

Allergy prevention starts before conception: Maternofetal transfer of tolerance protects against the development of asthma

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Background: Allergy prevention must start early because environmental exposures during pregnancy and young life determine disease risk.

Objective: In this study we analyzed whether prevention can start even earlier before conception by transfer of immunologic tolerance from the mother to the offspring preventing the offspring from having asthma.

Methods: BALB/c mice were orally tolerized with ovalbumin before conception by means of oral application of antigen. The offspring of tolerized and naive mothers were immunized with ovalbumin at 6 weeks and 4 months of age and analyzed in our murine asthma model.

Results: Although the offspring of naive mothers had an asthma-like phenotype, the offspring of tolerized mice were completely protected, even when immunized as late as 8 months after birth. Critically involved in the tolerance transfer was allergen-specific IgG, levels of which were increased in the sera of the mother, fetus, and pup and breast milk. FcRn^{-/-} mice, which cannot transport IgG through the placenta, transferred tolerance to the offspring only when the missing diaplacental IgG transfer was compensated by IgG transfer through breast milk from tolerant mothers but not when weaned by naive wet nurses. Inhibition of IFN- γ , produced by memory T cells in the offspring, abrogated the protective effect of maternal tolerance, demonstrating a crucial role for IFN- γ in the maintenance of allergen-specific tolerance.

Conclusion: Our data show that maternal immunologic memory has a significant and persistent effect on the immune response of the offspring. (*J Allergy Clin Immunol* 2008;122:1022-30.)

Key words: Asthma, tolerance, pregnancy, primary prevention

Asthma is a T_H2 lymphocyte-mediated inflammatory airway disease characterized by airway hyperreactivity (AHR), eosinophilic inflammation, increased mucus production, and increased levels of allergen-specific IgE.¹ Although pharmacotherapy in

Abbreviations used

AHR:	Airway hyperreactivity
BAL:	Bronchoalveolar lavage
BLG:	β -Lactoglobulin
GITR:	Glucocorticoid-induced tumor necrosis factor receptor
OVA:	Ovalbumin
TCR:	T-cell receptor

asthma effectively controls symptoms, curative therapies are missing so that prevention of this very common, chronic, and incurable disease is highly desirable.

In recent years a number of studies have shown that environmental exposures in the first 12 to 24 months of life (eg, in daycare centers or in a farming environment) might determine the development of childhood asthma and allergies.²⁻⁴ Therefore primary prevention strategies might ideally start in early childhood. There is increasing evidence that the temporal sequence of events might even be traced back to prenatal exposures, namely exposures of the mother. Recently, it has been shown that maternal exposure during pregnancy to an environment rich in microbial compounds might protect against the development of atopic sensitization in the children.⁵ This protective effect was confined to upregulation of receptors of the innate immune system⁵ and to T_H2-dependent immunoglobulin isotypes that were allergen and switch stage specific.⁶

In murine models it has been shown that endotoxin exposure of pregnant mice prevented the development of allergen-induced asthma in the offspring,⁷ whereas prenatal exposure to stress enhanced the susceptibility of the offspring toward airway inflammation.⁸ The observation that maternal, more than paternal, asthma is a significant risk factor for the development of asthma and atopy in children^{9,10} might indicate that preventive events can even be traced back to preconceptional exposures of the mother.

The aim of the present study was to analyze whether allergy prevention can start even before conception by means of transfer of immunologic tolerance from the mother to the offspring. If, for instance, tolerance to harmless antigens, such as allergens, that is acquired in female subjects before pregnancy is transferred to the offspring, primary prevention strategies would be effective for more than 1 generation and could terminate the increase of allergic diseases.

METHODS

Mice

Female BALB/cByJ, C57Bl/6J, and B6.129X1-Fcgrt^{tm1Dcr} mice (6-8 weeks of age) were obtained from the Jackson Laboratory (Bar Harbor, Me)

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Supported by the Deutsche Forschungsgemeinschaft (SFB 587, B14) and the Bundesministerium für Bildung und Forschung (FKZ 14/28).

Disclosure of potential conflict of interest: T. Polte, C. Hennig, and G. Hansen have received research funding from the German Research Foundation.

Received for publication February 6, 2008; revised September 5, 2008; accepted for publication September 8, 2008.

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0091-6749/\$34.00

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doi:10.1016/j.jaci.2008.09.014

and bred and maintained in our animal facility at the Martin Luther University in Halle/Saale (Germany). All animal experiments involved groups of 4 to 6 mice and were performed at least 2 times according to institutional and state guidelines. The Committee on Animal Welfare of Saxony-Anhalt approved the animal protocols used in this study.

