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BREAST MILK-MEDIATED TRANSFER OF AN ANTIGEN INDUCES TOLERANCE AND PROTECTION FROM ALLERGIC ASTHMA

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SUMMARY

Allergic asthma is a chronic disease characterized by airway obstruction in response to allergen exposure. It results from an inappropriate T helper (Th)-2 response to environmental airborne antigens and affects 300 million individuals. Its prevalence has dramatically increased in the recent decades most probably as a result from changes in environmental factors. Exposure to environmental antigens during infancy is critical for asthma development. Epidemiological studies on the relationship between breastfeeding, and allergic diseases have reached conflicting results. Here, we have investigated whether the exposure of lactating mice to an airborne allergen would impact asthma development in progeny. We found that airborne antigens are efficiently transferred from the mother to the neonate through milk and that tolerance induction does not require the transfer of immunoglobulins. Breastfeeding-induced tolerance relies on the presence of Transforming Growth Factor (TGF)-β during lactation, is mediated by regulatory $CD4^+$ T lymphocytes and depends on TGF-β signalling in T cells. In conclusion, breast milk-mediated transfer of an antigen to the neonate results in oral tolerance induction leading to antigen-specific protection from allergic airway disease. This study may pave the way for the design of new strategies to prevent the development of allergic diseases.

INTRODUCTION

Asthma and environmental factors

Asthma is a chronic respiratory disease characterized by reversible airway obstruction in response to specific and non specific stimuli. This lung inflammatory disease results from an inappropriate Th2 response against airborne antigens leading to pulmonary inflammation, airway eosinophilia, mucus hypersecretion and airway remodelling. The prevalence of asthma has increased steadily in the recent decades and is now one of the most common chronic diseases worldwide affecting around 300 million individuals (*Masoli* et al., 2004). The rapid increase in prevalence

of asthma is unlikely to result from genetic changes in populations but rather from changes in environmental factors such as exposure to pathogens, tobacco smoke, allergens, air pollution and changes in diet (*Devereux*, 2006; *Eder* et al., 2006). Furthermore, a large number of epidemiological studies have shown that exposure to environmental factors during infancy is critical for asthma development. Among these factors, exposure to airborne allergens may be of particular importance as allergen encounter is necessary for both sensitization in genetically prone individuals and for symptoms development in sensitized persons (*Holt* et al., 1999). This led to the hypothesis that reducing allergen exposure during infancy would lower the risk of being sensitized (*Arshad*, 2005; *Eder* et al., 2006; *Holt* and *Thomas*, 2005). This premise was tested in several allergen avoidance trials involving young children focusing on environmental control measures targeting a reduction in indoor allergen concentrations. Although allergen avoidance resulted in reduced symptoms in sensitized children, there was no convincing evidence that sensitization itself was reduced (*Arshad*, 2005; *Eder* et al., 2006; *Holt* and *Thomas*, 2005). In striking contrast, sensitization was actually increased in one study (*Woodcock* et al., 2004).

Breastfeeding and prevention of allergic disease

Breastfeeding is recognized as the main source of active and passive immunity in early life and is one of the most effective way of reducing death rate of children under five (*Brandtzaeg*, 2003; *Labbok* et al., 2004). Although the protective effect of breastfeeding on mortality is mainly observed in developing countries in which individuals are more exposed to infectious diseases, it is also beneficial for global health in developed countries. Many epidemiological studies have shown a protective effect of breastfeeding on asthma whether mothers were allergic or not (*Friedman* and *Zeiger*, 2005; *Gdalevich* et al., 2001; *Kull* et al., 2004; *van Odijk* et al., 2003). Although breast milk factors that are responsible for this protective effect have not yet been identified, it is noteworthy that breast milk contains high levels of immunosuppressive cytokines such as IL-10 and TGF-β (*Garofalo* et al., 1995; *Kalliomaki* et al., 1999; *Letterio* et al., 1994; *Oddy* et al., 2003; *Penttila* et al., 1998; *Saito* et al., 1993). Furthermore, epidemiological studies have shown a correlation between levels of TGF-β in breast milk and protection against wheeze and atopic dermatitis in breastfed children (*Kalliomaki* et al., 1999; *Oddy* et al., 2003). The presence of TGF-β in breast milk was also shown to prevent intestinal mucosa inflammation (*Penttila* et al., 2003) and to prevent allergy in allergic prone rat (*Penttila*, 2006).

Oral tolerance

In most cases, administration of antigen through oral route induces hypo-responsiveness to a subsequent challenge with the same antigen administered locally or systemically in an immunogenic form (*Mowat*, 2003; *Strobel* and *Mowat*, 2006). In animals, oral administration of autoantigen prevented and/or ameliorated various autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, and type 1 diabetes (*Mayer* and *Shao*, 2004). Oral tolerance was also protective in several animal models of allergy (*Keller* et al., 2006; *Mucida* et al., 2005; *van Halteren* et al., 1997) and in humans in the case of food allergy (*Patriarca* et al., 2003). Although the mechanisms underlying oral tolerance have not been entirely elucidated (re-

viewed in *Macpherson* and *Smith*, 2006; *Mayer* and *Shao*, 2004; *Mowat*, 2003), it is now clear that $CD4^+$ T lymphocytes play a critical role in this process and that several subsets of $CD4^+$ T cells, including TGF-β-producing Th3 cells, IL-10-producing Tr1 cells and naturally occurring $CD4^+$ $CD25^+$ T regulatory cells, could be involved. The mechanisms underlying oral tolerance have been extensively studied in animals, and more recently in children with cow's milk allergy (*Karlsson* et al., 2004). Several studies have shown that mesenteric lymph node (LN) dendritic cells (DC's) play a role in oral tolerance by promoting the differentiation of antigen-specific $CD4^+$ T regulatory cells (*Macpherson* and *Smith*, 2006; *Mowat*, 2003; *Strobel* and *Mowat*, 2006; *Worbs* et al., 2006). These DC's, that are likely to belong to the myeloid subset, probably originate from the lamina propria where they have acquired orally-administered proteins from intestinal lumen (*Strobel* and *Mowat*, 2006). Plasmacytoid DC's may also be involved in oral tolerance induction as suggested by accumulating evidence showing a cross talk between myeloid and plasmacytoid DC's in particular in intestinal mucosa (*Kuwajima* et al., 2006; *Lou* et al., 2007; *Yrlid* et al., 2006). Epithelial cells are also likely to play an important role in oral tolerance by interacting with neighbouring DC's (*Rimoldi* et al., 2005).

Immune response and oral tolerance in neonate

Early studies pioneered by Sir Peter Medawar have suggested that neonates are immunologically immature and prone to tolerance induction. Thus, neonates injected at birth with allogeneic splenocytes become tolerant and accept transplant from an allogeneic donor when they are adult. In contrast with these studies, several authors have recently shown that neonatal exposure to antigen can prime T cell responses and that inducing oral tolerance is more difficult in neonates than in adults (*Adkins* et al., 2004; *Hanson*, 1981; *Miller* et al., 1994; *Singh* et al., 1996; *Strobel*, 2001). For example, oral administration of Myelin Basic Protein (MBP) to rat neonates increased susceptibility to Experimental Autoimmune Encephalomyelitis (EAE) (*Miller* et al., 1994). Likewise, oral administration of OVA to mouse neonates increased delayed type hypersensitivity (DTH) and antibody responses in mice (*Hanson*, 1981; *Strobel*, 2001).

