/infant pairs recruited to the parent study were included in this study, and inclusion was based on the availability of paired day 7 and day 28 breast milk samples. Infants were followed prospectively to age 12 months. The aim of this study was to compare the levels of breast milk sCD14, total IgA and TGF- β 1 at day 7 and 28 in infants with eczema and in those without eczema, and in sensitized and non-sensitized infants during the first 12 months of life.

Clinical follow-up

As previously described (22), infants were evaluated at 3, 6 and 12 months for the presence of eczema via questionnaire and clinical assessment of visible eczema by a specialist allergy nurse or doctor. Eczema was defined according to the criteria of the modified version of the UK Working Party's diagnostic criteria for atopic dermatitis (23), namely, a history of itchy skin, scratching or rubbing plus at least three of the following: history of generally dry skin; history of skin rash affecting the flexures, cheeks or outer surfaces of the limbs; visible dermatitis at any study visit affecting the flexures, cheeks or outer surfaces of the limbs. Eczema severity was assessed using the Scoring Atopic Dermatitis (SCORAD). Skin prick tests (SPT) were performed on the back with house dust mite, cat, ryegrass

pollen, cow's milk, egg and peanut (Stallergens, Antony, France) at 12 months of age, and regarded as positive if the SPT wheal diameter was ≥ 3 mm larger than the negative control. We used 10% histamine chloride as a positive control, and glycerine-saline as a negative control. The infants were regarded as sensitized if they had at least one positive SPT to any tested allergen. IgE-associated eczema was defined as the presence of both positive SPT and eczema at any time during the first 12 months of life.

Sample collection and initial processing

Breast milk samples were collected at day 7 and day 28 post-partum. Breast milk was manually expressed into sterile polypropylene tubes by the lactating mothers at home at the end of a feed. The samples were placed immediately in a -20°C freezer where they were stored until delivery to the laboratory on an ice pack. Samples were then stored frozen at -80°C until analysed. Briefly, 1 ml of milk samples were thawed at room temperature and centrifuged at 450 G for 10 min at 4°C . The whey was removed and centrifuged at 1800 G for 30 min at 4°C . The aqueous milk phase was collected and used for analysis.

Measurement of sCD14 and TGF- β 1

Concentrations of TGF- β 1 and sCD14 in the aqueous-phase breast milk samples were measured in duplicate using commercial human TGF- β 1 and sCD14 DuoSet ELISA kits according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). For sCD14, samples were assayed at a dilution of 1:10,000; and for TGF- β 1, samples were measured undiluted. Recombinant human TGF- β 1 and sCD14 were used as positive controls, and the reagent diluent buffer provided by the manufacturer was used as a negative control. Seven-point standard curves using 1:2 serial dilutions were generated in the range of 2000–31.25 pg/ml for TGF- β 1 and 4000–62.5 pg/mL for sCD14. For TGF- β 1, samples were activated according to the manufacturer's instructions, by adding 1 N HCl for 10 min and neutralized with 1.2 N NaOH/0.5 M HEPES. The lower limit of detection for TGF- β 1 was 200 pg/ml and 250 ng/ml for sCD14. TGF- β 1 and sCD14 concentrations were determined based on standard curves generated by KCjunior v1.40.3 program (Bio-Tek Instruments, Winooski, VT, USA) using a four parameter equation and results reported in pg/ml for TGF- β 1 and $\mu\text{g}/\text{ml}$ for sCD14.

Measurement of total immunoglobulin A

Total IgA levels in breast milk were measured in undiluted aqueous-phase samples by nephelometry using an IM-MAGE[®] IgA kit (Beckman Coulter, Brea, CA, USA). Results were reported in g/l. The assay has an analytical range between 6.7 and 25,200 g/l with a sensitivity of 6.7 g/l and an inter-assay coefficient of variation (CV) of 5%. The level of total IgA in day 4 breast milk has previously been reported to be 1.0 g/l (24).

