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Breast milk–mediated transfer of an antigen induces tolerance and protection from allergic asthma

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Allergic asthma is a chronic disease characterized by airway obstruction in response to allergen exposure. It results from an inappropriate T helper type 2 response to environmental airborne antigens and affects 300 million individuals[1]. Its prevalence has increased markedly in recent decades, most probably as a result of changes in environmental factors[2]. Exposure to environmental antigens during infancy is crucial to the development of asthma[3]. Epidemiological studies on the relationship between breastfeeding and allergic diseases have reached conflicting results[4–8]. Here, we have investigated whether the exposure of lactating mice to an airborne allergen affects asthma development in progeny. We found that airborne antigens were efficiently transferred from the mother to the neonate through milk and that tolerance induction did not require the transfer of immunoglobulins. Breastfeeding-induced tolerance relied on the presence of transforming growth factor (TGF)-β during lactation, was mediated by regulatory CD4$^+$ T lymphocytes and depended on TGF-β signaling in T cells. In conclusion, breast milk–mediated transfer of an antigen to the neonate resulted 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.

We assessed the impact of airborne antigen exposure of lactating mice on the development of allergic asthma in their progeny (**Fig. 1a**). When they reached adulthood, the offspring were sensitized, challenged with ovalbumin (OVA) and analyzed for allergic airway disease. As compared to mice breastfed by unexposed mothers, those breastfed by OVA-exposed mothers showed decreased airway hyperreactivity (**Fig. 1b**), reduced numbers of eosinophils in bronchoalveolar lavage (BAL; **Fig. 1c**), milder peribronchial and perivascular cellular infiltration and decreased mucus deposition in the airways (**Fig. 1d,e**), lower collagen content in lungs (**Supplementary Fig. 1** online) and lower abundance of serum OVA-specific IgE, IgG1 and IgA (**Fig. 2a**). OVA-specific IgG2a levels were similar in both groups. Upon OVA re-stimulation, lung cells from mice breastfed by OVA-exposed mothers secreted smaller amounts of IL-4, IL-5, IL-10 and interleukin-13 (IL-13) as compared to cells from mice breastfed by unexposed mothers (**Fig. 2b**). Similar results were obtained with mediastinal lymph node cells (**Supplementary Fig. 2** online). The frequency of IL-4-, IL-5- and IL-10-secreting lung CD4$^+$ T cells dropped in mice breastfed by OVA-exposed mothers as compared to control mice (**Fig. 2c**). Interferon-γ– and TGF-β–secreting cells were not detected. In addition, the frequency of OVA-specific CD4$^+$ T cells in mice breastfed by OVA-exposed mothers was reduced by 75% as indicated by the frequency of CD40L$^+$CD4$^+$ T cells upon OVA re-stimulation[9] (**Fig. 2c**). Protection was also observed in BALB/c mice breastfed by mothers that had been exposed to OVA through the intranasal or the oral route and in C57BL/6 mice (**Supplementary Figs. 3** and **4** online). Antigen specificity was shown by experiments in which mice breastfed by OVA-exposed mothers were sensitized and challenged with the *Leishmania major* LACK antigen[10] (**Fig. 3a**).

Antigen-specific protection could result from the transfer of immunoglobulins[11], the antigen or both from the mother to the newborn through breast milk. To address this issue, wild-type (WT) newborns were breastfed by either B cell–deficient disrupted transmembrane μ exon (μMT) or lymphocyte-deficient RAG-2–knockout foster mothers. Mice breastfed by OVA-exposed μMT or RAG-2–knockout foster mothers showed reduced BAL eosinophilia, IL-13 secretion by lung cells and serum OVA-specific IgE abundance as compared to those breastfed by unexposed foster mothers (**Fig. 3b,c**). The degree of inhibition was similar to those observed in mice breastfed by WT mothers (**Fig. 1**). Therefore, tolerance did not require the transfer of immunoglobulin from the mother to the newborn and was independent of the mother's lymphocyte compartment.