Breast milk as a link between mother environment and breastfed child

Exposure of lactating mothers to pathogens impacts the immune status of the breastfed child. Breast milk contains antibodies that react to infectious agents that were present in the mother's environment. This is an ingenious mean to confer passive immunity to the breastfed child against infectious agents that he is likely to encounter shortly after birth (*Brandtzaeg*, 2003). In other studies, breast milk was shown to contain food protein such as OVA (egg), bovine β-lactoglobulin (cow's milk), gliadin (wheat) and Ara h1 and Ara h2 (peanut) (*Palmer* and *Makrides*, 2006). Therefore, it has been proposed that mothers' diet influences the immune response of the breastfed child towards dietary antigens. To test this hypothesis, lactating mothers were submitted or not to an eviction diet and the breastfed children were followed for development of allergic diseases (reviewed in *Palmer* and *Makrides*, 2006; *Sicherer*, 2002; *Zeiger*, 2003)). Unfortunately, consistency among these studies has been lacking (*Chandra* et al., 1989; *Hattevig* et al., 1989, 1999; *Herrmann* et al., 1996;

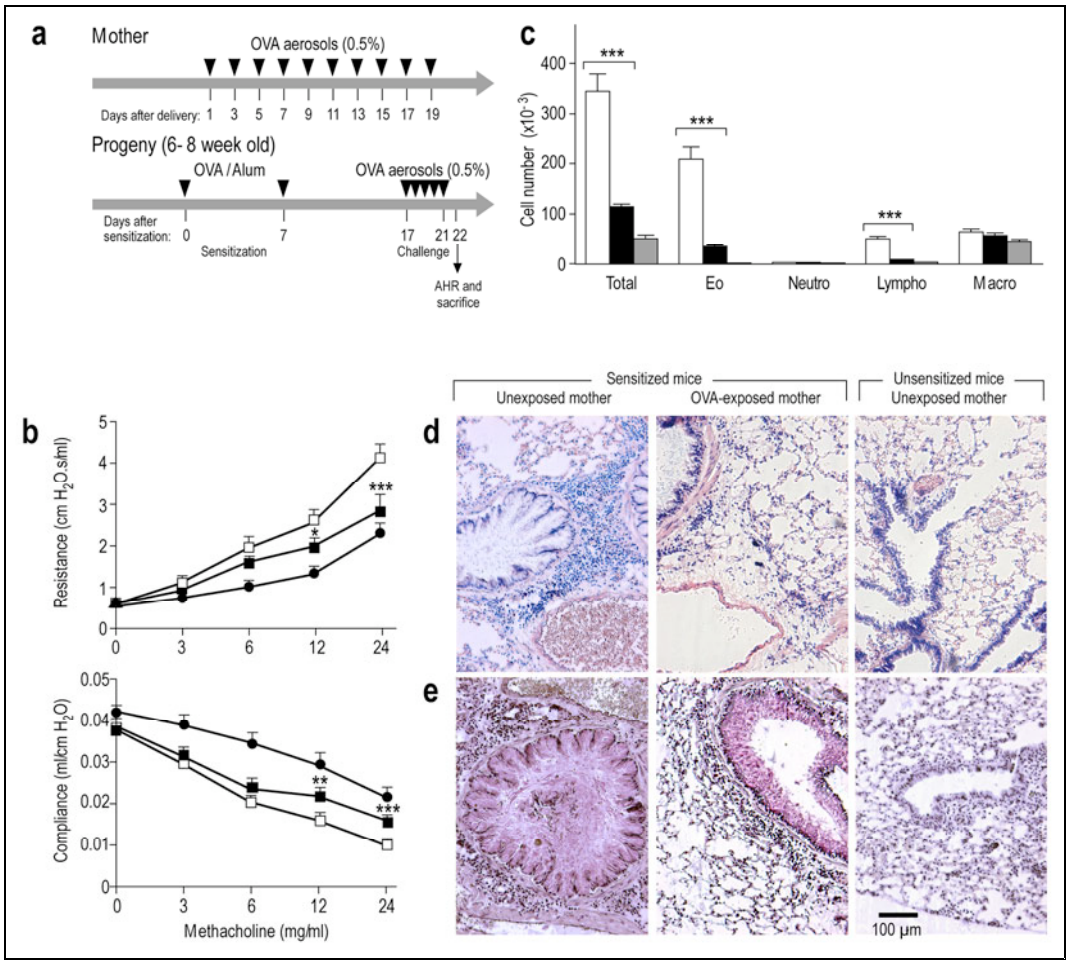

Figure 1: AHR and airway inflammation in mice breastfed on OVA-exposed mothers. (**a**) Experimental protocol. Lactating mothers were exposed or not to 0.5 % OVA aerosols during 20 min every other day from delivery until weaning. During aerosol exposure, pups were kept away from their mother. When 6-8 wk-old, offspring was sensitized with two injections of OVA in Alum, and challenged daily for 5 days with OVA aerosols. Mice were analyzed one day after the last aerosol. (**b**) AHR. Dynamic lung resistance and compliance were monitored in mice breastfed on OVA-exposed (filled squares) or unexposed mothers (empty squares) upon sensitization and challenge with OVA (n=6-7 mice per group in each experiment). Non-sensitized mice challenged with OVA (circles) were used as controls (n=3 in each experiment). Data are expressed as mean ± SEM of 2 independent experiments. **P*=0.03; ***P*=0.006 ****P*=0.0004. (**c**) Number and phenotype of BAL cells. BAL cells were analyzed by FACS in OVA-sensitized and challenged mice breastfed on OVA-exposed (black bars) and unexposed (empty bars) mothers. Non-sensitized mice challenged with OVA were used as controls (grey bars). Eosinophils, Eo; neutrophils, neutro; lymphocytes, lympho; macrophages, macro. Data are expressed as mean ± SEM of 5 experiments with n=6-8 mice per group for sensitized mice and of 2 experiments with n=5-6 mice for the non-sensitized group. ****P*<0.0001. (**d**, **e**) Histology of lung sections. Staining with May-Grünwald Giemsa (**d**) and PAS (**e**). Data show representative microscopic images at a 10-fold magnification.

Palmer and *Makrides*, 2006; *Sicherer*, 2002; *Zeiger*, 2003). Studies in rats and mice showed that oral administration of an antigen to the mother during lactation rendered the progeny tolerant towards that antigen as demonstrated by diminished immunoglobulins response and DTH (*Korotkova* et al., 2004; *Strobel*, 2001). Similar results were obtained in two studies in which an antigen was administered to the lactating mother through the parenteral route (*Fazekas de St Groth* et al., 1984; *Komatsu* et al., 1988).

AIM OF THE STUDY

The aim of this work was to understand how breastfeeding can confer protection towards asthma development. We formulated the hypothesis that an airborne antigen could be found in breast milk as described for dietary antigen and that the transfer of an airborne antigen to the neonate through breast milk could favour tolerance induction according to the numerous immunomodulatory factors presents in maternal milk.

METHODS

Mice

BALB/c mice and C57BL/6 mice were purchased from The Centre d'Elevage Janvier (France) and housed under SPF conditions. D0.11.10 TCR transgenic mice were provided from Dr. Fiona Powrie (University of Oxford). TGF-β DNRII (*Lucas* et al., 2000) were obtained from Dr. Lucas (NIH, USA), μMT and RAG-2-KO mice were obtained from the CDTA (France) and IL-10-deficient mice from Charles River (France). All non-transgenic mice used were on the BALB/c background unless indicated. TGF-β DNRII, μMT and IL-10-deficient mice were on the C57BL/6 background. In experiments using μMT and IL-10 deficient mice as foster mothers, adopted pups were wt BALB/c mice. In experiments in which TGF-βDNRII pups were adopted, mothers were wt C57BL/6.

Exposure of lactating mothers to antigen

Lactating mice were exposed or not to 0.5% OVA (Grade V, Sigma) aerosols for 20 min every other day starting 24 h after delivery until weaning using an ultrasonic nebulizer (Ultramed, Medicalia) connected to a 13000 cm^3 box that served as the deposition chamber for the mice (Figure 1a). Aerosols were given in groups of a maximum of 5-10 mothers. During aerosol exposure, mothers were separated from their progeny. Alternatively, lactating mothers received either intra-nasal or intra-gastric administrations of 0.1 mg and 0.5 mg of OVA, respectively, from delivery until weaning. OVA endotoxin content was determined using the QCL1000 chromogenic LAL kit assay (Cambrex). LPS content of OVA was below 10 ng/mg of protein. When indicated, mothers were treated with 1 mg of anti-TGF-β mAb (1D11 clone, ATCC) or isotype (GL113, rat IgG1, DNAX) twice a week from delivery until weaning. Upon anti-TGF-β treatment, TGF-β2 content in milk was partially reduced (20.7 ± 4.0 to 12.8 ± 4.9 ng/ml in isotype-treated and anti-TGF-β-treated mothers respectively; mean of 3

