The Journal of Immunology

Role of Breast Milk in a Mouse Model of Maternal Transmission of Asthma Susceptibility

Adriana S. Leme, Cedric Hubeau, Yuhong Xiang, Alejandra Goldman, Kaoru Hamada, Yasue Suzaki and Lester Kobzik

References   This article **cites 40 articles**, 6 of which you can access for free at:
http://www.jimmunol.org/content/176/2/762.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision

- **No Triage!** Every submission reviewed by practicing scientists

- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription   Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions   Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts   Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

Role of Breast Milk in a Mouse Model of Maternal Transmission of Asthma Susceptibility[1]

Adriana S. Leme,* Cedric Hubeau,* Yuhong Xiang,* Alejandra Goldman,[†] Kaoru Hamada,[‡] Yasue Suzaki,[‡] and Lester Kobzik[2]*

Epidemiologic data suggest a link between nursing by asthmatic mothers and increased risk of allergy in babies. We sought to experimentally test the potential contribution of breast milk mediator(s) in a mouse model of maternal transmission of asthma risk by evaluating the effect of adoptive nursing on asthma susceptibility in the offspring. We measured airway hyperresponsiveness (AHR) and allergic airway inflammation (AI) after an intentionally suboptimal OVA Ag sensitization, tested the allergen independence of the maternal effect by using a second allergen, casein, for sensitization of the baby mice, and tested the potential role of cytokines by measuring their levels in breast milk. Offspring of asthmatic, but not normal, mothers showed AHR and AI, indicating a maternal transfer of asthma risk. After adoptive nursing, both groups (litters born to asthmatic mothers and nursed by normal mothers, and normal babies nursed by asthmatic mothers) showed AHR (enhanced pause after methacholine aerosol, 50 mg/ml, 3.7 ± 0.7, 4.2 ± 0.5, respectively, vs 1.1 ± 0.1 normal controls, $n = 25$, $p < 0.01$) and AI, seen as eosinophilia on histology and bronchoalveolar lavage ($40.7 \pm 4.5\%$, $28.7 \pm 3.7\%$, vs $1.0 \pm 0.5\%$ normals, $n = 25$, $p < 0.01$) after OVA sensitization. Similar results using casein allergen were observed. Multiplex assays for cytokines (IFN-γ, IL-2, IL-4, IL-5, TNF-α, and IL-13) in breast milk were negative. Breast milk is sufficient, but not necessary, to mediate allergen-independent maternal transmission of asthma risk to offspring. *The Journal of Immunology,* 2006, 176: 762–769.

S usceptibility to asthma may be increased by factors present in early life (1–3). As reviewed by Busse and Rosenwasser (4), several findings support the concept of the maternal-mediated skewing of fetal and early neonatal immunity toward Th2 responses. The evidence includes the requirement of increased Th2 and decreased Th1 cytokines for a successful pregnancy (5). The synthesis of Th2 cytokines at the placental interface (6) and the observation of increased production of Th2 cytokines by fetal or neonatal T cells (7, 8) also support this view.

Epidemiologic data suggest that maternal, more than paternal, asthma is a significant risk factor for the development of asthma and atopy in children (9, 10). Breast milk is one potential way in which a mother may contribute to asthma susceptibility transmission to the child. In general, it has beneficial effects on neonatal nutrition and immunity (11, 12). However, epidemiologic studies of breastfeeding have provided conflicting results about whether it is protective (13–15) or a risk factor (16–18) for the development of asthma and atopy in children.

A remarkable finding is that the risk of asthma of children born to asthmatic mothers increases with breastfeeding (19). Specifically, the rate of active asthma in atopic children of asthmatic mothers that never breastfed, of mothers that breastfed for <4 mo, or of mothers that breastfed for >4 mo was found to be 9, 24, and 46%, respectively. In contrast, the rate in children of normal moth-

ers was ~12% for all three categories of nursing status. Nevertheless, the effect of breastfeeding on the asthma susceptibility transmission remains controversial, possibly because of individual variations in the composition of the breast milk (20–22). Given this variability, it is possible that the milk of asthmatic mothers differs from that of nonasthmatic mothers, resulting in distinct effects of breastfeeding on offspring susceptibility. In fact, Bottcher et al. (21) found higher concentrations of IL-4 in colostrum from allergic than nonallergic mothers, and similar trends for IL-5 and IL-13.

We have developed a mouse model of maternal transmission of asthma risk using female mice sensitized as neonates and exposed repeatedly to aerosolized Ag, OVA (23). Their offspring showed increased susceptibility to OVA after an intentionally suboptimal sensitization with this allergen (a single i.p. injection) followed by aerosolized allergen challenge, a protocol which failed to induce allergic responses in babies born to normal mothers. The increased susceptibility was manifested as allergen-induced airway hyperresponsiveness (AHR),[3] pulmonary allergic inflammation (AI), and serum OVA-specific IgE, which are features that recall human asthma. It was also shown in this model that the maternal effect is allergen independent: offspring of mother mice sensitized with OVA have increased susceptibility also to a second, unrelated allergen, casein (Cs). This finding suggests that the maternal effect is not mediated by specific Abs or Ags transferred from the mother to the offspring, leaving cytokines and immune cells as potential mediators.

In the present investigation, we sought to experimentally test the potential contribution of breast milk mediator(s) to maternal transmission of asthma risk. For this purpose, we 1) evaluated the effect of adoptive nursing on asthma susceptibility in the offspring by

*Department of Environmental Health, Harvard School of Public Health, Boston, MA 02115; [†]Escuela de Ciencia y Tecnologia, Universidad Nacional de General San Martin, Buenos Aires, Argentina; and [‡]Department of Internal Medicine II, Nara Medical University, Nara, Japan

[2] Address correspondence and reprint requests to Dr. Lester Kobzik, Department of Environmental Health, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115. E-mail address: lkobzik@hsph.harvard.edu

[3] Abbreviations used in this paper: AHR, airway hyperresponsiveness; AI, allergic inflammation; Cs, casein; Penh, enhanced pause; BAL, bronchoalveolar lavage; CBA, cytometric bead array; AsMo, asthmatic mother; AsBa, asthmatic baby; NoMo, normal mother; NoBa, normal baby.

measuring AHR and AI after intentionally suboptimal OVA sensitization; 2) tested the allergen independence characteristic of the maternal effect by using a second allergen, Cs, for the suboptimal sensitization of the baby mice; and 3) tested the potential role of breast milk cytokines as mediators of the maternal effect. This was accomplished by measuring cytokine levels directly in breast milk samples and indirectly in milk-rich stomach contents, and by quantifying cytokine mRNA expression in breast tissue of nursing mother mice.

Materials and Methods

Animals

Newborn BALB/c mice were obtained commercially from Charles River Laboratories as litters with their mother mouse at day 4 of age or by in-house breeding, as described below. Each mother mouse with its respective litter was housed separately, fed a commercially pelleted mouse feed, and given water ad libitum. The mice were housed in an animal facility that was maintained at 22–24°C with a 12-h dark/light cycle. All experimentation was conducted under a protocol approved by our institutional review board.