Western blotting analysis with monoclonal antibody (mAb) to OVA showed two bands in the milk of OVA-exposed mothers but not in that of unexposed mice, one at the level of OVA protein and one at a lower molecular weight that was probably a degradation product (**Fig. 3d**). The OVA concentration in the milk of OVA-exposed mothers was in the same range as that of dietary antigens in human milk[12], that is, 180 ± 20 ng/ml. As daily milk consumption by newborn mice is around 500 μl at day 10, mice breastfed by OVA-exposed mothers received about 100 ng of OVA daily. Breast milk contains dietary antigens[12], but the presence of airborne antigens has

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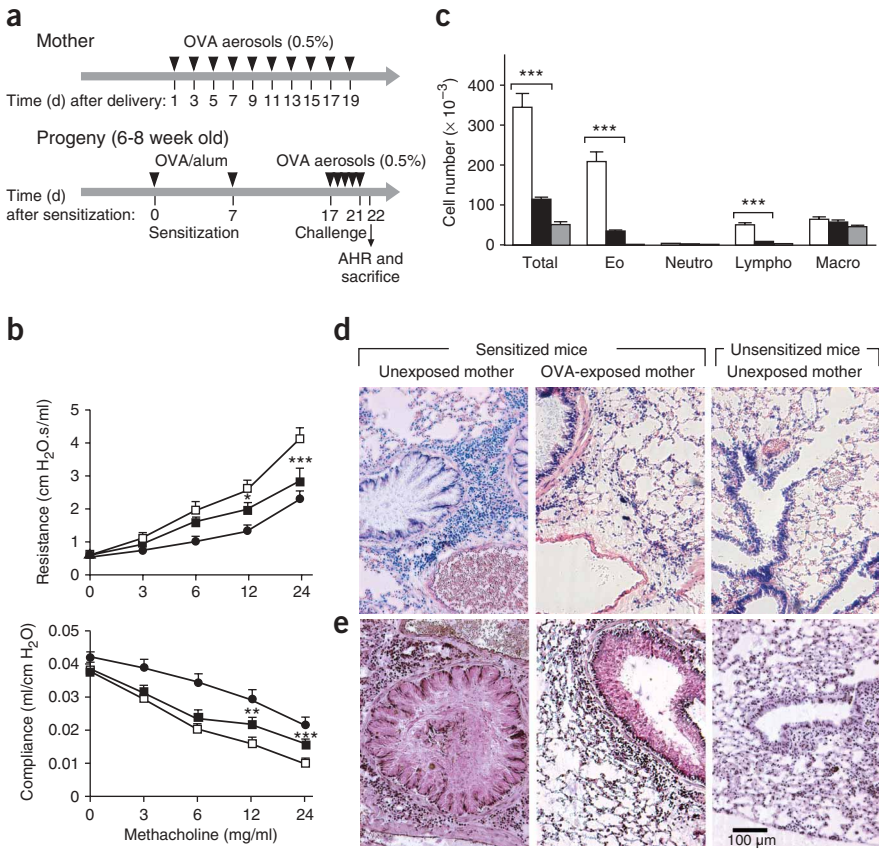

Figure 1 AHR and airway inflammation in mice breastfed by OVA-exposed mothers. (**a**) Experimental protocol. Lactating mothers were exposed or not exposed to 0.5% OVA aerosols for 20 min every other day from delivery until weaning. During aerosol exposure, pups were kept away from their mother. When 6–8 weeks old, offspring were sensitized with two injections of OVA in alum and challenged daily for 5 d with OVA aerosols. Mice were analyzed 1 d after the last aerosol exposure. (**b**) AHR. Dynamic lung resistance and compliance were monitored in mice breastfed by OVA-exposed (■) or unexposed mothers (□) upon sensitization and challenge with OVA ($n = 6$ or 7 mice per group in each experiment). Unsensitized mice challenged with OVA (○) were used as controls ($n = 3$ in each experiment). Data are expressed as means ± s.e.m. of two 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 by OVA-exposed (black bars) and unexposed (empty bars) mothers. Unsensitized mice challenged with OVA were used as controls (gray bars). Eo, eosinophils; neutro, neutrophils; lympho, lymphocytes; macro, macrophages. Data are expressed as means ± s.e.m. of five experiments with $n = 6$–8 mice per group for sensitized mice and of two experiments with $n = 5$ or 6 mice for the unsensitized group. $^{***}P < 0.0001$. (**d**,**e**) Histology of lung sections. Staining with May-Grünwald Giemsa (**d**) and periodic acid of Schiff (**e**).