Statistical analysis

Data were analysed as continuous data and dichotomous data (detected or not detected). Continuous data were assessed using histograms, and skewed data were $\log_{10}$ -transformed. Before transformation, the values below detection limit were replaced by the detection limit divided by 2. The Student's *t*-test was used to analyse normally distributed continuous data, and Mann–Whitney *U*-test was used for skewed data. Categorical data were analysed using chi-squared test or Fisher's exact test where expected frequencies were ≤ 5 . Data are presented as arithmetic mean $\pm$ 1 standard error of the mean (s.e.m.) or geometric means with 95% confidence intervals (CIs) or median with inter-quartile range (IQR). Potential confounding factors such as mode of delivery, duration of breastfeeding, yoghurt and antibiotic consumption during pregnancy, household pets and number of siblings were explored by including each of these factors singly in the logistic regression model, along with treatment group allocation and maternal history of allergy. Analyses were performed using Stata 11 (Stata Corp. LP, College Station, TX, USA). A *p* value < 0.05 was considered statistically significant for all analyses. Statistical power calculations revealed that our sample size of $n = 54$ in the non-eczema group and $n = 25$ in the eczema group allows 80% power at a 0.05 significance level to detect a 36% and 19% difference between groups for IgA at day 7 and day 28, respectively, a 20% difference between groups for sCD14 at days 7 and 28, and a 32% and 24% difference between groups for TGF- β 1 at day 7 and day 28, respectively. Our sample size of $n = 48$ in the non-sensitized group and $n = 22$ in the sensitized group allows 80% power at a 0.05 significance level to detect a 34% and 25% difference between groups for IgA at day 7 and day 28, respectively, a 20% and 22% difference between groups for sCD14 at day 7 and day 28, respectively, and a 36% and 24% difference between groups for TGF- β 1 at day 7 and day 28, respectively.

Results

Characteristic of study subjects

Paired day 7 and day 28 breast milk samples were available from 79 mothers whose infants were followed up prospectively to age 1 yr. Sixty-four (81%) mothers were allergic and 15 (19%) were not. Of 79 infants, 25 (31.6%) had eczema, while 54 infants (68.3%) had no eczema at any time in the first 12 months of life. Skin prick testing was performed in 70 of 79 infants. Of 70 infants, 22 (31.4%) had at least one positive SPT against tested allergens and therefore regarded as sensitized (atopic), while 48 infants (68.6%) were SPT negative (non-atopic). Of 25 infants who had eczema over the first 12 months of life, 12 (48%) had IgE-associated eczema. Information on breastfeeding was available on 53 infants; 42 infants were exclusively breastfed for at least 4 months (13 in the eczema and 29 in the non-eczema group), 5 were breastfed for 1–3 months, and six infants were never breastfed. The mean duration of exclusive breastfeeding was 6.5 months, and total breastfeeding was 10 months. Table 1 illustrates the

Table 1 Descriptive characteristics of participating infants who did or did not develop eczema and atopic sensitization during the first 12 months of life

	No eczema (n = 54)	Eczema (n = 25)	p Value*	Not sensitized (n = 48)	Sensitized (n = 22)	p Value*
Male, n (%)	27 (50)	13 (52)	0.9	22 (45.8)	11 (50)	0.7
Caesarean delivery, n (%)	12 (22.2)	6 (24)	0.9	9 (18.8)	7 (31.8)	0.2
Maternal allergy, n (%)	41 (75.9)	23 (92)	0.09	35 (72.9)	21 (95.4)	0.03
Paternal allergy, n (%)	30 (55.5)	15 (60)	0.6	29 (60.4)	11 (50)	0.5
Both parents allergy, n (%)	20 (37)	13 (52)	0.2	19 (39.6)	10 (45.4)	0.6
Sibling/s with eczema, n (%)	36 (66.7)	16 (64)	0.9	36 (75)	10 (45.4)	0.06
Commercial probiotic intake in pregnancy, n (%)	5 (9.2)	1 (4)	0.4	2 (4.2)	2 (9)	0.4
Yoghurt consumption during pregnancy wk), (g/wk), median (IQR)	400 (25600)	400 (100750)	0.3 [‡]	400 (0600)	300 (37.5600)	0.7
Antibiotic consumption during pregnancy, n (%)	16 (29.6)	6 (24)	0.6	13 (27)	7 (31.8)	0.7
Duration of breastfed >6 months, n (%)	43 (79.6)	19 (76)	0.7	42 (87.5)	20 (90.9)	0.7
Exclusive breastfed at least 4 months, n (%)	29 (53.7)	13 (52)	0.1	26 (54.1)	10 (45.4)	0.3
No. children in family >2, n (%)	3 (5.5)	1 (4)	0.8	2 (4.2)	1 (4.5)	0.9
Dog keeping, n (%)	20 (37)	10 (40)	0.8	19 (39.6)	9 (40.9)	0.9
Cat keeping, n (%)	10 (18.5)	4 (16)	0.8	10 (20.8)	2 (9)	0.2
Cigarette exposure, n (%)	7 (13)	3 (12)	0.9	6 (12.5)	3 (13.6)	0.9
SPT positive, n (%)	10 (18.5)	12 (48)	0.009	0	22 (100)	0.00

*Pearson's chi-squared test.