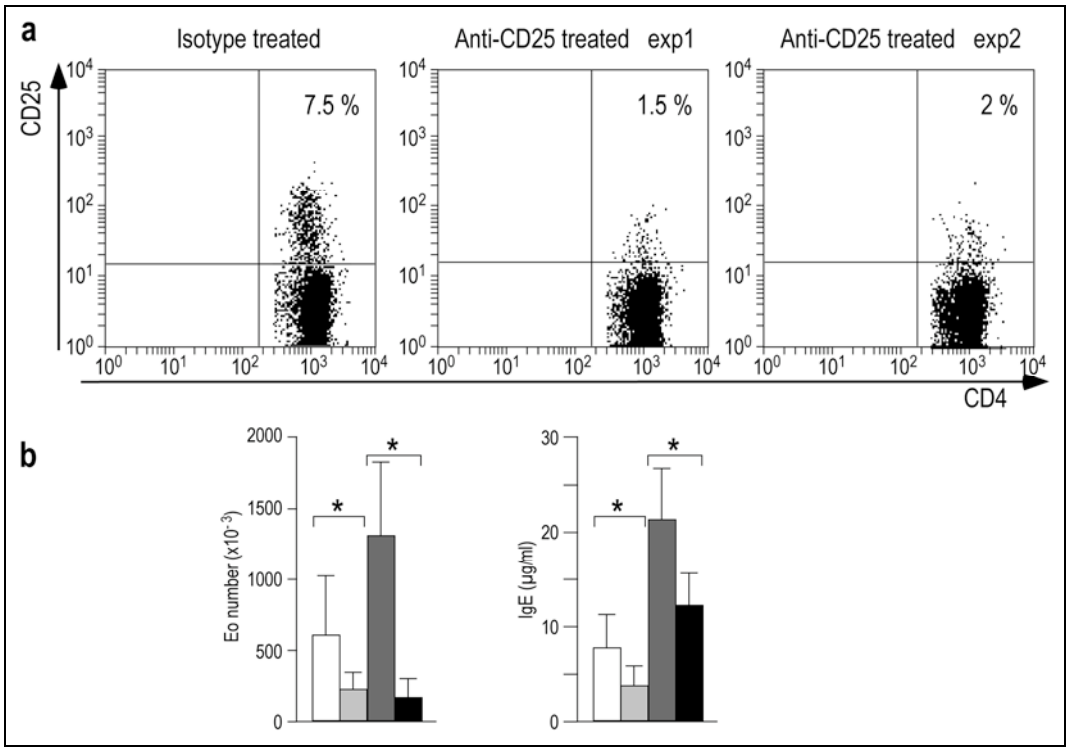

Figure 2: Role of $CD25^+$ cells in breastfeeding induced tolerance.
a: $CD25^+$ expression by $CD4^+$ T cells after anti-CD25 mAb administration. Spleens were collected one week after anti-CD25 mAb (PC61), or isotype (G113) administration. Cells were stained with anti-CD4, and anti-CD25 (7D4) mAbs and analyzed by FACS.
b: Breastfeeding-induced tolerance in anti-CD25 mAb~treated mice. 6-8 wk-old mice breastfed by OVA-exposed or unexposed mothers were treated with 0.5 mg of anti-CD25 mAb or control IgG 1 rat mAb. Mice were sensitized one week later and challenged with OVA. BAL eosinophil number and OVA-specific serum IgE levels are shown for isotype-treated mice breastfed by unexposed mothers (empty bars), or OVA-exposed mothers (light grey bars), anti-CD25-treated mice breastfed by unexposed mothers (dark grey bars) or OVA-exposed mothers (black bars). Data are expressed as mean + SD of values obtained in individual mouse for Eo and IgE and in pooled lung cells tor IL-13 secretion. n=5-7 mice per group, one representative experiment of two. *P=0.01.

independent experiments ± SD), and TGF-β-1 content in milk was reduced at least 6-fold (1.4±0.3 to 0.2±0.1 ng/ml; mean of 4 experiments ±SD). The administration of anti-TGF-β mAbs to lactating mothers also resulted in a partial depletion of TGF-β1 in newborn serum (56±13 to 26±12 ng/ml; mean of 3 experiments ± SD, n=14). TGF-β2 remained below the level of detection.

Induction of allergic asthma

Six to eight week old mice were used. Sensitization was performed by 2 i.p. injections of 10 µg of OVA in 2 mg of aluminium hydroxide (Alum) (Pierce) at day 0 and 7. From day 17 to day 21, mice were exposed to OVA (0.5%) aerosols for 20 min using an ultrasonic nebulizer (Ultramed, Medicalia) connected to a 13000cm^3 box that served as the deposition chamber for the mice (Figure 1a). When indicated, mice were injected with 0.5 mg of anti-CD25 mAb (PC61 clone, ATCC) or with rat IgG1 (GL113 clone, DNAX) one week before sensitization

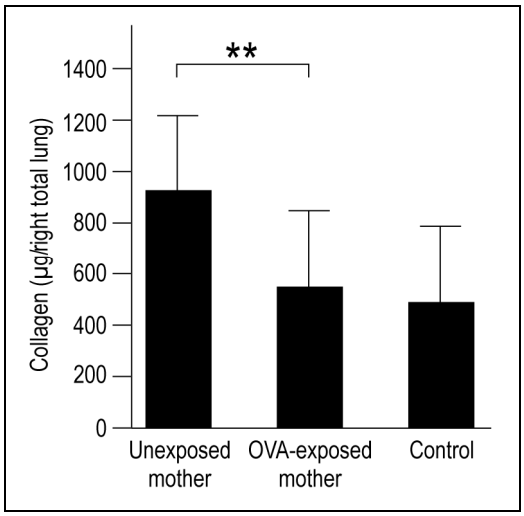

Figure 3: Collagen deposition in airways. Mice breastfed by OVA-exposed or unexposed mothers were sensitized with OVA in Alum and challenged with OVA aerosols (n=5-7 mice per group). Non-sensitized mice challenged with OVA aerosols were used as controls (n=3). One day after the last aerosol, lungs were collected and collagen content was assessed in right lungs. Data show means ± SD of two independent experiments. ***P*=0.005.

Treatment with anti-CD25 mAb resulted in a dramatic reduction in the frequency of $CD25^+$ $CD4^+$ T cells (1-2% versus 7.5%) (Figure 2a). In other experiments, mice were injected with 1 mg of anti-TGF-β mAb (1D11 clone, ATCC) or with rat IgG1 (GL113 clone, DNAX) one day before each sensitization. In some experiments, mice were sensitized with 10 µg LACK in 2 mg of Alum and further exposed to aerosols of 0.2% LACK as described (*Julia* et al., 2002). LACK was detoxified using an Endotrap column (Profos) according to the manufacturer's instructions to reach LPS amounts below 10 ng/mg of protein.

Airway hyperresponsiveness (AHR)

One day after the last aerosol, AHR was measured by invasive plethysmography (Emka Technologies) in response to inhaled methacholine (Sigma). For dynamic lung resistance and compliance, measurements were performed using a Flexivent apparatus (SCIREQ). Mice were anesthetized (5 ml/kg Dormitor 10 % (Medetomidine, Pfizer) - Imalgene 10% (Ketamine, Merial) tracheotomized, paralyzed (5 ml/kg Pavulon 1% (Pancuronium bromide, Organon) and immediately intubated with an 18-G catheter, followed by mechanical ventilation. Respiratory frequency was set at 150 breaths/min with a tidal volume of 0.2 ml, and a positive-end expiratory pressure of 2 ml H_2O was applied. Increasing concentrations of methacholine (0-24 mg/ml) were administered at the rate of 20 puffs per 10 seconds, with each puff of aerosol delivery lasting 10 ms, via a nebulizer aerosol system with a 2.5-4 µm aerosol particle size generated by a nebulizer head (Aeroneb, Aerogen). Baseline resistance was restored before administering the subsequent doses of methacholine.