not yet been assessed. Antigen distribution after aerosol administration has been previously assessed using radiolabeled ^{125}I-BSA or ^{125}I-OVA (refs. 13,14). Both studies demonstrated that 2–4% of antigen was found in the lung and 65–80% was found in the digestive tract 1–2 h 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 by 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[15,16].

To investigate whether milk-borne OVA is processed and presented to newborn lymphocytes, we injected OVA-specific T cell receptor

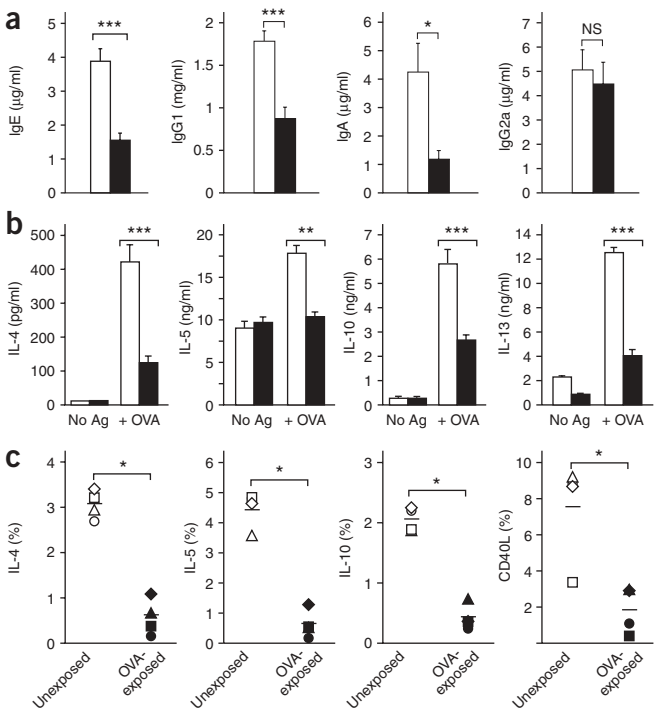

Figure 2 Immunoglobulin and T cell responses in mice breastfed by OVA-exposed mothers. (**a**) Serum levels of OVA-specific immunoglobulin. Sera from mice breastfed by OVA-exposed (filled bars) and unexposed (empty bars) mothers were analyzed for OVA-specific IgE, IgG1, IgA and IgG2a contents by ELISA. Histograms show the means ± s.e.m. of five independent experiments for IgE and IgG1 abundance and of two experiments for IgG2a and IgA abundance 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 by 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 means ± s.e.m. of five 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 by OVA-exposed (filled symbols) or unexposed mothers (empty symbols) were pooled in each group ($n = 5$–7) and incubated with OVA and mAb to CD28. 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. $^{*}P = 0.02$.

(TCR) transgenic KJ1-26$^+$ CD4$^+$ T cells into 2-week-old pups that were breastfed by OVA-exposed or unexposed mothers. Both the frequency of KJ1-26$^+$ CD4$^+$ T cells and the proportion of these cells that were CD69$^+$ or CD44^{high} were higher in mice breastfed by OVA-exposed mothers as compared to those breastfed by unexposed mice (**Fig. 3e**). 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, which both show immunosuppressive activities and favor tolerance induction[11,17–19]. However, mice breastfed by OVA-exposed IL-10–deficient mothers were protected from allergic airway inflammation as efficiently as those breastfed by OVA-exposed WT mothers, further suggesting that protection could occur in the absence of IL-10 in milk (data not shown). Because TGF-β–deficient mice die prematurely, we assessed the role of milk-borne TGF-β by injecting mAb to TGF-β into lactating mothers. Treatment with mAb to TGF-β did not result in growth retardation. Whereas mice breastfed by OVA-exposed isotypic control mAb–treated mothers were protected against allergic airway inflammation, mice breastfed by 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 serum IgE abundance (**Fig. 3f**). Therefore, both the reduced airway inflammation and the lower antigen-specific T helper type 2 (T_H2) response shown by mice breastfed by OVA-exposed mothers required the presence of TGF-β during lactation. This result is in agreement with a previous study that showed that oral induction of tolerance to a dietary antigen in formula-fed rats was achieved when the antigen was administered together with exogenous TGF-β[20].