Allergen sensitization and exposure

Maternal sensitization was accomplished by i.p. injections of 0.1 ml of PBS containing 5 μg of OVA and 1 mg of alum into mice at 5 and 9 days of age. After weaning, female mice were exposed to aerosols of OVA 3% (w/v) (OVA grade V; Sigma-Aldrich) in PBS (pH 7.4) for 10 min on 3 consecutives days at 4, 8, and 12 wk of age. The aerosol exposure was performed within individual compartments of a mouse pie chamber (Braintree Scientific) using a Pari IS2 nebulizer (Sun Medical Supply) connected to an air compressor (PulmoAID; DeVilbiss) (24). The efficacy of the protocol in creating allergic airway disease in the female (future mother) mice was evaluated after each of the aerosolized allergen challenges at 4, 8, and 12 wk of age (23). Immediately after the last aerosol exposure, the female mice were placed in cages with a normal male to allow mating. The mothers gave birth ~3 wk later, and the baby mice received a single i.p. injection of OVA and alum on day 4 of life (intentional suboptimal sensitization). On days 13–15, they were exposed to aerosolized OVA, as above. As previously observed, the suboptimal sensitization does not provoke any allergic response in babies born to normal mothers (23). Physiologic and pathologic analyses were performed on days 16 and 17, respectively. The experimental protocol is summarized in Fig. 1.

To test the effect of breast milk on the transmission of asthma susceptibility, some litters from asthmatic mother mice were adoptively switched within 48 h of birth for nursing to normal mothers who had just given birth. Similarly, the normal babies were adoptively switched for nursing to the asthmatic mother mice. The baby mice were then submitted to the suboptimal sensitization (above described) before aerosol challenge and evaluation 2 wk later. The groups of baby mice were coded according to two variables: the mother who nursed them (normal or "asthmatic"); and the mother from which they were born (normal or "asthmatic"). The coding system is summarized in Table I.

Similar protocols were used for studies with a second allergen, Cs (Sigma-Aldrich), in the suboptimal sensitization of the baby mice instead of OVA, followed by aerosol challenge with Cs (1%) for 20 min on days 13–15 and evaluation (days 16 and 17).

In some experiments, the litters were not adoptively switched as soon as they were born, but kept with their own mother for 3 days. On day 3 after birth, they were sensitized with a single i.p. injection of OVA (above described). One day later (day 4 after birth), litters from asthmatic mother mice were adoptively switched for nursing to normal mothers and vice

Table I. *Coding of experimental groups[a]*

Baby Mice	Breastfed by	Born from
NoMo/NoBa	Normal	Normal
AsMo/AsBa	Asthmatic	Asthmatic
NoMo/AsBa	Normal	Asthmatic
AsMo/NoBa	Asthmatic	Normal

[a] A summary of the coding system used to label different experimental groups is shown. The groups of baby mice were coded according to two variables: the mother who nursed them (normal or asthmatic), and the mother from which they were born (normal or asthmatic).

versa. Two weeks later, the babies were submitted to the OVA aerosol challenge and evaluation.

Pulmonary function testing

Airway responsiveness of baby mice to increasing concentrations of aerosolized methacholine was measured using whole body plethysmography (Buxco). Each mouse was placed in a chamber, and continuous measurements of box pressure/time wave were calculated via a connected transducer and an associated computer data acquisition system. The main indicator of airflow obstruction, enhanced pause (Penh), which shows strong correlation with the airway resistance examined by standard evaluation methods, was calculated from the box waveform (25). The use of Penh as a surrogate of airflow limitation is currently debated (26). Nevertheless, it has been reported that Penh strongly correlates with lung resistance in the BALB/c strain (27). Moreover, our analysis of very young animals precludes more invasive techniques.

After measurement of baseline Penh, either aerosolized PBS or methacholine in increasing concentrations (6, 12, 25, 50, and 100 mg/ml) was nebulized through an inlet of the chamber for 1 min, and Penh values for the first 5 min after each nebulization were averaged and used to compare results across treatment groups and individual mice.

Pathologic analysis

After physiologic testing, the baby mice were euthanized with sodium pentobarbital (Veterinary Laboratories), and the trachea was cannulated. Bronchoalveolar lavage (BAL) was performed five times with 0.3 ml of sterile PBS instilled and harvested gently. Lavage fluid (recovery ~90% of instilled) was collected and centrifuged at 4°C 1200 rpm for 10 min, and the cell pellet was resuspended in 0.25 ml of PBS. Total cell yield was quantified by hemocytometer. BAL differential cell counts were performed on cytocentrifuge slides prepared by centrifugation of samples at 800 rpm for 5 min (Cytospin 2; Shandon). These slides were fixed in 95% ethanol and stained with Diff-Quick (VWR), a modified Wright-Giemsa stain, and total of 100 cells were counted for each sample by microscopy. Macrophages, eosinophils, and lymphocytes were quantified.

After lavage, the chest wall was opened and the animals were exsanguinated by cardiac puncture for blood collection. Serum was prepared and stored at −20°C. The lungs were instilled with 10% buffered formalin, removed, and fixed in the same solution. After paraffin embedding, sections for microscopy were stained with H&E. An index of pathologic changes in coded H&E was derived by scoring the inflammatory cell infiltrates (eosinophils and mononuclear cells) around airways and vessels for greatest severity (0, normal; 1, <3 cell diameter thick; 2, 3–10 cells thick; 3, >10 cells thick) and overall extent (0, normal; 1, <10% of sample; 2, 10–25%; 3, >25%). The index was calculated by multiplying severity by prevalence, with a maximum possible score of 9.

Analysis of breast milk cytokines

In some experiments, normal mother mice and OVA-sensitized and exposed mothers were anesthetized with sodium pentobarbital (Veterinary Laboratories) on days 8–9 after birth to collect the breast milk. The mother mice were separated from the pups for 16 h before milk collection. To facilitate the collection, a single injection of 0.5 IU of oxytocin (Sigma-Aldrich) was given i.p. 5 min before milking. From each mouse 100–400 μl of milk was collected from several mammary glands. Some aliquots were centrifuged at $600 \times g$ for 5 min. The top lipid layer was discarded and the whey was collected and stored at −80°C until the evaluation of the cytokine levels.

The concentrations of IL-13 in the breast milk were detected by commercial ELISA kit (R&D Systems). The assay was performed according to the manufacturer's recommendation. The minimum detectable by this assay is 1.5 pg/ml. The levels of IFN-γ, IL-2, IL-4, IL-5, and TNF-α in the milk were measured by cytometric bead array (CBA) combined with flow cytometry (BD Biosciences), following instructions of the vendor. Its limit of detection is 1.0 pg/ml. IL-13 ELISA and CBA assays were performed in both the whey and the whole milk (aliquots that were not centrifuged). Positive control samples included normal milk to which known amounts of cytokines (either 25 or 50 pg/ml final) were added. Pilot studies where cytokines were added before or after separation of whey to test the effect, if any, of presence or absence of milk lipids showed no effect on the ability to detect the cytokines (data not shown).