Tolerance induction and immunization

For induction of oral tolerance, mice received 100 mg of ovalbumin (OVA, grade V; Sigma-Aldrich, Steinheim, Germany) on days –6 and –3 before mating by means of gastric intubation. Control mice received normal saline orally. Offspring mice (6–10 weeks or 7–8 months old) were immunized intraperitoneally with OVA (20 µg) adsorbed to 2 mg of an aqueous solution of aluminum hydroxide and magnesium hydroxide (Alum; Perbio Science, Bonn, Germany) on days 0 and 14, followed by 20 µg of OVA in 40 µL of normal saline administered intranasally on days 14 to 16 and 21 to 23. Control mice received Alum intraperitoneally and normal saline intranasally. One day after the last intranasal challenge (day 24), AHR was measured. Mice were killed on day 25 (Fig 1). For inhibition of IFN-γ, anti-IFN-γ mAb (XMG1.2, rat IgG1 anti-mouse IFN-γ mAb, 10 µg; eBioscience, San Diego, Calif) or control mAb (rat IgG1, eBioscience) was applied to the 6-week-old offspring on days 0, 6, 13, and 20.¹¹

Measurement of airway responsiveness

Airway responsiveness was assessed based on methacholine-induced airflow obstruction from conscious mice placed in a whole-body plethysmograph (model PLT UNR MS; EMKA Technologies, Paris, France), as described previously.¹²

Collection of bronchoalveolar lavage fluid

Cells in the lavage fluid were counted by using a hemocytometer, and bronchoalveolar lavage (BAL) cell differentials were determined, as described previously.¹¹

Lung histology and computer-based quantification of inflammation and mucus production

The left lung was fixed in 10% formalin and stained with hematoxylin and eosin (Merck, Darmstadt, Germany) or with periodic acid–Schiff (Sigma, Taufkirchen, Germany). For objective evaluation and quantification of the degree of pulmonary inflammation and mucus production, lung sections were scanned with a digital camera (VisiTron Systems, Puchheim, Germany; 5 shots per lung) and analyzed with HistoClick software based on morphometric image analysis developed in our laboratory. The degree of inflammation and mucus production is expressed by the number of pixels.

Immunoglobulin assay

OVA-specific IgG1 and IgE serum levels were measured by using a sandwich ELISA according to a standard protocol, as described previously.¹¹ Milk was extracted from stomachs from 4-day-old neonates, diluted 1:2 with RPMI, and centrifuged at 200g. The top layer was discarded, and the whey was collected and stored at –20°C until measurement of immunoglobulin levels, as described above.

Cytokine production

Splenocytes (5×10^6 cells per well) were isolated and restimulated *in vitro* with 200 µg/mL OVA in culture medium (RPMI medium supplemented with 10% FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin) 1 day after airway function testing. Cytokines were measured in supernatants from restimulated spleen cells after 3 days by using DuoSet ELISA kits (R&D Systems, Minneapolis, Minn), according to the manufacturer's instructions. For measurement of IFN-γ levels in supernatants of naive splenocytes after OVA stimulation, CD4⁺ and CD8⁺ T cells were isolated by means of magnetic cell