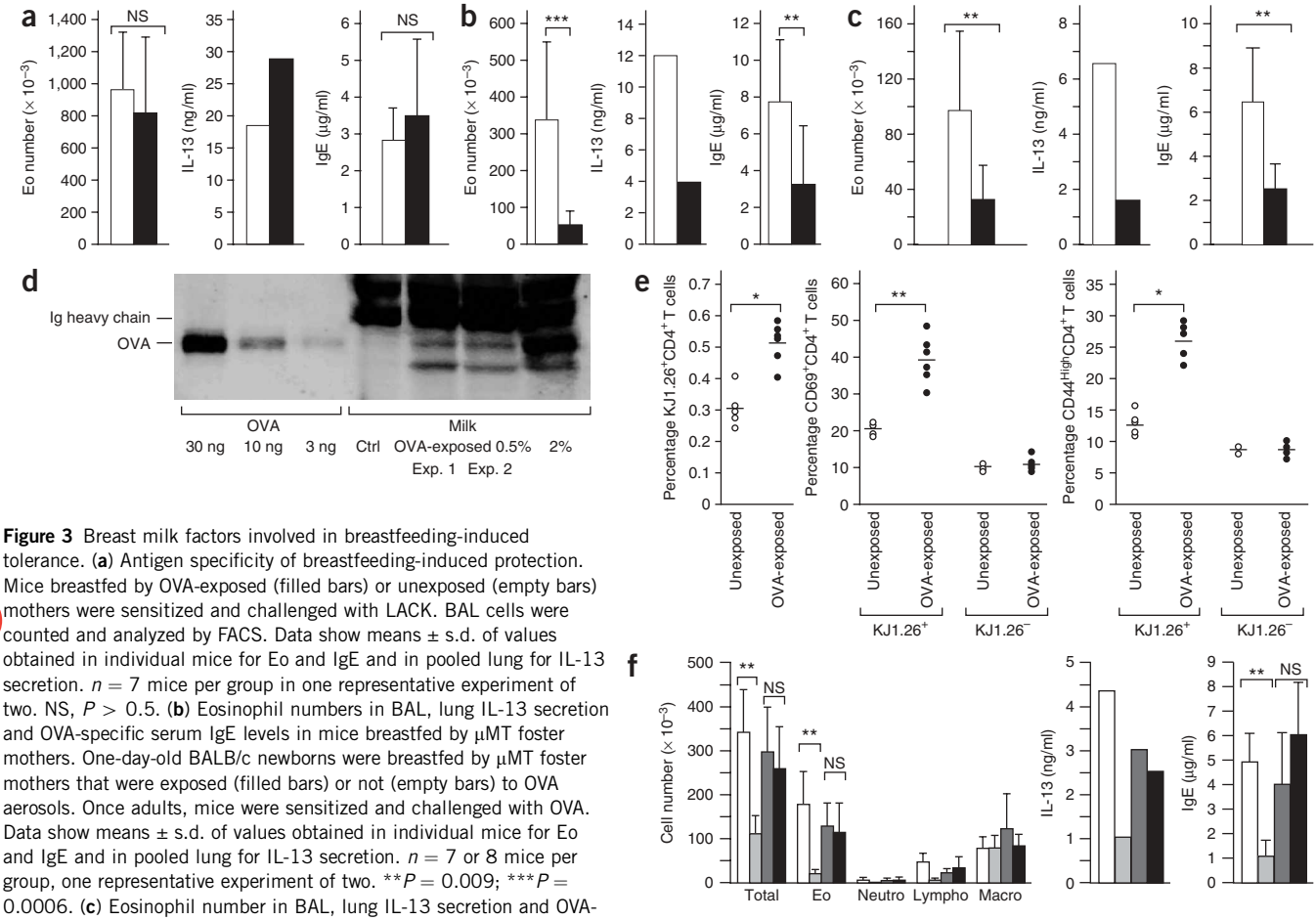

Figure 3 Breast milk factors involved in breastfeeding-induced tolerance. (**a**) Antigen specificity of breastfeeding-induced protection. Mice breastfed by OVA-exposed (filled bars) or unexposed (empty bars) mothers were sensitized and challenged with LACK. BAL cells were counted and analyzed by FACS. Data show means ± s.d. 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 by μMT foster mothers. One-day-old BALB/c newborns were breastfed by 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 ± s.d. of values obtained in individual mice for Eo and IgE and in pooled lung for IL-13 secretion. $n = 7$ or 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–knockout mothers. BALB/c newborns were breastfed by RAG-2–knockout 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 ± s.d. 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. (**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 blotting using a mouse mAb to OVA followed by a mAb to mouse immunoglobulin. One representative experiment of three. (**e**) Antigen-driven T cell activation in breastfed newborns. CD4$^+$ T cells from DO11.10 TCR transgenic mice (3×10^6 per mouse) were injected into 2-week-old BALB/c pups that were breastfed by OVA-exposed (filled dots) or unexposed BALB/c mothers (open dots). We analyzed peripheral lymph node cells by flow cytometry 72 h later, after staining with mAbs to CD4, CD69, CD44 and KJ1-26. 