Analysis of breast milk cytokines from baby stomachs

Normal babies nursed by normal mothers (NoMo/NoBa, Table I), and babies born to and nursed by asthmatic mothers (AsMo/AsBa, Table I) were euthanized with sodium pentobarbital (Veterinary Laboratories) on day 4

after birth without sensitization. Their milk-filled stomachs were harvested and homogenized in a solution of protease inhibitors (1 ml/200 mg of tissue; Protease Inhibitor Cocktail, Sigma-Aldrich), by using a tissue homogenizer (Power Gen 125; Fisher Scientific). The homogenates were centrifuged at 2000 rpm at 4°C for 10 min, and the supernatant was frozen at −20°C for posterior measurement of cytokine levels (28).

As described for assay of cytokines in breast milk, the concentrations of IFN-γ, IL-2, IL-4, IL-5, and TNF-α in stomach contents were determined by CBA combined with flow cytometry. Positive control samples included stomachs from normal babies to which known amounts of cytokines (25 pg/ml) were added before homogenization and collection of supernatants.

Analysis of cytokine mRNA expression in breast tissue

Normal and asthmatic mother mice breast tissue were harvested on days 3–5 after giving birth under anesthesia with sodium pentobarbital (Veterinary Laboratories). Samples from different mammary glands were collected and kept in RNA*later* solution (10 μl/mg tissue; Qiagen), a RNA stabilization reagent, at 4°C for a few days. Total RNA was extracted from 30-mg samples of the harvested breast tissue using an RNeasy Mini kit (Qiagen) as directed by the manufacturer. Total RNA (1 μg/sample) was reverse transcribed using M-MLV Reverse Transcriptase (Promega) at 37°C for 60 min.

Expression of mRNA for IL4, IL-5, and IL-13 was quantified using real-time RT-PCR. A 4-μl aliquot of the diluted cDNA mixture was amplified by real-time RT-PCR using iQ SYBER green Supermix (Bio-Rad) under the following conditions: 94°C × 2 min, 59°C × 1 min, 65°C × 1 min for 45 cycles in the iCycler iQ (Bio-Rad) real-time detection system. The mRNA expression for IL4, IL-5, and IL-13 were presented as copy numbers, which were calculated based on standard curves for the three cytokines measured.

The following primers were used: IL-4 forward primer, 5′-ACAG GAGAAGGGACGCCAT-3′, IL-4 reverse primer, 5′-GAAGCCCTACA GACGAGCTCA-3′; IL-5 forward primer, 5′-AGCAGTGGTGAAAGAG ACCTT-3′, IL-5 reverse primer, 5′-TCAATGCATAGCTGGTGATTT-3′; IL-13 forward primer, 5′-AGACCAGACTCCCCTGTGCA-3′, IL-13 reverse primer, 5′-TGGGTCCTGTAGATGGCATTG-3′.

Lung homogenate samples from OVA-sensitized and -challenged adult mice, where IL-4, IL-5, and IL-13 were previously detected, were used as positive controls. The quantification of the housekeeping gene β-actin in the breast tissue samples was also done to verify the conditions of tissue and RNA preparations.

Statistical analysis

Data are expressed as mean ± SEM. To test the significance between the different groups, one-way ANOVA with Newman-Keuls multiple comparison posttest was performed using GraphPad Prism version 3.0 for Windows (GraphPad Software). Statistical significance was accepted when $p \leq 0.05$.

Results

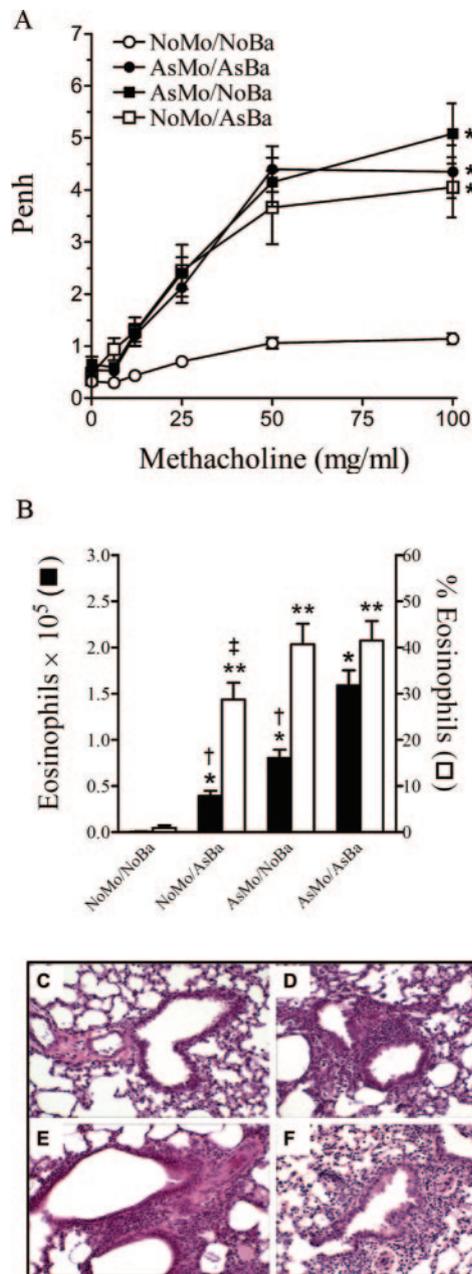*Nursing and susceptibility to OVA-induced allergic airways disease*

The basic protocol for these studies (summarized in Fig. 1) was performed on mice derived from either normal or OVA-sensitized/exposed mothers. We subjected the offspring from asthmatic or normal mice to an intentionally suboptimal sensitization with OVA (a single i.p. injection on day 4 after birth) before aerosolized allergen challenge on days 13–15 of life. The role of breast milk was tested by adoptive switching of litters shortly after birth (around 24–48 h, see below), resulting in an experimental design comprised of four major groups of baby mice. The coding system used to designate them is summarized in Table I.

Two groups represent the positive and negative controls for analysis of breast milk effects. The positive control group is made up of babies from asthmatic mother mice (AsMo/AsBa), which showed: 1) AHR to methacholine (increased Penh; Fig. 2*A*); 2) increased eosinophils on BAL (Fig. 2*B*); and 3) robust pathologic changes of AI (eosinophils and mononuclear cell infiltration around airways and vessels) (Fig. 2*C*). Results of semiquantitative scoring of histology support the qualitative changes illustrated in Fig. 2*D*, and are presented in Table II. These results contrast to those found in the negative control group, babies from normal mother mice (NoMo/NoBa), in which absence of AHR and AI was seen (Fig. 2).