[‡]MannWhitney *U*-test.

characteristics of the infants, including variables which are known to influence breast milk immune markers and eczema development. There was little evidence of differences between the infants who developed and did not develop allergic disease in the first 12 months of life.

Concentrations of total IgA, TGF- β 1 and sCD14 in breast milk of allergic and non-allergic mothers

There was a decline in the levels of sCD14, TGF- β 1 and total IgA measured from day 7 (early milk) to day 28 (mature milk), as shown in Table 2. We examined the effects of maternal history of allergic disease on the levels of breast milk immune parameters. We found that mothers with allergic disease (*n* = 64) had higher levels of sCD14 in their early milk compared with non-allergic mothers (*n* = 15) (median sCD14 16.74 vs. 13.74 μ g/ml, respectively; *p* = 0.05). A similar association was seen for total IgA, with allergic mothers having higher early milk total IgA levels than mothers without allergic disease (median IgA 0.78 vs. 0.51 g/l, respectively; *p* = 0.05). There were no associations between mature milk sCD14 or total IgA levels and maternal history of allergic disease. Maternal allergic status did not influence the levels of TGF- β 1 in early or mature breast milk.

The relationship between breast milk immune markers and allergic disease outcomes at 12 months

The levels of breast milk sCD14, total IgA and TGF- β 1 were compared in infants who subsequently developed eczema in the first year of life and those who did not. No significant

Table 2 Levels (median with 25th/75th percentiles) of breast milk immune markers at day 7 (early milk) and day 28 (mature milk)

Breast milk immune markers	Day 7 (n = 79)	Day 28 (n = 79)
Total IgA (g/l)	0.7 (0.50.9)	0.4 (0.30.6)
sCD14 (μ g/ml)	15.8 (12.119.3)	11.2 (8.213.4)
TGF- β 1 (pg/ml)	451.3 (338.0737.3)	200 (280.8410.4)

differences were found in the levels of breast milk sCD14, TGF- β 1 or total IgA between infants who had eczema at any time in the first 12 months of life (cumulative incidence) and those who never had eczema (Fig. 1). There were also no significant differences in the levels of breast milk sCD14, TGF- β 1 and total IgA between infants who had eczema at 12 months of age (point prevalence) and those who did not have eczema (Fig. 2). None of the analysed breast milk immunoactive factors were different between infants with or without atopic sensitization (data not shown). We were unable to perform a similar analysis for IgE-associated eczema as the number of infants with IgE-associated eczema was small (*n* = 12).

Similar results were obtained when we examined the relationship between breast milk immune parameters and eczema outcomes using logistic regression with adjustment for maternal allergy and study treatment (Table 3). Additional adjustment (singly) for paternal and sibling's allergic status, duration of breastfeeding, yoghurt and commercial probiotic consumption during pregnancy and breastfeeding, maternal