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 by mothers injected with mAb to TGF-β or with isotype control rat IgG1. Newborns were breastfed by isotype-treated unexposed mother (empty bars), isotype-treated OVA-exposed mother (light gray bars), antibody to TGF-β–treated, unexposed mothers (dark gray bars) or antibody to TGF-β–treated, OVA-exposed mothers (black bars). Data are expressed as means ± s.d. of values obtained in individual mice for Eo and IgE and in pooled lung cells for IL-13 secretion. $n = 6$ or 7 mice per group, one representative experiment of two. $^{**}P = 0.001$; NS, $P > 0.1$.

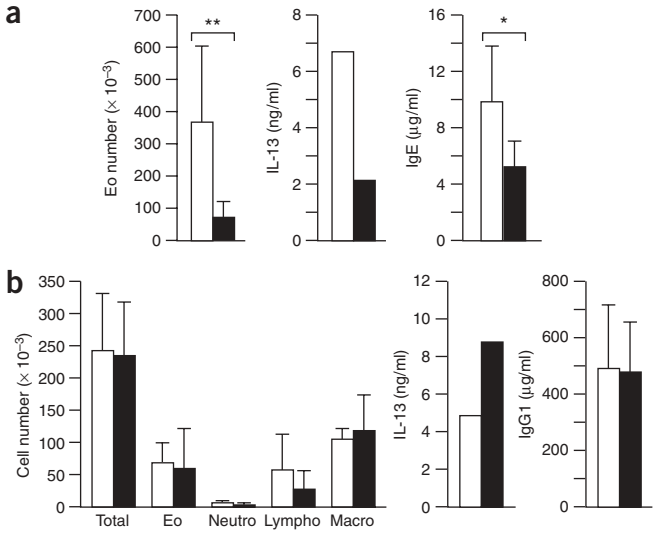

Figure 4 T_{reg} cells in mice breastfed by OVA-exposed mothers. (**a**) Tolerance transfer by $CD4^+$ T cells. Spleen $CD4^+$ T cells were purified from mice breastfed by OVA-exposed (filled bars) or unexposed (empty bars) mice and injected into adult naive recipients that were further sensitized and challenged with OVA. Data are expressed as means ± s.d. 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 by WT mice. TGF-DNRII mice were breastfed by C57BL/6 WT foster mothers that were exposed (filled bars) or not (empty bars) to OVA aerosols. Data show means ± s.d. 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.