After adoptive nursing, increased susceptibility to allergic airway disease was observed in babies from both groups: normal
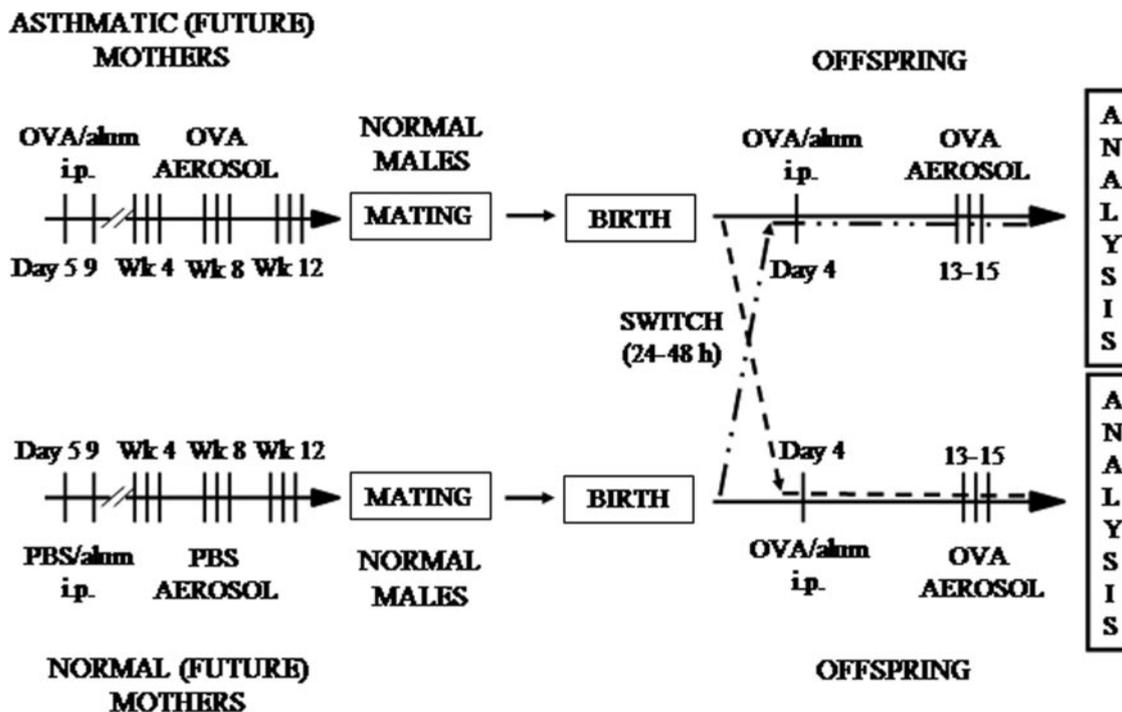
FIGURE 1. Schematic of main protocol. After birth, some litters from asthmatic mother mice (OVA sensitized and challenged before mating) were adoptively transferred on days 1–2 for nursing by normal mothers which had also just given birth, or vice versa. Baby mice received a single i.p. injection of OVA and alum (day 4), followed by exposure to aerosolized OVA on days 13–15 of life and analysis on days 16 and 17.

FIGURE 2. Nursing and maternal transmission of susceptibility to OVA-induced allergic airways disease. Babies from the groups AsMo/AsBa (●, n = 29), AsMo/NoBa (■, n = 25), and NoMo/AsBa (□, n = 23), but not NoMo/NoBa (○, n = 24) showed: *A*, increased AHR (Penh); *, p < 0.01 vs NoMo/NoBa at 12–100 mg/ml methacholine. *B*, Increased eosinophils (×10⁵) in BAL samples: *, **, p < 0.001 vs NoMo/NoBa; †, p < 0.01 vs AsMo/AsBa; ‡, p < 0.05 vs AsMo/AsBa and AsMo/NoBa. Histopathologic changes such as accumulation of eosinophils and mononuclear cells around airways and vessels were also observed in *D* (AsMo/AsBa), *E* (AsMo/NoBa), and *F* (NoMo/AsBa), but not in *C* (NoMo/NoBa); original magnification, ×200. See also scoring of changes in Table II.

baby mice suckled by asthmatic mothers (AsMo/NoBa), and babies of asthmatic mothers breastfed by normal mice (NoMo/AsBa). These groups showed: 1) AHR to methacholine (increased Penh; Fig. 2*A*); 2) increased eosinophils on BAL (Fig. 2*B*); and 3) intense pathologic changes of AI (Fig. 2, *C–F* and Table II). Control switches of babies between asthma mothers did not disrupt their increased susceptibility (data not shown). Similarly, switches between normal mothers also did not change the negative results

Table II. *Quantitative analysis of histopathologic changes in babies sensitized and exposed to OVA[a]*

Group	n =	Inflammation Index
NoMo/NoBa	12	0
AsMo/AsBa	14	2.68 ± 0.28*
NoMo/AsBa	6	3.75 ± 1.03*
AsMo/NoBa	11	2.36 ± 0.41*

[a] An index of pathologic changes in coded H&E slides was derived by scoring the inflammatory cell infiltrates around airways and vessels for greatest severity (0, normal; 1, <3 cell diameter thick; 2, 3–10 cells thick; 3, >10 cells thick) and overall extent (0, normal; 1, <10% of sample; 2, 10–25%; 3, >25%). The index was calculated by multiplying severity by prevalence, with a maximum possible score of 9. *, p < 0.01 vs NoMo/NoBa.

(data not shown), indicating that merely switching the babies does not cause a nonspecific effect. These data suggest that breast milk contains factors which are sufficient, but not necessary, to increase susceptibility of offspring to development of allergic airway disease in this model.

Susceptibility to respiratory allergy to a second allergen

To more directly test the role of specific allergen and/or Ab in maternal transfer of susceptibility, we modified the previous protocol (Fig. 1) by replacing the OVA allergen used for sensitization and challenge of baby mice with an unrelated allergen, Cs, which is a bovine protein Ag. In this way, we tested the response of babies born to OVA-allergic and -exposed mothers, to sensitization with a single i.p. injection of Cs, followed by challenge with Cs aerosols (days 13–15) and evaluation (days 16 and 17). Babies from OVA-asthmatic (AsMo/AsBa) and adoptively nursed groups (AsMo/NoBa and NoMo/AsBa), but not normal (NoMo/NoBa), mother mice showed increased susceptibility to sensitization by the single i.p. treatment with Cs. This was manifested as: 1) AHR to methacholine (increased Penh; Fig. 3*A*); 2) increased eosinophils on bronchoalveolar lavage (Fig. 3*B*); and 3) characteristic pathologic changes of AI (Fig. 3, *C–F*). Results of semiquantitative scoring of histology support the qualitative changes illustrated in Fig. 3, *C–F*, and are presented in Table III. These data indicate that allergens and/or specific Abs are not responsible for the transmission of susceptibility through breast milk.