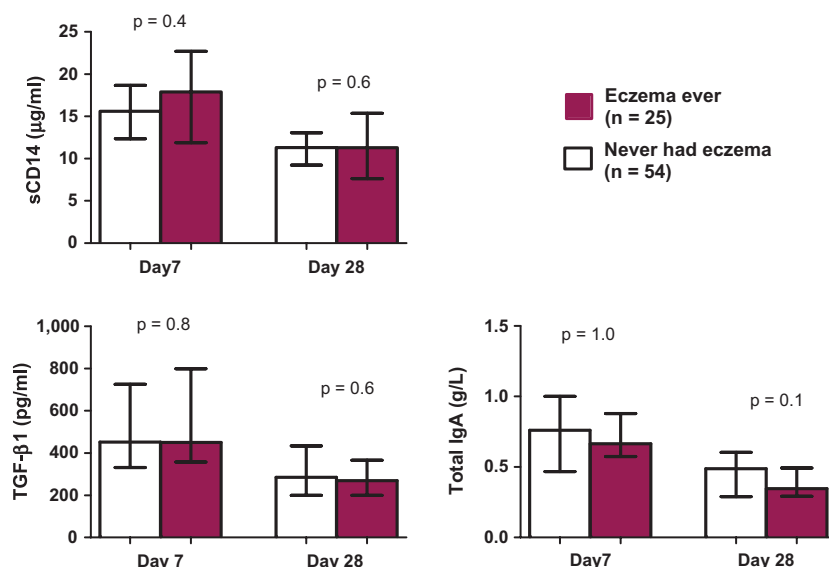


Figure 1 Comparison of maternal breast milk immune markers in infants who did or did not develop eczema in the first year of life. The levels of sCD14, TGF- β 1 and total IgA in early (day 7) and mature (day 28) milk from mothers whose infants developed eczema ever (red bars) and those who never had eczema (clear bars) during the first 12 months of life. The levels are shown as median with inter-quartile ranges, and groups were compared by Mann–Whitney *U*-tests.

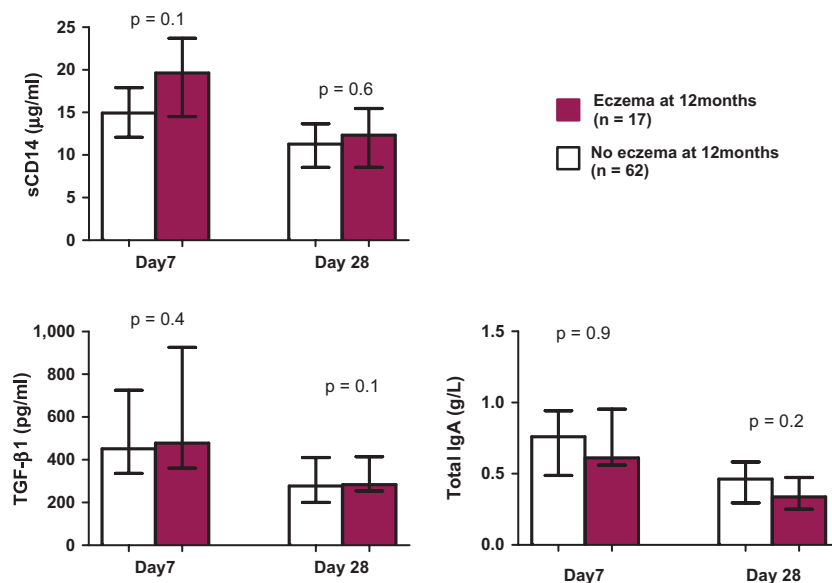


Figure 2 Comparison of maternal breast milk immune markers in infants who did or did not have eczema at 12 months of age (point prevalence). The levels of sCD14, TGF- β 1 and total IgA in early (day 7) and mature (day 28) breast milk from mothers whose infants had eczema (red bars) and did not have eczema (clear bars) at age 12 months. The levels are shown as median with inter-quartile ranges, and groups were compared by Mann–Whitney *U*-tests.

antibiotic use (indicating maternal infection) during breast-feeding, and number of siblings did not influence any of the findings (data not shown), signifying no significant association of these potential confounding factors with the immune markers in breast milk at 1 wk and 3 months post-partum (data not shown).

Discussion

Immunomodulatory factors in breast milk are thought to influence the development and maturation of the mucosal immune system of the infants (2). However, the relationship between breast milk immune factors and infant allergic disease

Table 3 Development of eczema in the first 12 months of life in relation to breast milk immune markers, in a logistic regression model controlling for possible confounders

Breast milk immune markers	No eczema (n = 54)	Eczema (n = 25)	p Value*	Adjusted p value†
Total IgA (g/l)				
Day 7	0.8 (0.51.0)	0.7 (0.60.9)	0.8	0.7
Day 28	0.5 (0.30.6)	0.3 (0.305)	0.2	0.1
sCD14 (μ g/ml)				
Day 7	15.6 (12.517.9)	17.5 (11.822.4)	0.4	0.7
Day 28	11.3 (9.012.9)	10.1 (7.614.8)	0.7	0.4
TGF- β 1 (pg/ml)				
Day 7	451.3 (330.4725.4)	450.4 (357.2798.7)	0.7	0.9
Day 28	284.9 (200433.6)	269.3 (200366.0)	0.7	0.6

Levels are shown as median with inter-quartile range.