Analysis of BAL cells

Mice were bled and a canula was inserted into the trachea. Lungs were

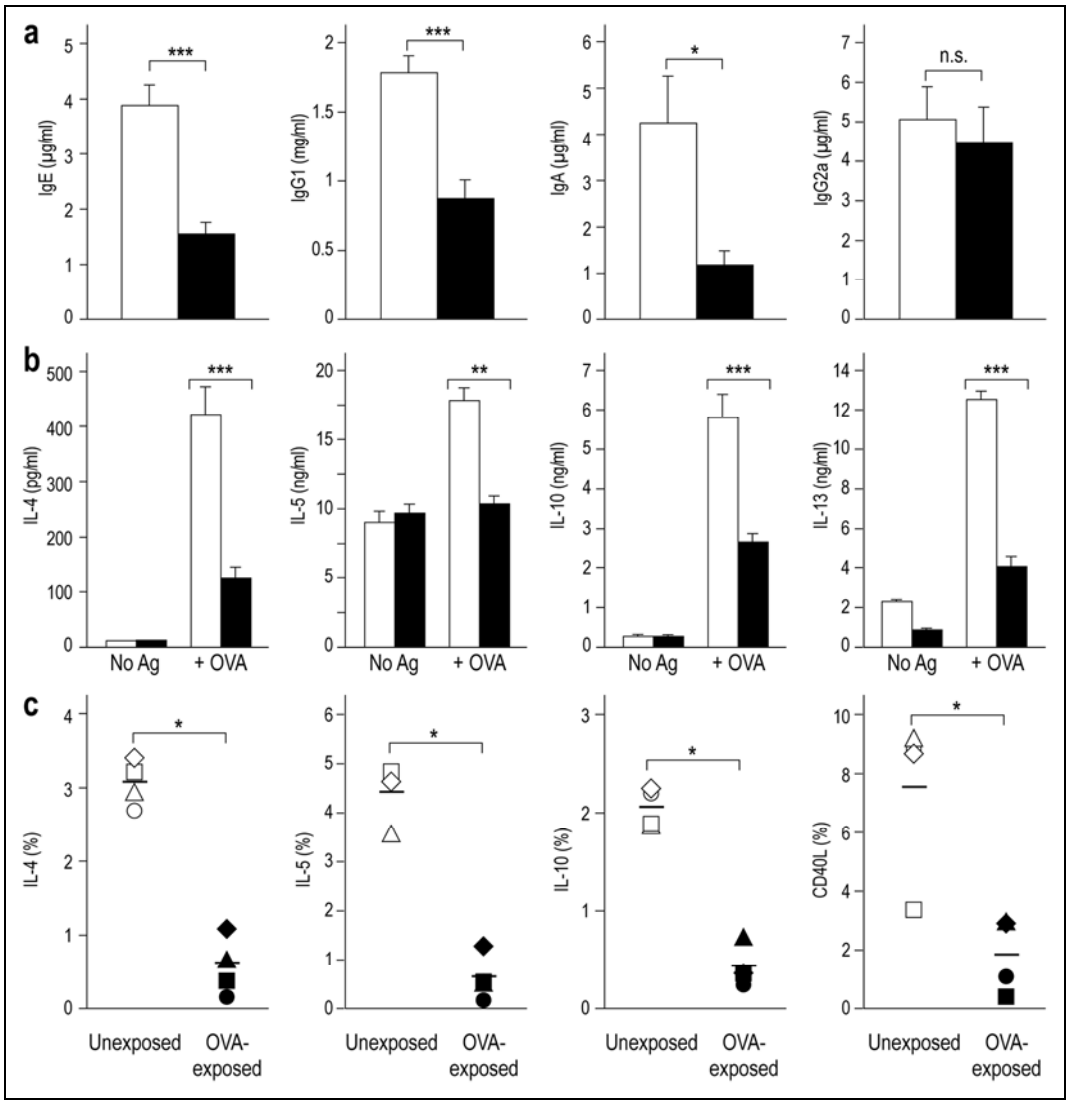

Figure 4: Immunoglobulin and T cell responses in mice breastfed on OVA-exposed mothers.
a: Serum levels of OVA-specific Ig. Sera from mice breastfed on OVA-exposed (filled bars) and unexposed (empty bars) mothers were analyzed for OVA-specific IgE, IgG1, IgA and IgG2a contents by ELISA. Histograms show the mean ± SEM of five independent experiments for IgE and IgG1 levels, and of two experiments for IgG2a and IgA levels with 6-8 mice per group. **P* = 0.01; ****P* <0.0001; ns *P*=0.4.
b: Cytokine secretion by lung cells. Lung cells of mice breastfed on OVA-exposed (filled bars) or unexposed (empty bars) mothers were pooled in each group (n=6-8) and cultured in triplicate with or without 100 µg/ml of OVA. Supernatants were analyzed 72 h later for IL-4, IL-5, IL-13 and IL-10 contents by ELISA. Data are expressed as mean ± SEM of 5 independent experiments. ***P*=0.004; ****P*=0.0005.
c: Frequency of cytokine-secreting and OVA-specific lung $CD4^+$ T cells. Lung cells of mice breastfed on OVA-exposed (filled symbols) or unexposed mothers (empty symbols) were pooled in each group (n=5-7) and incubated with OVA and anti-CD28 mAb. Data show the frequency of IL-4-, IL-5- and IL-10-secreting cells and $CD40L^+$ cells after gating on $CD4^+$ T cells in four independent experiments.**P*=0.02

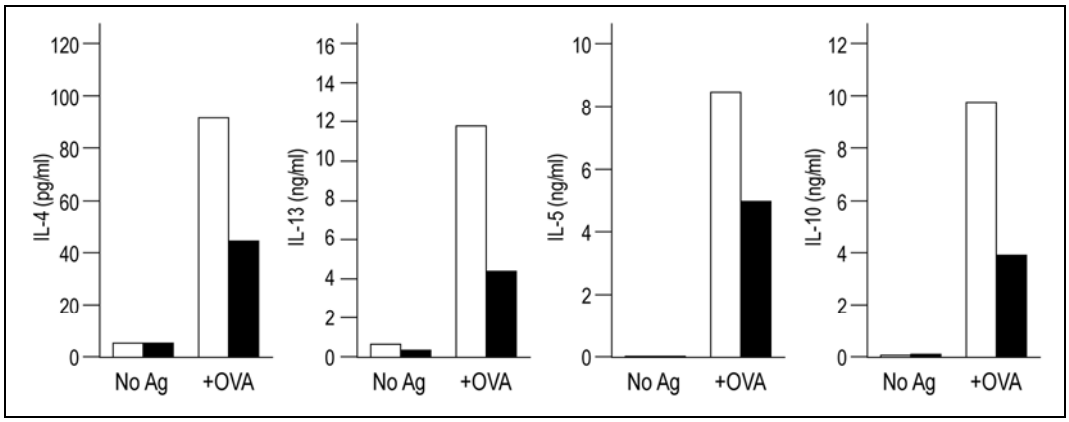

Figure 5: Cytokine secretion by mediastinal LN cells. Mice breastfed by OVA-exposed (filled bars) or unexposed (empty bars) mothers were sensitized and challenged with OVA. Pooled mediastinal LN cells were incubated in triplicate with or without OVA. Data show means of concentration of the indicated cytokine in supernatants of two independent experiments in which LN cells of each groups were pooled. n=5-7 mice per group.

washed 3 times with 1 ml of PBS. For differential BAL cell counts, cells were stained with mAb anti-CCR3 (R&D), anti-Gr1, anti-CD3 and anti-CD19 mAbs (Becton Dickinson**,** BD) and analyzed by FACS using a FACScalibur flow cytometer and Cellquest software. Eosinophils were defined as $CCR3^{+}$ $CD3^{-}CD19^{-}$, neutrophils as $Gr\text{-}1^{high}$ $CD3^{-}CD19^{-}$, lymphocytes as $CD3^{+}CD19^{+}$ and alveolar macrophages as large autofluorescent cells.

Serum antibody measurements

Serum OVA-specific or LACK-specific IgG1, IgG2a, IgA and IgE were measured by ELISA. For IgG1 quantification, antigen-coated Maxisorp plates (Nunc) were incubated with serial dilution of sera and biotinylated anti-IgG1 mAb (BD). For antigen-specific IgE, IgG2a, IgA, plates were first coated with the respective capture mAb (BD), and incubated with serum dilutions. Biotinylated-OVA or LACK antigen was then added. HRP-conjugated streptavidin (BD) and TMB (KPL) were used for detection.