Effect of age at time of adoptive nursing on maternal transmission of asthma susceptibility

A few studies have described differences in cytokine levels in colostrum, but not in mature milk, from asthmatic compared with normal mothers (20–22). To test the postulate that there is a "susceptibility window" in the first 24–48 h after birth for the transfer of milk factors from mothers to offspring, we investigated the role of this early milk on the transmission of maternal effect. In these experiments, the litters were not switched as soon as they were born, but instead kept with their own mothers for 3 days. On day 3 after birth, the baby mice were sensitized with a single i.p. injection of OVA (as described above). One day later (day 4 after birth), some litters from asthmatic mother mice were adoptively switched for nursing to normal mothers. Similarly, the normal babies were adoptively switched for nursing to the asthmatic mother mice. Two weeks later, the babies were submitted to aerosol challenge with allergen and evaluation. Babies from OVA-asthmatic (AsMo/AsBa, the positive control), but not normal (NoMo/NoBa, the negative control), mother mice showed marked susceptibility to sensitization by the single i.p. treatment with OVA, manifest as: 1) AHR to methacholine (increased Penh; Fig. 4*A*); 2) increased

A

B
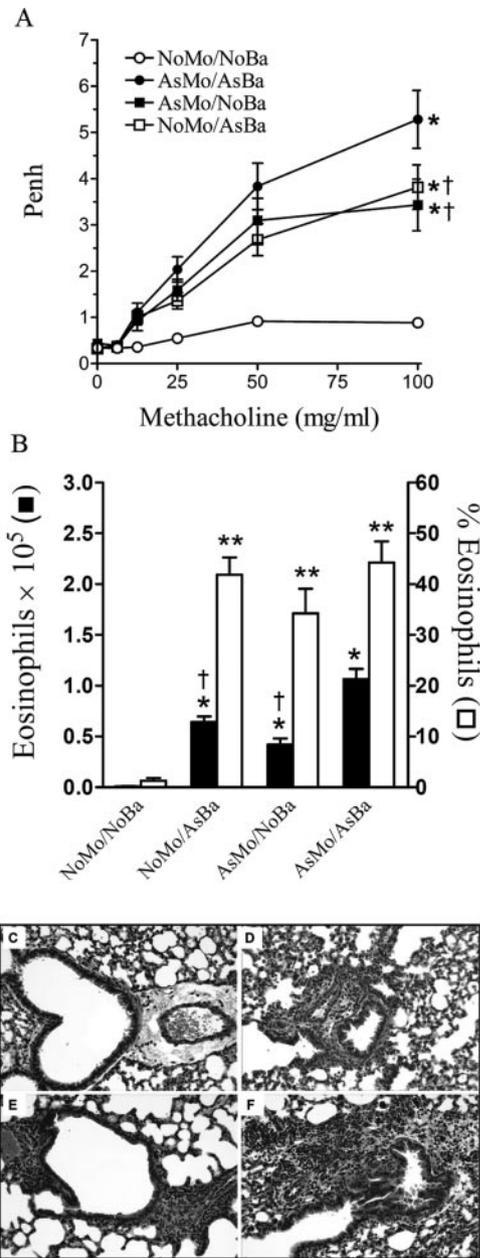
FIGURE 3. Nursing and allergen independence of maternal effect. Babies from the groups AsMo/AsBa (●, $n = 24$), AsMo/NoBa (■, $n = 30$), and NoMo/AsBa (□, $n = 26$), but not NoMo/NoBa (○, $n = 30$), after sensitization with a single i.p. injection of casein (Cs) at 4 days of age, and exposed to aerosols of Cs on days 13–15 of age, showed: *A*, increased AHR (Penh), *, $p < 0.01$ vs NoMo/NoBa at 12–100 mg/ml methacholine; †, $p < 0.05$ vs NoMo/NoBa at 12–100 mg/ml methacholine. *B*, Increased eosinophils (×10^5) in BAL samples: *, **, $p < 0.001$ vs NoMo/NoBa; †, $p < 0.05$ vs AsMo/AsBa. Accumulation of eosinophils and mononuclear cells around airways and vessels were also seen in *D* (AsMo/AsBa), *E* (AsMo/NoBa), and *F* (NoMo/AsBa), but not in *C* (NoMo/NoBa); original magnification, ×200. See also pathologic scoring changes in Table III.

eosinophils on bronchoalveolar lavage (Fig. 4*B*). The groups resulting from adoptive transfer (AsMo/NoBa and NoMo/AsBa) showed the same results as before: an increased susceptibility to the single injection of OVA, expressed as increased AHR and increased eosinophils on BAL (Fig. 4, *A* and *B*). Histopathologic evaluation also showed allergic inflammation in the positive control and adoptively transferred groups (Fig. 4, *C–F*). Semiquantitative scoring showed an increased inflammation index in the

Table III. *Quantitative analysis of histopathologic changes in babies sensitized and exposed to Cs*[a]

Group	$n =$	Inflammation Index
NoMo/NoBa	9	0
AsMo/AsBa	9	3.67 ± 0.58*
NoMo/AsBa	9	3.38 ± 0.59*
AsMo/NoBa	9	2.39 ± 0.45*

[a] An index of pathologic changes in coded H&E slides was derived by scoring the inflammatory cell infiltrates around airways and vessels for greatest severity (0, normal; 1, <3 cell diameter thick; 2, 3–10 cells thick; 3, >10 cells thick) and overall extent (0, normal; 1, <10% of sample; 2, 10–25%; 3, >25%). The index was calculated by multiplying severity by prevalence, with a maximum possible score of 9. *, $p < 0.01$ vs NoMo/NoBa

groups AsMo/AsBa (2.58 ± 0.27), NoMo/AsBa (3.08 ± 0.29), and AsMo/NoBa (0.75 ± 0.27) compared with NoMo/NoBa (0, $n = 6$, $p < 0.05$). These data indicate that colostrum does not play a special role in the transfer of the factor(s) responsible for the maternal transmission of asthma susceptibility, because mature breast milk received by baby mice after adoptive switching on day 4 after birth can also mediate this effect.

Analysis of breast milk cytokines

We used three related approaches to test the potential role of cytokines in milk to act as mediators of the maternal effect. We measured cytokine levels directly in breast milk samples by either ELISA or CBA methods and indirectly by analysis of milk-rich stomach contents, and we also quantified the cytokine mRNA expression in breast tissue of nursing mother mice.

Breast milk was collected from normal and OVA-sensitized and- exposed mother mice on days 8–9 after they gave birth. The concentration of IL-13 in the milk was measured by ELISA technique, while the levels of IFN-γ, IL-2, IL-4, IL-5, and TNF-α were measured by the CBA method. The levels of all the cytokines measured in breast milk are shown in Fig. 5*A*. These assays did not detect significant levels for any of the six cytokines, neither in the samples collected from normal mother mice, nor in the samples from OVA-asthmatic mothers. The positive control samples showed that the methods used were able to measure the cytokines.

In another approach, NoMo/NoBa and AsMo/AsBa baby mice were sacrificed on day 4 after birth without sensitization. Their stomachs were harvested and processed for measurement of cytokine levels. Because the only nutritional source of babies at this age is the mother's breast milk, it was assumed that the analysis of cytokines in their stomach contents reflects the milk levels of cytokines. The levels of cytokines (IFN-γ, IL-2, IL-4, IL-5, and TNF-α) in their stomach contents measured by CBA are shown in Fig. 5*B*. No significant levels for any of the five cytokines were measured in the samples from both NoMo/NoBa and AsMo/AsBa babies. The positive control samples again showed that the CBA technique was able to measure cytokines, if present, within these samples.