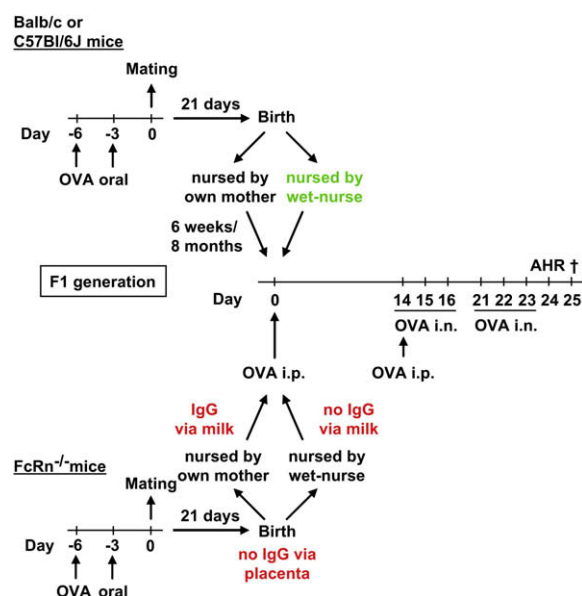


FIG 1. Tolerization and immunization protocol. For induction of oral tolerance, BALB/c, C57Bl/6J, or FcRn-deficient mice, which cannot transport immunoglobulin through the placenta, received OVA orally on days –6 and –3 before mating. Some neonates were given to naive wet nurses immediately after birth. The offspring at the age of 6 to 10 weeks or at the age of 7 to 8 months were immunized intraperitoneally with OVA on days 0 and 14, followed by OVA administered intranasally on days 14 to 16 and 21 to 23. One day after the last intranasal challenge (day 24), AHR was measured. Mice were killed on day 25. *i.n.*, Intranasal; *i.p.*, intraperitoneal.

sorting (T-cell isolation kits; Miltenyi Biotec, Bergisch Gladbach, Germany) from offspring of tolerant or naive mothers. CD4⁺ or CD8⁺ T cells (1×10^6) were cultured with 2×10^6 irradiated splenocytes from BALB/c mice and stimulated with OVA (200 µg/mL).

Iterative slide-based cytometry

For detection of antigen-specific cells, splenocytes (5×10^6 cells per well) of pups of tolerized mothers and control animals were restimulated in culture plates with OVA (200 µg/mL) or mock control for 72 hours. Brefeldin A (1 µL/mL; GolgiBlock, BD Biosciences, Mississauga, Ontario, Canada) was added 2 hours before harvesting the cells. Before analysis, a fraction enriched for activated cells was prepared by depleting naive B cells by means of MACS (B cell isolation kit, Miltenyi Biotec). The B cell-depleted fraction was washed and resuspended in 1 µL of PBS. Iterative slide-based cytometry was performed by using cell-adhesive microfluidic channels (1-µL sample volume; NanoSky, Hannover, Germany) on a motorized Axioskop 2 (Zeiss, Berlin, Germany) controlled by CellHopper software (NanoSky, Germany). Spleen cells of samples and control animals were immobilized on the chip surface, fixed with CellFix (BD Bioscience), and subjected to comprehensive immunophenotyping with the following marker set (all antibodies were phycoerythrin conjugated): surface markers, CD3, CD4, CD8, CD11b, CD11c, CD19, CD25, CD30, CD44, CD45, CD45R, CD49b, CD69, CD70, CD80, CD86, CD154, CD152, CD137, CD134, glucocorticoid-induced tumor necrosis factor receptor (GITR), T-cell receptor (TCR) 2, TCR1, MHC-II, and F4/80; intracellular markers, IFN-γ, IL-4, IL-5, IL-10, IL-2, and TNF-α.

Statistical analysis

At least 3 female mice per group per experiment were tolerized before conception. The offspring of each tolerized mouse was individually marked, and at least 2 mice per littermate were combined to 1 group (4–6 mice per group per experiment) and analyzed in our murine asthma model. The