Cytokine assays

One day after the last aerosol, lungs and mediastinal LN were removed separately, minced, and digested with collagenase I (Gibco) and DNAse (Roche) for 30 minutes at 37°C. Cell suspensions were filtered through a 70 µm cell strainer and depleted of red blood cells using red blood cell lysis buffer. Cells from each group were pooled and 4 X 10^6 lung and 1.5 X 10^6 LN cells were cultured in triplicate for 72 h in medium containing OVA (100µg/ml) or not in 48 well-plates, or 96 well-plates, respectively. Culture medium was RPMI 1640 (Gibco) containing 5% heat-inactivated FCS (Perbio), 50 µM β2-mercaptoethanol (Gibco), and penicillin/streptomycin (Gibco). Supernatants were analyzed by ELISA for IL-4, IL-5, and IL-10 using antibody pairs from BD and for IL-13 contents using kit from R&D Systems. Detection levels were 15 pg/ml (IL-4), 300 pg/ml (IL-5) and 150 pg/ml (IL-10 and IL-13). For intracellular staining, cells were incubated with 100 µg/ml OVA and 1 µg/ml of anti-CD28 (BD) for 6 h. Brefeldin A

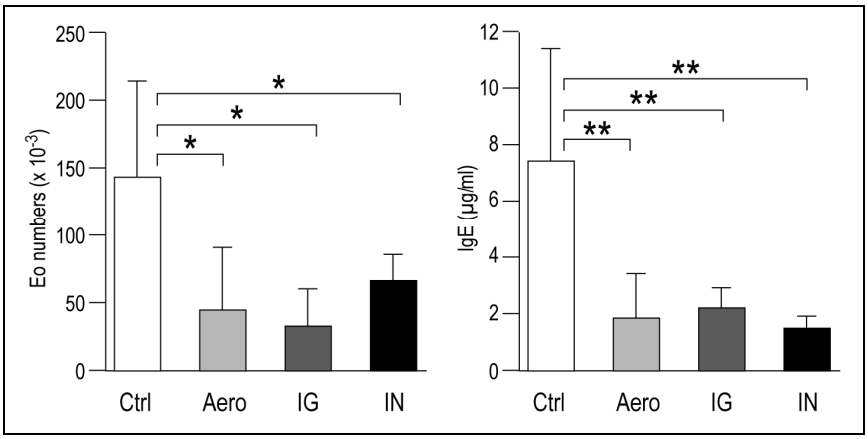

Figure 6: Eosinophil numbers in HAL and OVA-specific serum IgE in mice breastfed on mothers exposed to OVA via aerosol, intragastric or intranasal route. Newborns were breastfed by mothers that were left unexposed (empty bar), exposed to OVA by aerosols (light grey bar), intragastrie (dark grey bar) or intra-nasal administration (black bar). Once adult, mice were sensitized and challenged with OVA Data show means ± SD of values obtained in individual mice, one representative experiment of two. n=5-6 mice. **P* =0.03 and ***P* =0.004.

(5 µg/ml, Sigma) was added during the last 4 h. Cells were then stained with anti-CD4 mAb, fixed, permeabilized using cytofix/cytoperm reagent (BD), stained with anti-CD40L (BD) and anti-IL-4, IL-5 or IL-10 mAbs (BD) and analyzed by FACS. TGF-β1 and TGF-β2 amounts were determined in milk and sera using kits from R&D and Promega, respectively.

Histology

Left lungs were harvested and fixed with Immunohistofix and embedded in Immunohistowax (Infertiles). 4-µm sections were performed and stained with May Grünwald Giemsa or Periodic Acid of Schiff (Sigma).

Lung collagen content

Collagen lung content was determined by quantifying soluble collagen with the Sircoll Collagen assay kit (Biocolor), according to the manufacturer's instructions.

OVA content in milk

Four to six hours after OVA aerosol exposure of lactating mothers, breast milk was collected after ocytocin (Sigma) injection or from the stomach of 2 wk-old pups. Samples were spun down at 3500g for 10 min. Proteins from the aqueous phase were analyzed onto a 10% acrylamide SDS-PAGE followed by standard immunoblotting techniques. OVA was detected using a mouse mAb anti-OVA (Abcam), followed by a goat anti-mouse HRP-Ab (Jackson). Blots were developed using the super signal West femto kit (Pierce) and chemiluminescence was recorded using a luminescence image analyser LAS-3000 (Raytest, France). Quantification of captured images was performed using the Aida Image Analyzer software (Raytest).

Antigen-driven T cell activation in breastfed newborns

$CD4^+$ T cells were purified from DO11.10 TCR transgenic mice and 3 X 10^6 cells were injected into 2 wk-old BALB/c pups that were breastfed on OVA-exposed or unexposed BALB/c mothers. Pups were sacrificed 72 h later. Axillary and inguinal LN cells were analyzed by FACS after staining

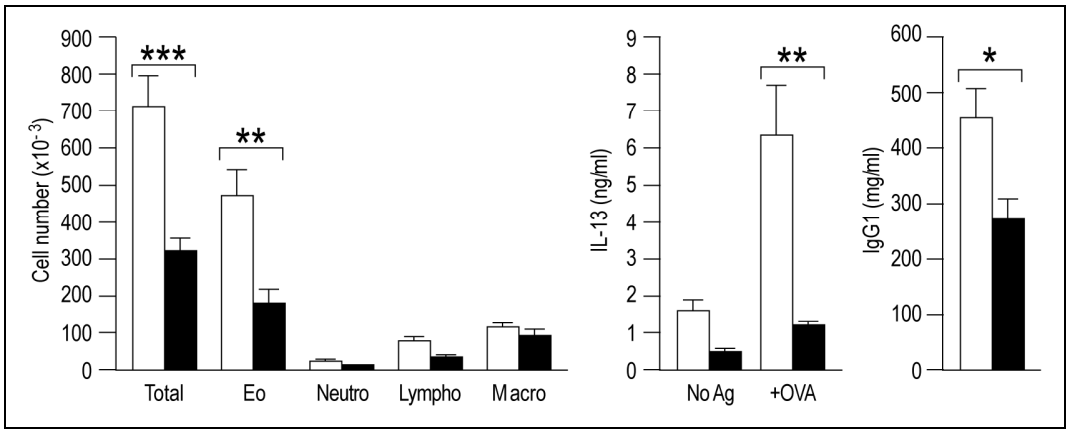

Figure 7: BAL cell numbers, IL-13 secretion and OVA-specific serum IgGI in C57BL/6 mice breastfed by OVA-exposed C57BL/6 mothers. C57BL/6 lactating mice were exposed (filled bars) or not (empty bars) to OVA aerosols. Once adult, offspring was sensitized and challenged with OVA. Data are expressed as means ± SEM of value obtained in individual mice for Eo and IgG1 and in pooled lung cells for IL-13 secretion in three independent experiments with n=5-7 mice per group. OVA-specific IgE was not detectable in sera of C57BL/6 mice. **P*=0.01; ***P*= 0.002; ****P*= 0.0004.

with anti-CD4, anti-CD69, anti-CD44 and KJ1-26 mAbs (BD).

CD4 T cell transfer

Spleens from 6-8 wk old BALB/c mice that had been breastfed on OVA-exposed or unexposed mothers were collected. $CD4^+$ T cells were enriched by negative depletion using CD4 isolation kit (Dynal) and further sorted using a high-speed sorter VANTAGE $SETLO^+$ flow cytometer (BD) after staining with anti-CD4 mAb. Cells were pure to more than 98% as demonstrated by staining with anti-CD4 mAbs. $5x10^6$ purified $CD4^+$ T cells were injected i.v. into 6-8 wk old BALB/c naive recipients. Mice were sensitized and further challenged with OVA 24 h later.

Statistical analysis

In all experiments, statistical significance was assessed using a two-tail p-value calculated with Mann-Whitney non-parametric test. P-values were calculated by comparing mice breastfed on OVA-exposed mother to those breastfed on unexposed mothers.