Breast tissue samples of normal and asthmatic mother mice were also collected. Total RNA was extracted and reverse-transcribed to cDNA. Expression of mRNA for IL4, IL-5, and IL-13 was quantified using real-time RT-PCR. Breast tissue harvested from both normal and asthmatic mother mice did not show significant mRNA expression for IL4, IL-5, and IL-13 (Fig. 5*C*). Detection of the mRNA of these cytokines in control samples (lung homogenates from OVA-sensitized and -challenged adult mice) where IL-4, IL-5, and IL-13 were previously detected (data not shown), validated the sensitivity of the technique. The quantification of mRNA for the housekeeping protein β-actin (Fig. 5*C*) in
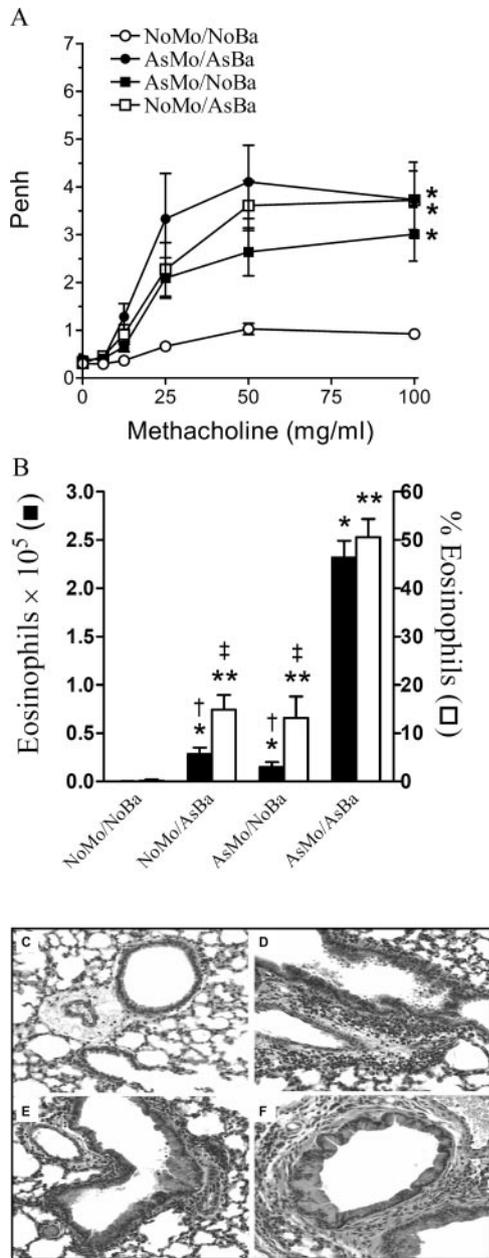
FIGURE 4. Effect of age at time of adoptive nursing on maternal transmission of asthma susceptibility. Baby mice subjected to adoptive switching for nursing 4 days after birth, AsMo/NoBa (■, $n = 21$), and NoMo/AsBa (□, $n = 22$) show: *A*, increased AHR (Penh), *, $p < 0.05$ vs NoMo/NoBa at 50–100 mg/ml methacholine. *B*, Increased eosinophils ($\times 10^5$) in BAL samples, *, **, $p < 0.05$ vs NoMo/NoBa; †, ‡, $p < 0.05$ vs AsMo/AsBa. Accumulation of eosinophils and mononuclear cells around airways and vessels were also seen in *D* (AsMo/AsBa), *E* (AsMo/NoBa), and *F* (NoMo/AsBa), but not in *C* (NoMo/NoBa); original magnification, $\times 200$.

breast tissue collected from either normal or asthmatic mother mice indicates that both tissue and RNA preparations were in appropriate conditions, also supporting our data.

Discussion

Epidemiologic studies suggest that maternal asthma is a significant risk factor for the development of asthma and atopy in children (9, 10). Although the literature remains controversial, there are data suggesting that the breast milk is one potential way in which a mother may contribute to asthma susceptibility (16–18). In the
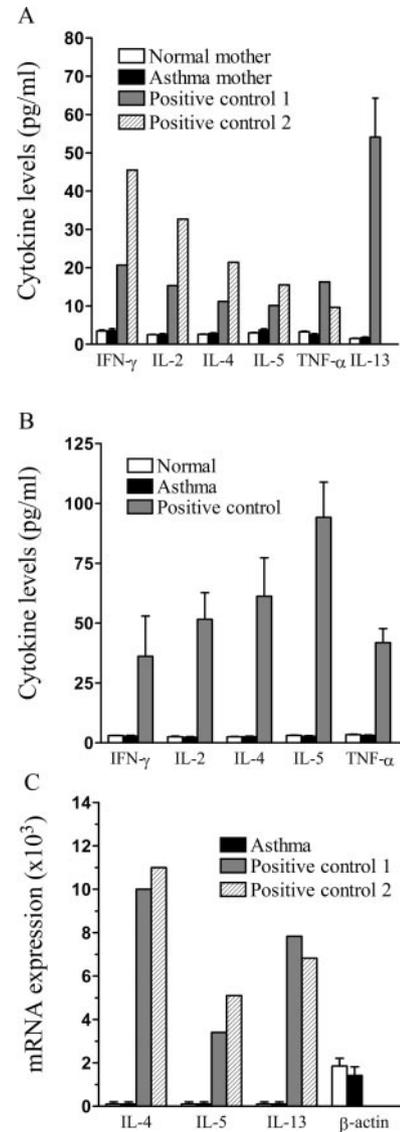
FIGURE 5. Analysis of breast milk cytokines. *A*, No significant levels of IFN-γ, IL-2, IL-4, IL-5, TNF-α, and IL-13 were detected in normal ($n = 7$) or asthmatic mother milk ($n = 6$), although these cytokines were detected in positive control samples (normal milk "spiked" with cytokines). *B*, No significant levels of IFN-γ, IL-2, IL-4, IL-5, and TNF-α were detected in homogenates of milk-laden stomach samples from offspring of normal ($n = 10$) or asthmatic mothers ($n = 17$). *C*, Both normal ($n = 5$) and asthmatic ($n = 5$) mother mice did not show significant mRNA expression for IL4, IL-5, and IL-13 in breast tissue samples as quantified using real-time RT-PCR. Cytokine mRNA was detected in positive control samples (lungs from OVA-sensitized and -challenged mice).

present investigation, we sought to experimentally test the potential contribution of breast milk mediator(s) in the maternal transmission of asthma risk. We used the mouse model previously developed in our laboratory, in which female mice are sensitized as neonates, exposed repeatedly to aerosolized OVA, and mated (23). In this model, their offspring show increased susceptibility to OVA after intentionally suboptimal sensitization with this allergen followed by aerosolized allergen challenge.