To assess whether protection from allergic airway disease relies on the presence of regulatory T (T_{reg}) cells, we injected $CD4^+$ T cells from mice breastfed by OVA-exposed mothers to adult naive mice. As compared to mice injected with control $CD4^+$ T cells, animals injected with $CD4^+$ T cells from mice breastfed by OVA-exposed mothers showed reduced BAL eosinophil numbers, IL-13 secretion by lung cells and serum abundance of OVA-specific IgE (**Fig. 4a**). 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[21]. To assess whether $CD25^+$ T_{reg} cells are necessary for breastfeeding-induced protection, we injected mice breastfed by OVA-exposed or unexposed mothers with antibody to CD25 or isotypic control mAb once they had reached adulthood and then sensitized them and challenged them with OVA. As previously reported[22], this treatment resulted in increased allergic airway inflammation in mice breastfed by unexposed mothers (**Supplementary Fig. 5b** online). In this setting, the amounts of inhibition of airway eosinophilia and OVA-specific IgE induced by mother exposure to OVA were similar in mice treated with mAb to CD25 and in those treated with rat IgG1: 87% versus 61% inhibition for eosinophilia and 47% versus 53% inhibition for OVA-specific IgE, (**Supplementary Fig. 5b**). Therefore, whereas $CD4^+$ 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 requires TGF-β signaling in T cells, we used TGF-β DNRII mutant mice, in which T cells do not respond to TGF-β (ref. 23). TGF-β DNRII newborns were breastfed by 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 (**Fig. 4b**). Therefore, the reduced airway inflammation and antigen-specific T cell responses observed in mice breastfed by OVA-exposed mothers are dependent on TGF-β signaling in T cells. We next investigated whether TGF-β was required for protection when mice were adults. Mice breastfed by OVA-exposed mothers were treated with either antibody to TGF-β or isotypic control mAb 1 d 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 the T_{reg} cells had already been induced during the neonatal period (**Supplementary Fig. 6** online).

In conclusion, we have shown that an antigen can be transferred from lactating mice to their progeny through breast milk, eventually resulting in antigen-specific tolerance and prevention of allergen-induced lung pathology. Whereas the oral administration of an antigen to adult rodents results in tolerance induction[24], the induction of oral tolerance in neonates is far more difficult[25–28]. Neonates are biased toward T_H2 responses as compared to adults[25]. 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 toward a T_H2-mediated disease. Breastfeeding-induced tolerance may rely on both the chronic administration of an antigen at low dose, a setting known to promote tolerance induction[29,30], and the presence of milk-borne TGF-β. It remains to be determined whether tolerance is also observed when lactating mothers are allergic to the antigen being used. Epidemiological studies on the relationship between breastfeeding and the development of allergic diseases have yielded conflicting results, whether or not the atopic status of the mothers was taken into account[4–8]. However, maternal airborne allergen exposure and antigen content in milk were not recorded in these studies. Our work may provide a rationale for new epidemiological studies assessing the presence of airborne antigens in human milk and the prevalence of allergic diseases in children breastfed by 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–mediated antigen transfer as a crucial factor in this process.

METHODS

Mice. We purchased BALB/c mice and C57BL/6 mice from The Centre d'Elevage Janvier (France) and housed them under specific pathogen–free conditions. D0.11.10 TCR transgenic mice were provided by F. Powrie (University of Oxford). We obtained TGF-β DNRII mice (ref. 23) from P.J. Lucas (US National Institutes of Health), μMT and RAG-2–knockout mice from the Centre de Distribution, Typage et Archivage animal (France) and IL-10–deficient mice from Charles River (France). All nontransgenic mice were on the BALB/c background, unless indicated otherwise. 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, the 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. We exposed or did not expose lactating mice to 0.5% OVA (grade V, Sigma) aerosols for 20 min every other day, from 24 h after delivery until weaning, using an ultrasonic nebulizer (Ultramed, Medicalia) connected to a 13,000-cm^3 box that served as the deposition chamber for the mice (**Fig. 1a**). We gave aerosols to groups of a maximum of 5–10 mothers. During aerosol exposure, we separated the