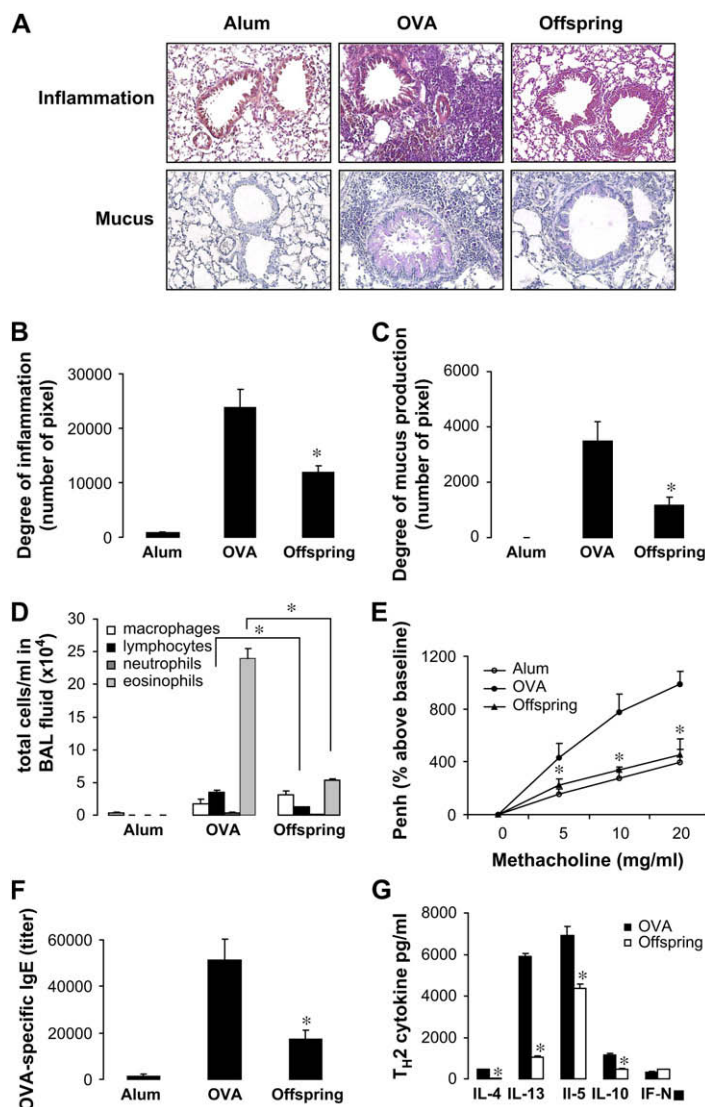


FIG 2. Tolerization of BALB/c mice before conception prevents the development of an asthma-like phenotype in the offspring. Lung tissues from offspring of tolerized mothers revealed reduced inflammation (hematoxylin and eosin stain, original magnification $\times 200$) and mucus hypersecretion (periodic acid-Schiff stain, original magnification $\times 200$) in the OVA-immunized group compared with that seen in the OVA-immunized offspring from naive mothers (**A**). This was confirmed by an objective, investigator-independent, computer-based quantification of lung inflammation (**B**) and mucus hypersecretion (**C**). Cell numbers in the BAL fluid (**D**), AHR (**E**), OVA-specific IgE levels (**F**), and T_H2 cytokine production (**G**) of splenocytes from the OVA-immunized offspring of tolerized mothers were significantly reduced compared with those seen in the OVA-immunized offspring of naive mothers. Data are expressed as means $\pm$ SEMs ($n \geq 9$ animals per group). $*P < .05$, 1-way ANOVA and the Bonferroni multiple comparisons test. *Penh*, Enhanced pause.

experiment was repeated in this way at least once. One-way ANOVA and the Bonferroni multiple comparisons test was used to determine the statistical significance of differences between groups.

RESULTS

Tolerization of BALB/c mice before conception prevents the development of an asthma-like phenotype in the offspring

In a murine model we have observed that tolerance to a specific antigen can be induced by means of oral administration of the

antigen, preventing the development of an asthma-like phenotype (see Fig E1 in this article's Online Repository at www.jacionline.org). The aim of the present study was to investigate whether tolerance to a specific antigen and its protective effect can be transferred from the mother to the pup when tolerance is induced in the mother before pregnancy. For this purpose, female mice were tolerized before pregnancy, and the offspring of these mothers (F1 generation) were immunized with the same antigen at the age of 6 to 10 weeks in a 3-week protocol (Fig 1). Afterward, these mice were analyzed in our well-established murine asthma model^{11,12} and compared with the offspring of naive mothers.