RESULTS

We have assessed the impact of airborne antigen exposure of lactating mice on the development of allergic asthma in their progeny (Figure 1a). When adults, the offspring was sensitized, challenged with OVA and analyzed for allergic airway disease. As compared to mice breastfed on unexposed mothers, mice breastfed on OVA-exposed mothers exhibited decreased airway hyperreactivity (Figure 1b), reduced numbers of eosinophils in bronchoalveolar lavage (BAL) (Figure 1c), milder peribronchial and perivascular cellular infiltration and decreased mucus deposition in the airways (Figure 1d,e), lower collagen contents in lungs (Figure 3) and reduced levels of

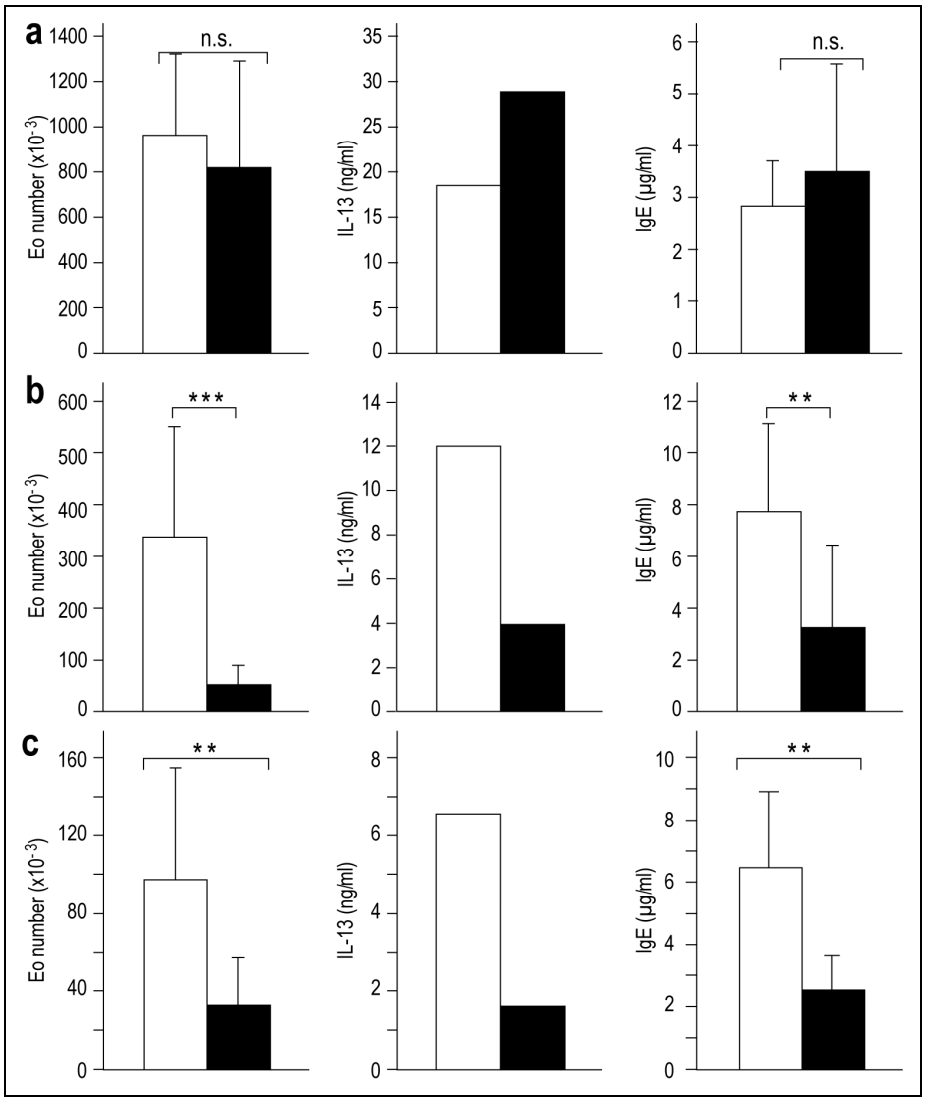

Figure 8: Breast milk factors involved in breastfeeding-induced tolerance.
a: Antigen-specificity of breastfeeding-induced protection. Mice breastfed on OVA-exposed (filled bars) or unexposed (empty bars) mothers were sensitized and challenged with LACK. BAL cells were counted and analyzed by FACS. Data show means ± SD of values obtained in individual mice for Eo and IgE and in pooled lung for IL-13 secretion. n=7 mice per group in one representative experiment of two. ns: $P > 0.5$.
b: Eosinophil numbers in BAL, lung IL-13 secretion and OVA-specific serum IgE levels in mice breastfed on µMT foster-mothers. One day-old BALB/c newborns were breastfed on µMT foster-mothers that were exposed (filled bars) or not (empty bars) to OVA aerosols. Once adults, mice were sensitized and challenged with OVA. Data show means ± SD of values obtained in individual mice for Eo and IgE and in pooled lung for IL-13 secretion. n=7-8 mice per group, one representative experiment of two. $^{**}P=0.009$; $^{***}P=0.0006$.
c: Eosinophil number in BAL, lung IL-13 secretion and OVA-specific serum IgE in mice fostered by RAG-2-KO mothers. BALB/c newborns were breastfed on RAG-2-KO foster mothers that were exposed (filled bars) or not (empty bars) to OVA aerosols. Once adult, mice were sensitized and challenged with OVA Data show means ± SD of values obtained in individual mice for Eo and IgE and in pooled lung for IL-13 secretion in one representative experiment of two. n=5-7 mice. $^{**}P=0.008$ for Eo and $^{**}P=0.004$ for IgE.

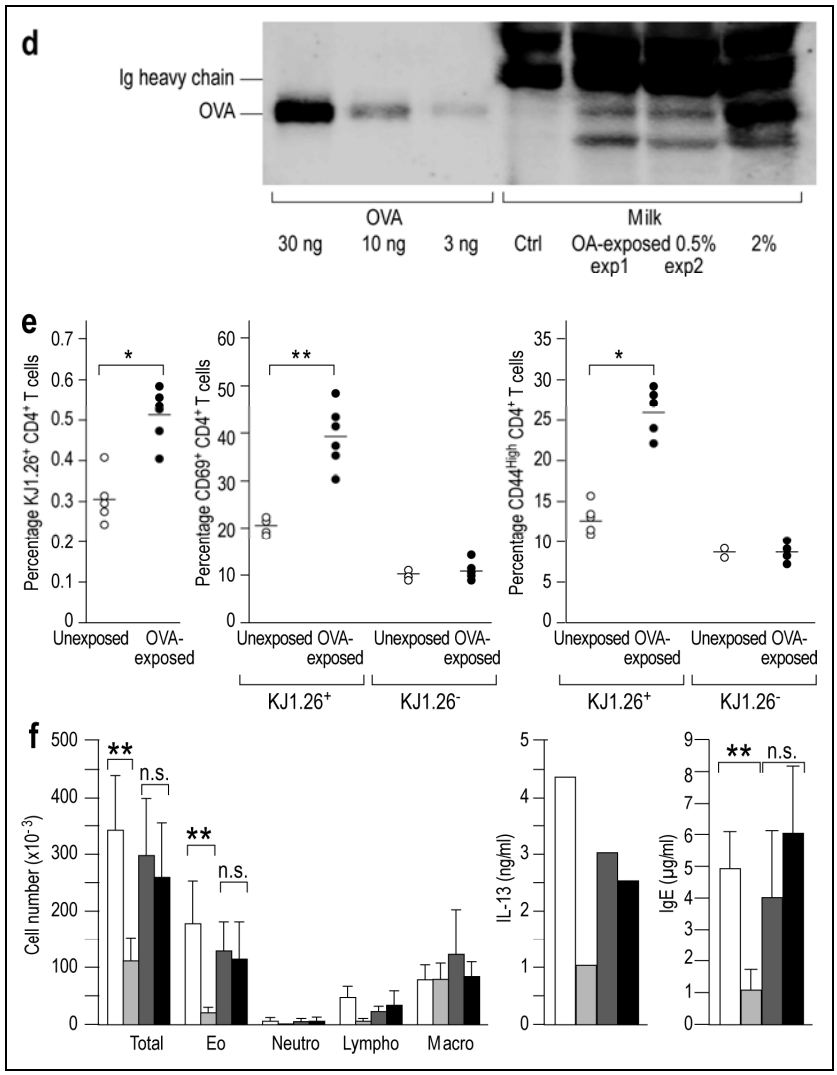

Figure 8 (continued): Breast milk factors involved in breastfeeding-induced tolerance.
d: OVA in breast milk. Lactating mothers were exposed to aerosols of the indicated concentration of OVA for 20 min. Breast milk was harvested 6 h later and analyzed for OVA content by Western Blot using a mouse anti-OVA mAb followed by an anti-mouse Ig mAb. One representative experiment of three.
e: Antigen-driven T cell activation in breastfed newborns. $CD4^+$ T cells from DO11.10 TCR transgenic mice (3 X 10^6 per mouse) were injected into 2 wk-old BALB/c pups that were breastfed on OVA-exposed (filled dots) or unexposed BALB/c mothers (empty dots). 72 h later, peripheral LN cells were analyzed by flow cytometry after staining with anti-CD4, anti-CD69, anti-CD44 and KJ1-26 mAb. Data show the results obtained in individual mice. n=4-6 in one representative experiment of three. **P*=0.01; ***P*=0.004.
f: Role of TGF-β during lactation in breastfeeding-induced tolerance. BAL cell numbers, lung IL-13 secretion and OVA-specific serum IgE levels in mice breastfed on mothers injected with anti-TGF-β mAb or with isotype rat IgG1. Newborns were breastfed on isotype-treated unexposed mother (empty bars), isotype-treated OVA-exposed mother (light grey bars), anti-TGF-β-treated unexposed mothers (dark grey bars) or anti-TGF-β-treated OVA-exposed mothers (black bars). Data are expressed as means ± SD of values obtained in individual mice for Eo and IgE and in pooled lung cells for IL-13 secretion. n=6-7 mice per group, one representative experiment of 2. ***P*=0.001; ns *P*>0.1.