mothers from their progeny. Alternatively, we gave lactating mothers either intranasal or intragastric administrations of 0.1 mg and 0.5 mg of OVA, respectively, from delivery until weaning. We determined the OVA endotoxin content with the QCL1000 chromogenic LAL kit assay (Cambrex). The LPS content of OVA was below 10 ng/mg of protein. Where indicated, we treated the mothers with 1 mg of mAb to TGF-β (clone 1D11, American Type Culture Collection (ATCC)) or isotype control antibody (GL113, rat IgG1, DNAX) twice a week from delivery until weaning. After treatment with antibody to TGF-β, the TGF-β2 content in milk was partially reduced (20.7 ± 4.0 to 12.8 ± 4.9 ng/ml in isotype-treated and antibody to TGF-β–treated mothers respectively; mean of three independent experiments ± s.d.), and the TGF-β1 content in milk was reduced at least sixfold (1.4 ± 0.3 to 0.2 ± 0.1 ng/ml; mean of 4 experiments ± s.d.). The administration of TGF-β–specific mAb to lactating mothers also resulted in a partial depletion of TGF-β1 in newborn serum (56 ± 13 to 26 ± 12 ng/ml; mean of three experiments ± s.d., $n = 14$). TGF-β2 remained below the level of detection.

Induction of allergic asthma. We used 6–8-week-old mice. We performed sensitization by intraperitoneally injecting 10 μg of OVA in 2 mg of aluminum hydroxide (alum, Pierce) on days 0 and 7. From day 17 to day 21, we exposed mice to OVA (0.5%) aerosols for 20 min using an ultrasonic nebulizer (Ultramed, Medicalia) connected to a 13,000-cm^3 box that served as the deposition chamber for the mice (**Fig. 1a**). Where indicated, we injected mice with 0.5 mg of mAb to CD25 (PC61 clone, ATCC) or with rat IgG1 (GL113 clone, DNAX) 1 week before sensitization. Treatment with mAb to CD25 resulted in a large reduction in the frequency of $CD25^+CD4^+$ T cells (1–2% versus 7.5%; **Supplementary Fig. 5a**). In other experiments, we injected mice with 1 mg of mAb to TGF-β (clone 1D11, ATCC) or with rat IgG1 (GL113 clone, DNAX) 1 d before each sensitization. In some experiments, we sensitized mice with 10 μg LACK in 2 mg of alum and further exposed them to aerosols of 0.2% LACK, as described previously[10]. We detoxified LACK with an Endotrap column (Profos) according to the manufacturer's instructions to reach lipopolysaccharide amounts below 10 ng/mg of protein.

Airway hyperreactivity (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 per kg body weight (ml/kg) of 10% medetomidine (Pfizer) and 10% ketamine (Merial)), tracheotomized, paralyzed (5 ml/kg pancuronium bromide 1% (Organon)) and immediately intubated with an 18-gauge 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 s, 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 administration of the subsequent doses of methacholine.

Analysis of bronchoalveolar lavage cells. We bled mice and inserted a cannula into the trachea. We washed the lungs three times with 1 ml of PBS. For differential BAL cell counts, we stained cells with mAbs to CCR3 (R&D Systems), Gr1, CD3 and CD19 (Becton Dickinson) and analyzed them by FACS using a FACSCalibur flow cytometer and CellQuest software. We defined eosinophils as $CCR3^+CD3^-CD19^-$, neutrophils as $Gr\text{-}1^{hi}CD3^-CD19^-$, lymphocytes as $CD3^+CD19^+$ and alveolar macrophages as large autofluorescent cells.

Serum antibody measurements. We measured serum OVA-specific or LACK-specific IgG1, IgG2a, IgA and IgE by ELISA. For IgG1 quantification, we incubated antigen-coated Maxisorp plates (Nunc) with serial dilutions of sera and biotinylated mAb to IgG1 (Becton Dickinson). For antigen-specific IgE, IgG2a and IgA, we first coated the plates with the respective capture mAb (Becton Dickinson) and incubated with the serum dilutions. We then added biotinylated OVA or LACK antigen. We used horseradish peroxidase–conjugated streptavidin (Becton Dickinson) and tetramethylbenzidine (KPL) for detection.