*Groups were compared by MannWhitney *U*-test.

†Logistic regression analyses, adjusted for treatment group and maternal allergic status.

outcomes remains controversial. In addition, more recent studies suggest that probiotic effects on breast milk immunoactive factors may not be relevant to modulation of allergic disease outcomes. Previously, we showed that supplementation with LGG during the last 4 wks of pregnancy modulated immunoactive factors (sCD14 and total IgA, but not TGF- β) in breast milk (22). Nevertheless, LGG supplementation failed to modify the risk for development of eczema in the first year of life (23). In this present study, we investigated whether these breast milk factors were associated with development of eczema, as such an association had been suggested by previous studies (21, 25, 26). We found that breast milk sCD14 and total IgA concentrations were not associated with the development of subsequent allergic disease in the infants at age 1 yr. We also found no relationship between breast milk TGF- β 1 and allergic disease development. Nevertheless, maternal history of allergic disease was associated with higher levels of total IgA and sCD14 in early but not mature milk, while no difference was observed for TGF- β 1. These findings suggest that differences in breast milk immune factors may not be critical determinants of allergic disease.

Several studies have explored the relationship between levels of TGF- β , sCD14 and IgA in breast milk and the development of allergic manifestations in infants. A large prospective study demonstrated a significant reduction in infantile wheeze with high concentrations of TGF- β 1 in breast milk (17). Low concentrations of breast milk sCD14 were found to be associated with increased risk of developing allergic disease and/or atopic sensitization up to 4 yrs of age (18, 27, 28). Low levels of total IgA and/or cow's milk-specific IgA in breast milk are associated with an increased risk of later allergies and atopic sensitization in infants (14, 15, 18). In contrast, other studies have reported no association between levels of TGF- β 1 (29, 30), TGF- β 2 (29), sCD14 (30) and secretory IgA (29) in breast milk and development of atopic sensitization and/or allergic symptoms during the first 2 yrs of life. This is supported by a large birth cohort study involving more than 4000 infants that found no significant associations between TGF- β 1 or TGF- β 2 levels in colostrum and the risk of atopic sensitization or allergic disease by the age of 4 yrs, suggesting

that the effect of TGF- β may not extend beyond infancy (18). In addition, two recent studies reported no consistent associations between total and food-specific IgA levels in breast milk and development of allergic disease in children at age 5, 11 and 20 yrs (31, 32). Nevertheless, a systematic review by Oddy et al. (33) that included 12 cohort studies of infants at risk of developing allergic disease found an inverse association between breast milk TGF- β and allergy-related outcomes in infancy and early childhood. Our findings are consistent with previous studies (29, 30) that found no significant association between breast milk immune markers and allergy development in infants.

There are several plausible reasons that may explain these inconsistent findings. First, differences in definitions of allergic disease; the definitions of eczema/atopic dermatitis, atopic eczema/IgE-associated eczema and wheeze/asthma vary between studies, making a conclusive statement on the protective effect of immunoactive factors in breast milk difficult. Second, the concentrations of cytokines and other immune factors are known to vary with the course of lactation and also between individuals. Higher TGF- β 2 concentrations are demonstrated in the colostrum than mature milk (34), and lower levels of TGF- β 1 and TGF- β 2 are found in the breast milk of allergic mothers than mothers without allergy (35), although the largest study to date did not show any difference in these breast milk factors between allergic and non-allergic mothers (18). Third, the use of different assay methods may yield variability in the measured levels, contributing to the discrepant results between studies and preventing the identification of a putative protective level for these breast milk immune factors against allergic disease. Fourth, in our study, the majority of infants, regardless of atopic manifestation, were exclusively breastfed for at least 4 months and continued to be breastfed until the age of 12 months. Breastfeeding has been shown to modulate gut microbiota and hence infant immune responses (36). Taken together, our findings suggest that breast milk immune markers may not be a critical determinant of infant immune modulation.