serum OVA-specific IgE, IgG1 and IgA (Figure 4a). OVA-specific IgG2a levels were similar in both groups. Upon OVA restimulation, lung cells from mice breastfed on OVA-exposed mothers secreted reduced amounts of IL-4, IL-5, IL-10 and IL-13 as compared to cells from mice breastfed on unexposed mothers (Figure 4b). Similar results were obtained with mediastinal lymph node cells (Figure 5). The frequency of IL-4, IL-5 and IL-10-secreting lung $CD4^+$ T cells dropped in mice breastfed on OVA-exposed mothers as compared to control animals (Figure 4c). IFN-γ- and TGF-β-secreting cells were not detected. In addition, the frequency of OVA-specific $CD4^+$ T cells in mice breastfed on OVA-exposed mothers was reduced by 4-fold as demonstrated by the frequency of $CD40L^+CD4^+$ T cells upon OVA restimulation (*Frentsch* et al., 2005) (Figure 4c). Protection was also observed in BALB/c mice breastfed on mothers that have been exposed to OVA through the intranasal or the oral route and in C57BL/6 mice (Figures 6 and 7). Antigen-specificity was demonstrated by experiments in which mice breastfed on OVA-exposed mothers were sensitized and challenged with the *Leishmania* LACK antigen (*Julia* et al., 2002) (Figure 8a).

Antigen-specific protection could result from the transfer of immunoglobulins (Ig) (*Labbok* et al., 2004) and/or the antigen from the mother to the newborn through breast milk. To address this issue, wild type (wt) newborns were breastfed on either B cell-deficient μMT or lymphocyte-deficient RAG-2 KO foster-mothers. Mice breastfed on OVA-exposed μMT or RAG-2 KO foster-mothers exhibited reduced BAL eosinophilia, IL-13 secretion by lung cells, and serum OVA-specific IgE levels as compared to those breastfed on unexposed foster-mothers (Figure 8b,c). The levels of inhibition were similar to those observed in mice breastfed on wt mothers (Figure 1). Therefore, tolerance did not require the transfer of Ig from the mother to the newborn and was independent of the mother's lymphocyte compartment.

Western blotting analysis using anti-OVA mAbs showed two bands in milk of OVA-exposed mothers but not in unexposed animals, one at the level of OVA protein and one at a lower molecular weight that was likely to be a degradation product (Figure 8d). OVA concentration in milk of OVA-exposed mothers was in the same range as for dietary antigens in human milk (*Palmer* and *Makrides*, 2006), i.e. 180±20 ng/ml. Knowing that daily milk consumption by newborn mice is around 500 μl at day 10, mice breastfed on OVA-exposed mothers received about 100 ng of OVA daily.

To investigate whether milk-borne OVA was processed and presented to newborn lymphocytes, we injected OVA-specific TCR transgenic $KJ1\text{-}26^+$ $CD4^+$ T cells into 2 wk-old pups that were breastfed on OVA-exposed or unexposed mothers. Both the frequency of $KJ1\text{-}26^+$ $CD4^+$ T cells and the proportion of these cells that were $CD69^+$ or $CD44^{high}$ were higher in mice breastfed on OVA-exposed mothers as compared to those breastfed on unexposed mice (Figure 8e). Therefore, airborne OVA was transferred from the mother to the newborn through the milk and presented to $CD4^+$ T cells in the breastfed newborn.

Breast milk contains IL-10 and TGF-β that both exhibit immunosuppressive activities and favour tolerance induction (*Labbok* et al., 2004; *Letterio* et al., 1994; *Penttila* et al., 1998; *Saito* et al., 1993). However, mice breastfed on OVA-exposed IL-10-deficient mothers were protected from allergic

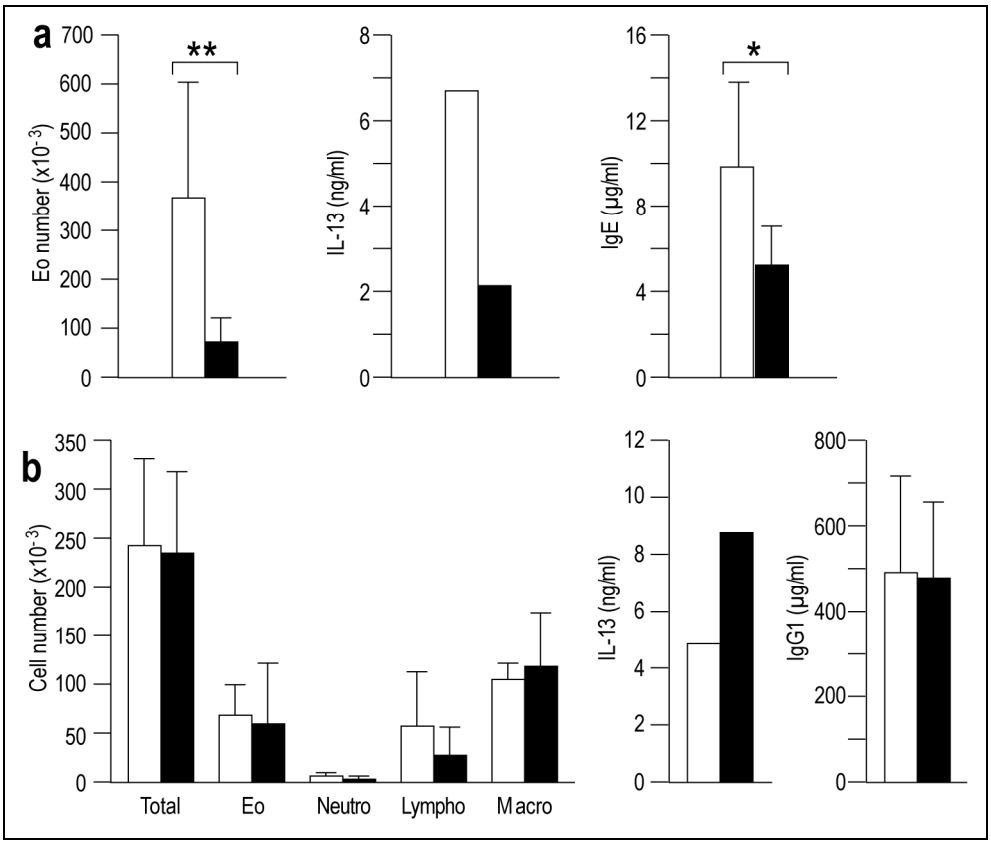

Figure 9: T_{reg} cells in mice breastfed on OVA-exposed mother
a: Tolerance transfer by $CD4^+$ T cells. Spleen $CD4^+$ T cells were purified from mice breastfed on OVA-exposed (filled bars) or unexposed (empty bars) mice and injected into adult naïve recipients that were further sensitized and challenged with OVA. Data are expressed as mean ± SD of values obtained in individual mice for Eo and IgE and in pooled lung for IL-13 secretion. n=6-8 mice per group, one representative experiment of three. ** P=0.001; * P=0.01.
b: BAL cell numbers, lung IL-13 secretion and OVA-specific IgG1 levels in TGF-DNRII mice breastfed on wt mice. TGF-DNRII mice were breastfed on C57BL/6 wt foster-mothers that were exposed (filled bars) or not (empty bars) to OVA aerosols. Data show means ± SD of values obtained in individual mice for Eo and IgG1 and in pooled lung cells for IL-13 secretion. OVA-specific IgE were not detectable in sera of TGF-DNRII mice. n=5-7 mice per group, one representative experiment of two.

airway inflammation as efficiently than those breastfed on OVA-exposed wt mothers further suggesting that protection could occur in the absence of IL-10 in milk (data not shown). Since TGF-β-deficient mice die prematurely, we assessed the role of milk borne TGF-β by injecting anti-TGF-β mAb to lactating mothers. Anti-TGF-β mAb treatment did not result in growth retardation. While mice breastfed on OVA-exposed isotypic control mAb-treated mothers were protected against allergic airway inflammation, mice breastfed on OVA-exposed TGF-β-depleted mothers developed OVA-induced airway inflammation as assessed by BAL eosinophilia, IL-13 secretion by lung cells and OVA-specific IgE serum level (Figure 8f). Therefore, both the reduced airway inflammation and lower antigen-specific Th2 re-

sponse exhibited by mice breastfed on OVA-exposed mothers required the presence of TGF-β during lactation. This result is in agreement with a previous study that showed that oral tolerance induction towards a dietary antigen in formula-fed rats was achieved when it was administered together with exogenous TGF-β (*Penttila*, 2006).