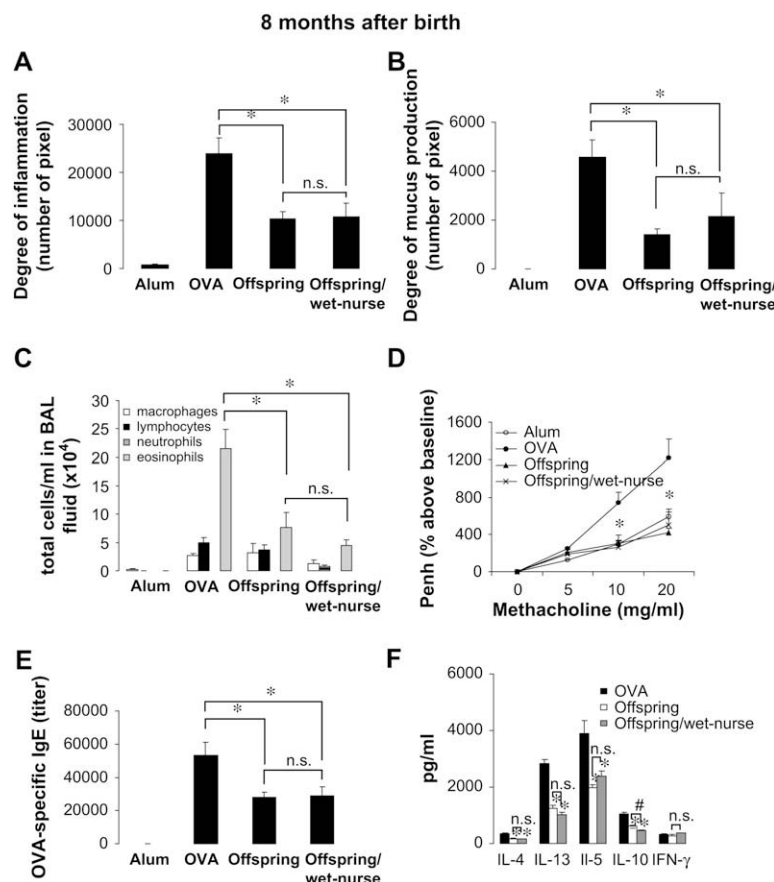


FIG 3. Transfer of tolerance from mothers to offspring is long-lasting and not crucially dependent on breast-feeding. BALB/c mice were tolerized to OVA before mating. Some neonates were given to naive wet nurses immediately after birth. The offspring were immunized with OVA at the age of 7 to 8 months (see Fig 1). Inflammation (A) and mucus production (B) quantified by using a computer-based image-analyzing program, total cell number in BAL fluid (C), AHR (D), OVA-specific IgE serum levels (E), and T_H2 cytokine production (F) in spleen cell cultures were significantly reduced, even when the offspring was immunized as late as 7 to 8 months after birth. Data are expressed as means $\pm$ SEMs ($n \geq 9$ animals per group). * $P < .05$, 1-way ANOVA and the Bonferroni multiple comparisons test. n.s., Not significant; Penh, enhanced pause.

Tolerance induction in the mothers before pregnancy with OVA almost abrogated the development of an asthma-like phenotype in the F1 generation, whereas the offspring of naive mothers had an asthma-like phenotype in response to OVA immunization (Fig 2). Airway inflammation and AHR were not induced by OVA immunization in the offspring of tolerized mothers, as demonstrated by substantially less inflammatory infiltrates in hematoxylin and eosin-stained lung sections and low cell numbers in the BAL fluid (Fig 2, A, B, and D) and by reduced AHR (Fig 2, E). In addition, periodic acid-Schiff-stained lung sections showed a significant reduction of mucus production in the offspring of tolerized mothers compared with the offspring of naive mothers (Fig 2, A and C). Furthermore, the offspring of tolerized mothers produced significantly less allergen-specific IgE (Fig 2, F) and IgG1 (OVA-specific IgG1 [titer]: offspring of naive mothers, $471,725 \pm 101,847$; offspring of OVA-tolerized mothers, $121,267 \pm 24,388$; $P < .05$), as well as T_H2 cytokines, in spleen cell culture supernatants compared with the offspring of naive mothers (Fig 2, G).

To investigate whether the maternofetal transfer of tolerance is allergen specific, we sensitized the offspring of mothers that were tolerized to OVA with β -lactoglobulin (BLG), a heterologous antigen. AHR, eosinophilic inflammation, and BLG-specific

serum IgE levels were comparably increased in both the offspring of naive mothers and of OVA-tolerized mothers, demonstrating that the transferred tolerance is allergen specific (see Fig E2 in this article's Online Repository at www.jacionline.org).

Transfer of tolerance from mothers to offspring is long-lasting and not crucially dependent of breast-feeding

The offspring of tolerized mothers and naive mothers were grown in our animal facility for several months without any contact with OVA. Seven to eight months after birth, these mice were immunized systemically and intranasally with OVA, according to our routinely used protocol (see the Methods section). In the offspring of tolerized mothers, the asthma phenotype was still significantly reduced compared with that seen in the offspring of naive mothers, demonstrating the long-lasting protective effect of tolerance transfer (Fig 3). Some of the neonates of tolerant mothers did not receive breast milk from their own mothers but were cross-fostered immediately after birth by naive mice to analyze the role of breast-feeding. These animals were equally protected against the development of an asthma-