Cytokine assays. One day after the last aerosol treatment, we removed lungs and mediastinal lymph nodes separately, minced them and digested them with collagenase I (Gibco) and DNase (Roche) for 30 min at 37 °C. We filtered cell suspensions through a 70-μm cell strainer and depleted them of red blood cells using red blood cell lysis buffer. We pooled cells from each group and cultured 4×10^6 lung and 1.5×10^6 lymph node cells in triplicate for 72 h in medium containing or not containing OVA (100μg/ml) in 48-well plates or 96-well plates, respectively. The culture medium was RPMI 1640 (Gibco) containing 5% heat-inactivated FCS (Perbio), 50 μM 2-mercaptoethanol (Gibco) and penicillin-streptomycin (Gibco). We analyzed the supernatants by ELISA for IL-4, IL-5 and IL-10 using antibody pairs from Becton Dickinson and for IL-13 contents using a kit from R&D Systems. The 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, we incubated cells with 100 μg/ml OVA and 1 μg/ml of antibody to CD28 (Becton Dickinson) for 6 h. We added brefeldin A (5 μg/ml, Sigma) during the last 4 h. We then stained the cells with mAb to CD4, fixed them, permeabilized them with Cytofix/Cytoperm reagent (Becton Dickinson), stained them with antibody to CD40L (Becton Dickinson) and mAbs to IL-4, IL-5 or IL-10 (Becton Dickinson), and analyzed them by FACS. We determined TGF-β1 and TGF-β2 amounts in milk and sera with kits from R&D and Promega, respectively.

Histology. We harvested left lungs, fixed them with Immunohistofix and embedded them in Immunohistowax (Infertiles). We cut 4-μm sections and stained them with May Grünwald Giemsa or periodic acid of Schiff (Sigma).

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

OVA content in milk. We collected breast milk after oxytocin (Sigma) injection or from the stomachs of 2-week-old pups, 4–6 h after OVA aerosol exposure of lactating mothers. We spun down samples at 3,500*g* for 10 min. We analyzed proteins from the aqueous phase by 10% acrylamide SDS-PAGE followed by standard immunoblotting techniques. We detected OVA with a mouse mAb to OVA (Abcam), followed by a horseradish peroxidase–conjugated goat antibody to mouse IgG (Jackson). We developed blots with the Supersignal West Femto kit (Pierce) and recorded chemiluminescence with a LAS-3000 luminescence image analyzer (Raytest, France). We performed quantification of captured images with Aida Image Analyzer software (Raytest).

Antigen-driven T cell activation in breastfed newborns. We purified $CD4^+$ T cells from DO11.10 TCR transgenic mice and injected 3×10^6 cells into 2-week-old BALB/c pups that were breastfed by OVA-exposed or unexposed BALB/c mothers. We killed them 72 h later. We analyzed axillary and inguinal lymph node cells by FACS after staining with mAbs to CD4, CD69, CD44 and KJ1-26 (Becton Dickinson).

$CD4^+$ T cell transfer. We collected the spleens from 6–8-week-old BALB/c mice that had been breastfed by OVA-exposed or unexposed mothers. We enriched for $CD4^+$ T cells by negative depletion using a CD4 isolation kit (Dynal) and further sorted the cells after staining with mAb to CD4 with a high-speed sorter, the VANTAGE $SETLO^+$ flow cytometer (Becton Dickinson). The cells were >98% pure, as shown by staining with mAb to CD4. We injected 5×10^6 purified $CD4^+$ T cells intravenously into 6–8-week-old BALB/c naive recipients. We sensitized the mice and further challenged them with OVA 24 h later.

Statistical analysis. In all experiments, we assessed statistical significance using a two-tailed *P* value calculated with the Mann-Whitney nonparametric test. We calculated *P* values by comparing mice breastfed by an OVA-exposed mother to those breastfed by unexposed mothers.

Note: Supplementary information is available on the Nature Medicine website.

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