The major finding in the present study is that breast milk contains factors which are sufficient, but not necessary, to increase susceptibility of offspring to development of allergic airway disease. To test the role of breast milk on the transmission of asthma risk, the baby mice were adoptively switched between normal and

asthmatic mothers, and then nursed by the foster mother. AHR to methacholine, increased eosinophils on BAL, and pulmonary histopathologic changes were observed in normal baby mice breast-fed by asthmatic mothers, indicating that breastfeeding is sufficient for the transmission of asthma susceptibility. Our results also resemble data from an important epidemiologic study, in which the risk of asthma within the group of children born to asthmatic mothers increases with breastfeeding (19). Because the babies born to asthmatics and nursed by normal mothers also showed an increase in asthma susceptibility, we deduce that asthmatic breast milk is not the only way in which the transmission of the risk occurs. Another likely mechanism is the transference of immune mediators, e.g., cytokines or cells, such as lymphocytes, during intrauterine life. There is evidence that maternal leukocytes cross the placenta into fetal tissue, as reviewed by Papadogiannakis (29), and this represents an alternative mechanism for maternal influence on the developing immune system.

We demonstrated here that the nursing effect is allergen independent: offspring of mother mice sensitized with OVA present increased susceptibility also to Cs, a second, unrelated allergen. This finding indicates that the maternal effect is not mediated by specific Abs or Ags transferred from the mother to the offspring through the breast milk, leaving cytokines and immune cells as potential mediators. Nevertheless, the data do not exclude the possibility that Ag-specific immunity can also be transferred to the baby mice. In our study, the colostrum did not show a special role in the transfer of the factor(s) responsible by the maternal transmission of asthma susceptibility to the offspring, as previously observed in some epidemiologic studies (20–22). The mature breast milk received by the baby mice after adoptive switching at day 4 after birth caused the same effect as observed in babies switched in the first 24–48 h of life.

We initially postulated that cytokines present in the breast milk would have an important role in the observed transmission of asthma susceptibility, especially the Th2-related mediators such as IL-4, IL-5, and IL-13. To test this hypothesis, we used three related approaches: we measured cytokines in breast milk samples directly, indirectly by analysis of milk-rich stomach contents, and by quantification of cytokine mRNA expression in breast tissue of nursing mother mice. However, both the direct analysis of breast milk samples and the investigation of milk-rich stomach contents did not show significant levels of IFN-γ, IL-2, IL-4, IL-5, TNF-α, and IL-13, despite the presence of maternal asthma. In addition, breast tissue specimens harvested from both normal and asthmatic mother mice did not present significant mRNA expression for IL-4, IL-5, and IL-13 in our study. It is noteworthy that there is a large variation in the reported composition of breast milk in terms of cytokine content (20–22).

Because we were not able to detect cytokines in the breast milk of either normal or asthmatic mother mice in our model, two possibilities can be considered. First, the cytokines studied may be important but present in milk earlier than we were able to study due to technical limitations in sample volumes obtained. Because milk from asthmatic mothers at day 4 still has an effect on normal babies (Fig. 4), it is unlikely, but not excluded, that cytokine levels drop to undetectable levels from days 4 to 8, the time point when we harvested samples to increase yield. We also cannot formally exclude that cytokines present at levels below the detection sensitivity of our assays are sufficient to cause the effects seen, but consider this unlikely. The second possibility is that other mediators merit consideration. These include transfer of maternal leukocytes, which may influence the neonatal immune response (30–32). Another class of mediator(s) to consider is the fatty acids present in milk.

This speculation is based on reports correlating types of fatty acids present in breast milk and the development of asthma and atopy (33–35). The human breast milk is rich in long-chain polyunsaturated fatty acids, especially *o*-3 (such as eicosapentaenoic acid and docoexaenoic acid) and *o*-6 fatty acids (for example, γ-linoleic acid and arachidonic acid), which have immunomodulatory actions (36–38). However, the exact role of breast milk fatty acids on the development of allergies is not clear. Depending on their types and amounts presented in breast milk, the balance between Th1 and Th2 responses may change, either favoring or suppressing the development of atopy in the child. A higher ratio of *o*-6 to *o*-3 fatty acids in breast milk seems to favor an increase in the Th1 responses, whereas a decrease in the same ratio may skew the neonatal immunity toward Th2 responses (37, 39, 40).

In conclusion, the data indicate that breast milk contains factors which are sufficient, but not necessary, to increase susceptibility of offspring to the development of allergic airway disease in this mouse model. The nursing effect was shown to be allergen independent, suggesting that it is not mediated by specific Abs or Ags transferred from mother to offspring. Both colostrum and the mature breast milk seem to have the same importance on the maternal transmission of asthma susceptibility to the offspring. These experimental findings support the epidemiologic observation of increased risk of asthma in babies of nursing asthmatic mothers. The mediator(s) in milk responsible for increasing susceptibility to asthma remain to be identified.

Disclosures

The authors have no financial conflict of interest.

References

1. Gern, J. E., R. F. Lemanske, and W. W. Busse. 1999. Early life origins in asthma. *J. Clin. Invest.* 104: 837–843.
2. Holt, P. G., and C. A. Jones. 2000. The development of the immune system during pregnancy and early life. *Allergy* 55: 688–697.
3. Wright, A. L. 2004. The epidemiology of the atopic child: who is at risk for what? *J. Allergy Clin. Immunol.* 113: S2–S7.
4. Busse, W. W., and L. J. Rosenwasser. 2003. Mechanisms of asthma. *J. Allergy Clin. Immunol.* 111: S799–S804.
5. Wegmann, T. G., H. Lin, L. Guilbert, and T. R. Mosmman. 1993. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunol. Today* 14: 353–356.
6. Lin, H., T. R. Mosmman, L. Guilbert, S. Tuntipopipant, and T. G. Wegmann. 1993. Synthesis of T helper 2-type cytokines at the maternal-fetal interface. *J. Immunol.* 151: 4562–4573.
7. Warner, J. A., A. C. Jones, E. A. Miles, and J. O. Warner. 1996. Prenatal sensitization. *Ped. Allergy Immunol.* 7: 98–101.
8. Prescott, S., C. Macaubas, B. Holt, T. Smallacombe, R. Loh, P. Sly, and P. Holt. 1998. Transplacental priming of the human immune system to environmental allergens: universal skewing of initial T cell responses toward the Th2 cytokine profile. *J. Immunol.* 160: 4730–4737.
9. Ruiz, R. G., D. M. Kemeny, and J. F. Price. 1992. Higher risk of infantile atopic dermatitis from maternal atopy than from paternal atopy. *Clin. Exp. Allergy* 22: 762–766.
10. Litonjua, A., V. Carey, H. Burge, S. Weiss, and D. Gold. 1998. Parental history and the risk of childhood asthma: does mother confer more risk than father? *Am. J. Resp. Crit. Care Med.* 158: 176–181.
11. Hanson, L. A., M. Korotkova, L. Haversen, I. Mattsby-Baltzer, M. Hahn-Zoric, S. A. Silfverdal, B. Strandvik, and E. Telemo. 2002. Breast-feeding, a complex support system for the offspring. *Pediatr. Int.* 44: 347–352.
12. Brandtzaeg, P. 2003. Mucosal immunity: integration between mother and the breast-fed infant. *Vaccine* 21: 3382–3388.
13. Oddy, W. H., P. G. Holt, P. D. Sly, A. W. Read. L. I. Landau, F. J. Stanley, G. E. Kendall, and P. R. Burton. 1999. Association between breast feeding and asthma in 6 year old children: findings of a prospective birth cohort study. *Br. Med. J.* 319: 815–819.
14. Oddy, W. H., and J. K. Peat. 2002. Maternal asthma, infant feeding, and the risk of asthma in childhood. *J. Allergy Clin. Immunol.* 110: 65–67.
15. Kull, I. K., C. Almqvist, G. Lilja, G. Pershagen, and M. Wickman. 2004. Breast-feeding reduces the risk of asthma during the first 4 years of life. *J. Allergy Clin. Immunol.* 114: 755–760.
16. Bergmann, R. L., T. L. Diepgen, O. Kuss, K. E. Bergmann, J. Kujat, J. W. Dudenhausen, and U. Wahn. 2002. Breastfeeding duration is a risk factor for atopic eczema. *Clin. Exp. Allergy* 32: 205–209.
17. Sears, M. R., J. M. Greene, A. R. Willan, D. R. Taylor, E. M. Flannery, J. O. Cowan, G. P. Herbison, and R. Poulton. 2002. Long-term relation between