To assess whether protection from allergic airway disease relied on the presence of regulatory T cells (T_{reg}), we injected $CD4^+$ T cells from mice breastfed on OVA-exposed mothers to adult naïve mice. As compared to mice injected with control $CD4^+$ T cells, animals injected with $CD4^+$ T cells from mice breastfed on OVA-exposed mothers exhibited reduced BAL eosinophil numbers, IL-13 secretion by lung cells and serum levels of OVA-specific IgE (Figure 9a). These data suggested that a mechanism of active immune suppression by $CD4^+$ T cells was responsible for the breastfeeding-induced tolerance.

$CD25^+$ T_{reg} cells are involved in the regulation of allergic disease (*Robinson* et al., 2004). To assess whether $CD25^+$ T_{reg} cells were necessary for breastfeeding-induced protection, mice breastfed on OVA-exposed or unexposed mothers were injected with anti-CD25 or isotypic control mAb once adults and sensitized and challenged with OVA. As previously reported (*Lewkowich* et al., 2005), this treatment resulted in increased allergic airway inflammation in mice breastfed on unexposed mothers (Figure 2b). In this setting, the levels of inhibition of airway eosinophilia and OVA-specific IgE induced by mother exposure to OVA were similar in mice treated by anti-CD25 mAb and in those treated by rat IgG1: 87 versus 61% inhibition for eosinophilia; 47% versus 53% inhibition for OVA-specific IgE, (Figure 2b). Therefore, while $CD4^+$ T_{reg} cells are involved in breastfeeding-induced protection from allergic airway disease, $CD4^+$ $CD25^+$ T cells are not required.

To assess whether breastfeeding-induced protection required TGF-β signalling in T cells, we used TGF-β DNRII mutant mice in which T cells do not respond to TGF-β (*Lucas* et al., 2000). TGF-β DNRII newborns were breastfed on wt mothers that were exposed or not to OVA aerosols. Exposure of foster mothers to OVA did not induce protection in TGF-β DNRII mutant mice (Figure 9b). Therefore, the reduced airway inflammation and antigen-specific T cell responses observed in mice breastfed on OVA-exposed mothers were dependent on TGF-β signalling in T cells. We next investigated whether TGF-β was required for protection when mice were adults. Mice breastfed on OVA-exposed mothers were treated with either anti-TGF-β or isotypic control mAb one day before sensitization, challenged with OVA aerosols and analyzed for allergic airway inflammation. Neutralization of TGF-β before sensitization did not prevent breastfeeding-induced protection demonstrating that TGF-β was no longer required once T_{reg} cells have already been induced during the neonatal period (Figure 10).

DISCUSSION

We have demonstrated that an airborne antigen can be transferred from lactating mice to their progeny through breast milk eventually resulting in antigen-specific tolerance and prevention from asthma.

Breast milk contains dietary antigens (*Palmer* and *Makrides*, 2006) but

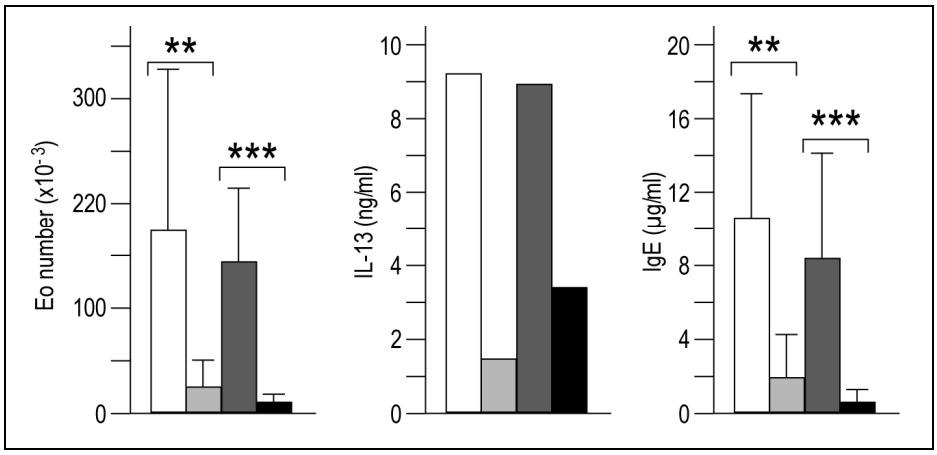

Figure 10: Breastfeeding-induced tolerance in anti-TGF-β mAb-treated offspring.
6-8 wk-old mice breastfed by OVA-exposed or unexposed mothers were treated with 1 mg of anti-TGF-β or isotype mAb before sensitization to OVA, and challenged with OVA. BAL eosinophil numbers, IL-13 secretion by lung T cells and OVA-specific serum IgE levels are shown for isotype-treated mice breastfed by unexposed mothers (empty bars), or OVA-exposed mothers (light grey bars), anti-TGF-β-treated mice breastfed by unexposed mothers (dark grey bars) or OVA-exposed mothers (black bars). Data are expressed as mean ± SD of values obtained in individual mice for Eo and IgE and in pooled lung cells for IL-13 secretion. n=6-7 mice per group, one representative experiment of two. ***P*=0.004 ****P*=0.0006.

the presence of airborne antigen has not yet been assessed. Antigen distribution after aerosol administration has been previously assessed using radiolabelled ^{125}I-BSA or ^{125}I-OVA (*Holt* et al., 1981; *Willoughby* and *Willoughby*, 1977). Both studies demonstrated that 2-4% of antigen was found in the lung and 65-80% in the digestive tract 1-2 hr after aerosol exposure. Therefore, although some airborne antigens penetrate into the distal alveoli, the bulk of inhaled antigen is found in the gut. Indeed, inhaled antigens are either trapped in the nasal passage and swallowed or deposited to the lung and cleared via the mucociliary escalator to the digestive tract. Therefore, the presence of airborne OVA in milk most likely results from the transfer of OVA from the airways to the mammary gland mainly through the gut and for a small proportion through the alveolar-capillary barrier of the lung (*Bensch* et al., 1967; *Braley* et al., 1978).

While the oral administration of an antigen to adult rodents results in tolerance induction (*Faria* and *Weiner*, 2005), inducing oral tolerance in neonates is far more difficult (*Adkins* et al., 2004; *Hanson*, 1981; *Miller* et al., 1994; *Strobel* and *Ferguson*, 1984). Neonates are biased for Th2 responses as compared to adults (*Adkins* et al., 2004). This is in apparent contrast with our data showing that the transfer of an antigen from the mother to the newborn via the milk induces tolerance towards a Th2-mediated disease. Breastfeeding-induced tolerance may rely on the chronic administration of an antigen at low dose, a setting known to promote tolerance induction *(Apostolou and von Boehmer*, 2004; *Faria* et al., 2003) together with the presence of milk-borne TGF-β. It remains to be determined whether tolerance is also observed when lactating mothers are allergic.

Epidemiological studies on the relationship between breastfeeding and the

development of allergic diseases have reached conflicting results whether the atopic status of the mothers was taken into account or not (*Friedman* and *Zeiger*, 2005; *Gdalevich* et al., 2001; *Guilbert* et al., 2007; *Kramer* et al., 2007; *van Odijk* et al., 2003). However, maternal airborne allergen exposure and antigen content in milk were not recorded in these studies. Our work may confer a rationale for new epidemiological studies assessing the presence of airborne antigens in human milk and the prevalence of allergic diseases in children breastfed on mothers exposed to airborne allergens. This report gives new insights into the mechanisms underlying tolerance induction in neonates and pinpoints maternal influence through breast milk antigen transfer as a critical factor in this process.

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