Probiotics have recently been advocated for the prevention of allergic disease. A few of these studies have also examined

the effects of supplemented probiotics on immune markers in breast milk in an attempt to determine the mechanisms through which probiotics mediate their beneficial effects. However, the relationship between breast milk factors and allergic disease outcomes remains controversial. Recent probiotic studies revealed that changes in breast milk immune markers were associated with varied and inconsistent clinical effects (19, 21, 25, 26, 37, 38), suggesting that probiotic effects on breast milk immune factors may not be relevant to modulation of allergic disease outcomes. Previously, we have shown that prenatal supplementation with LGG to pregnant mothers can influence sCD14 and total IgA in breast milk, but failed to modulate TGF- β 1 (22). Here, we demonstrate that breast milk sCD14, TGF- β 1 and total IgA did not relate to later development of allergic disease. Taken together, our findings and those of others suggest that these breast milk immune factors may not be critical determinants of allergic disease in infants, and also that probiotic treatment can modulate immune markers in breast milk without necessarily modifying risk of eczema development or sensitization. This is consistent with a recent study reporting that while two different strains of probiotic *L. rhamnosus* HN001 and *Bifidobacterium lactis* HN019 both modulated breast milk TGF- β 1 and IgA (26), only one of these (HN001) conferred a protective effect against eczema (39).

It has been suggested that the specific titre of breast milk TGF- β concentrations are important for protective effects based on the observation that those studies that reported beneficial effects of TGF- β against eczema/allergic disease detected higher concentrations of breast milk TGF- β compared with the studies that found no associations (33). The median concentration of TGF- β 1 in our study was 451.3 pg/ml in early milk and 200 pg/ml in mature milk (Table 2) as opposed to the mean concentration of 787 pg/ml reported by Oddy et al. (17) that was associated with decreased risk of wheeze at age 1 yr. In addition, the median concentration of TGF- β 2 that was shown to confer protection against allergic disease was 3325 pg/ml in colostrum and 1644 pg/ml in mature milk (16). In contrast, Snijders et al. (30) who reported mean TGF- β 1 of 221 pg/ml found no significant association between levels of TGF- β 1 and allergic disease outcomes during the first 2 yrs of life. Therefore, the level of breast milk TGF- β may be an important factor for protective effects.

It has been speculated that higher concentrations of TGF- β are influenced by maternal allergy and use of probiotics (33). Our second aim was to assess whether milk levels of sCD14, TGF- β 1 and total IgA reflect the mother's allergic status. The role of maternal allergic status in influencing breast milk immunomodulatory factors remains controversial; several studies have documented lower concentrations of TGF- β 1 and TGF- β 2 in breast milk of allergic mothers compared with non-allergic mothers (35), while the largest study to date did not identify any significant association between maternal allergic status and breast milk immunomodulatory factors (30). Some studies reported lower levels of OVA-specific IgA in colostrum of allergic as compared with non-allergic mothers (40). In contrast, a large population-based study found no difference in the concentrations of either total or cow's

milk-specific IgA between atopic and non-atopic mothers (18). The concentrations of sCD14 were found to be lower in colostral samples of atopic mothers compared with non-atopic mothers using a definition of atopy based on a single symptom, but not when asthma or two or more symptoms were included (18). We found increased levels of total IgA and sCD14, but similar TGF- β 1, in breast milk of allergic mothers compared with non-allergic mothers, although the p values were only just significant (borderline) and the non-allergic group comprised a small sample size. Our findings are consistent with Snijders et al. (30) that reported higher levels of sCD14, but similar TGF- β 1, in breast milk of mothers with a history of allergy or sensitized mothers. These findings suggest that maternal allergic status may not be an important determinant in modulating breast milk immune markers and hence the development of allergic disease in recipient infants.

Our study has several strengths. First, we examined immunoactive factors in breast milk at two different time points as opposed to previous studies that collected only one milk sample (17, 18, 27, 28, 30). Second, we used a robust classification of allergic disease by the UK Working Party criteria and clinical examination performed by trained allergy specialist personnel. Third, data were analysed by adjusting for maternal allergic status, a factor that we and others have found can influence the levels of immunoactive factors in breast milk and also can influence infant allergic disease risk. We acknowledge that variability of breast milk collection by the participating mothers could have led to variation of the measured breast milk immune factors in the present study; however, mothers were provided with clear instructions on method of collection.

In conclusion, we found no evidence for a relationship between sCD14, TGF- β 1 and total IgA in breast milk and allergic manifestations in infants during the first 12 months of life, although we have previously found modulating effects of probiotics on breast milk immune markers. Studies involving larger sample size and a longer period of observation are warranted to confirm these findings. The results of the present and previous studies suggest that apparent immunological effects on breast milk immunoactive factors may not lead to clinical benefits, and these immune markers may not be critical determinants of allergic disease in children.

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