breastfeeding and development of atopy and asthma in children and young adults: a longitudinal study. *Lancet* 360: 901–907.

18. Miyake, Y., A. Yura, and M. Iki. 2003. Breastfeeding and the prevalence of symptoms of allergic disorders in Japanese adolescents. *Clin. Exp. Allergy* 33: 312–316.

19. Wright, A. L., C. J. Holberg, L. M. Taussig, and F. D. Martinez. 2001. Factors influencing the relation of infant feeding to asthma and recurrent wheeze in childhood. *Thorax* 56: 192–197.

20. Rudloff, S., T. Niehues, M. Rutsch, C. Kunz, and H. Schroten. 1999. Inflammation markers and cytokines in breast milk of atopic and nonatopic women. *Allergy* 54: 206–211.

21. Bottcher, M. F., M. C. Jenmalm, R. P. Garofalo, and B. Bjorksten. 2000. Cytokines in breast milk from allergic and nonallergic mothers. *Pediatr. Res.* 47: 157–162.

22. Bottcher, M. F., M. C. Jenmalm, and B. Bjorksten. 2003. Cytokine, chemokine and secretory IgA levels in human milk in relation to atopic disease and IgA production in infants. *Pediatr. Allergy Immunol.* 14: 35–41.

23. Hamada, K., Y. Suzaki, A. Goldman, Y. Y. Ning, C. Goldsmith, A. Palecanda, B. Coull, C. Hubeau, and L. Kobzik. 2003. Allergen-independent maternal transmission of asthma susceptibility. *J. Immunol.* 170: 1683–1689.

24. Rudmann, D., M. Moore, J. Tepper, M. Aldrich, J. Pfeiffer, H. Hogenesch, and D. Tumas. 2000. Modulation of allergic inflammation in mice deficient in TNF receptors. *Am. J. Physiol.* 279: L1047–L1057.

25. Hamelmann, E., J. Schwarze, K. Takeda, A. Oshiba, G. Larsen, C. Irviv, and E. W. Gelfand. 1997. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am. J. Respir. Crit. Care Med.* 156: 766–775.

26. Bates, J., C. Irvin, V. Brusasco, J. Drazen, J. Fredberg, S. Loring, D. Eidelman, M. Ludwig, P. Macklem, J. Martin, et al. 2004. The use and misuse of Penh in animal models of lung disease. *Am. J. Respir. Cell Mol. Biol.* 31: 373–374.

27. Adler, A., G. Cieslewicz, and C. G. Irvin. 2004. Unrestrained plethysmography is an unreliable measure of airway responsiveness in BALB/c and C57BL/6 mice. *J. Appl. Physiol.* 97: 286–292.

28. Rijneveld, A. W., S. Weijer, S. Florquin, P. Speelman, T. Shimizu, S. Ishii, and T. van der Poll. 2004. Improved host defense against pneumococcal pneumonia in platelet-activating factor receptor-deficient mice. *J. Infect. Dis.* 189: 711–716.

29. Papadogiannakis, N. 1997. Traffic of leukocytes through the maternofetal placental interface and its possible consequences. *Curr. Top. Microbiol. Immunol.* 222: 141–157.

30. Letterio, J. J., A. G. Geiser, A. B. Kulkarni, N. S. Roche, M. B. Sporn, and A. B. Roberts. 1994. Maternal rescue of transforming growth factor-β 1 null mice. *Science* 264: 1936–1938.

31. Goldman, A. S., and R. M. Goldblum. 1997. Transfer of maternal leukocytes to the infant by human milk. *Curr. Top. Microbiol. Immunol.* 222: 205–213.

32. Hawkes, J. S., D.-L. Bryan, and R. A. Gibson. 2002. Cytokine production by human milk cells and peripheral blood mononuclear cells from the same mothers. *J. Clin. Immunol.* 22: 338–344.

33. Duchen, K. 2001. Are human milk polyunsaturated fatty acids (PUFA) related to atopy in the mother and her child? *Allergy* 56: 587–592.

34. Reichardt, P., D. Muller, U. Posselt, B. Vorbeg, U. Diez, U. Schlink, W. Reuter, and M. Borte. 2004. Fatty acids in colostrum from mothers of children at high risk of atopy in relation to clinical and laboratory signs of allergy in the first year of life. *Allergy* 59: 394–400.

35. Dunstan, J. A., J. Roper, L. Mitoulast, P. E. Hartmann, K. Simmer, and S. L. Prescott. 2004. The effect of supplementation with fish oil during pregnancy on breast milk immunoglobulin A, soluble CD14, cytokine levels and fatty acid composition. *Clin. Exp. Allergy* 34: 1237–1242.

36. Thijs, C., A. van Houwelingen, I. Poorterman, A. Mordant, and P. van den Brandt. 2000. Essential fatty acids in breast milk of atopic mothers: comparison with non-atopic mothers, and effect of borage oil supplementation. *Eur. J. Clin. Nutr.* 54: 234–238.

37. Das, U. N. 2002. Essential fatty acids as possible enhancers of the beneficial actions of probiotics. *Nutrition* 18: 786–789.

38. Laiho, K., A.-M. Lampi, M. Hamalainen, E. Moilanen, V. Piironen, T. Arvola, S. Syrjanen, and E. Isolauri. 2003. Breast milk fatty acids, eicosanoids, and cytokines in mothers with and without allergic disease. *Pediatr. Res.* 53: 642–647.

39. Das, U. N. 2004. Perinatal supplementation of long-chain polyunsaturated fatty acids, immune response and adult diseases. *Med. Sci. Monit.* 10: HY19-HY25.

40. Stoney, R. M., R. K. Woods, C. S. Hosking, D. J. Hill, M. J. Abramson, and F. C. K. Thien. 2004. Maternal breast milk long-chain n-3 fatty acids are associated with increased risk of atopy in breastfed infants. *Clin. Exp. Allergy* 34: 194–200.