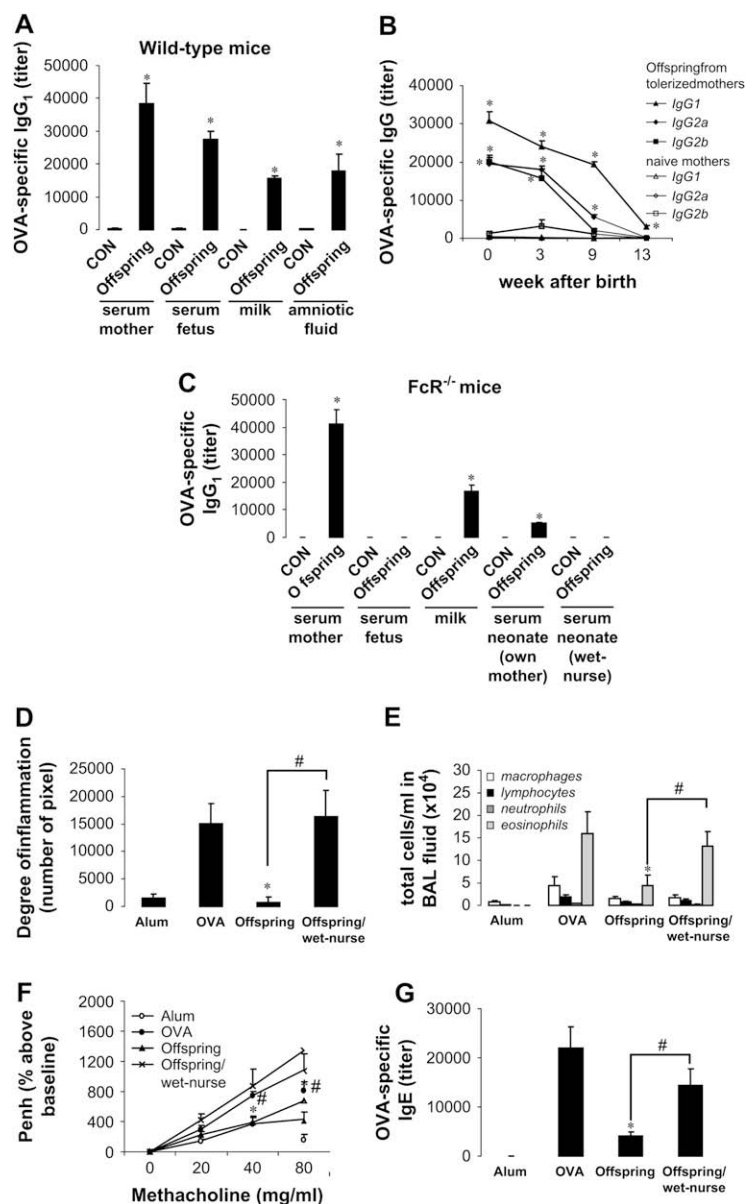


FIG 4. Maternofetal transfer of tolerance is mediated by antigen-specific IgG antibodies. In wild-type animals OVA-specific IgG₁ levels were increased in the sera of pregnant mice and the fetus, the amniotic fluid, and the milk when mice were tolerized before conception (**A**). The decrease of antigen-specific antibodies after birth was time dependent (**B**). FcRn^{-/-} mice were tolerized before mating, and some neonates were weaned by naive wet nurses immediately after birth. OVA-specific IgG₁ levels were increased in the sera of pregnant mice, in the milk, and in the sera of the wet-nursed neonates when mice were tolerized before conception but not in the sera of the fetus and of the cross-fostered neonate (**C**). Lung inflammation (**D**), total cell numbers in the BAL fluid (**E**), AHR (**F**), and OVA-specific IgE serum levels (**G**) were significantly reduced in the offspring of tolerized 6.129X1-Fcgrt^{tm1Dcr} mothers but not in their offspring nurtured by wet nurses. For Fig 4, A through C, data are expressed as means $\pm$ SEMs ($n \geq 5$ animals per group). * $P < .05$. For Fig 4, D through G, data are expressed as means $\pm$ SEMs ($n \geq 9$ animals per group). * $P < .05$, 1-way ANOVA and the Bonferroni multiple comparisons test. CON, Control; Penh, enhanced pause.

like phenotype as their siblings. These data show that breast milk is not obligatory for maternofetal tolerance transfer (Fig 3).

Maternofetal transfer of tolerance is mediated by antigen-specific IgG antibodies

To further analyze possible mechanisms of maternofetal tolerance transfer, we investigated the role of immunoglobulins

because most of them (eg, IgG antibodies) can pass the placental barrier and are therefore possible mediators of the tolerance transfer. Sera from the mother and fetus, as well as amniotic fluid, were taken for IgG analysis from pregnant mice that had been tolerized before conception 3 to 1 days before delivery (Fig 1). Breast milk was extracted 4 days after birth. OVA-specific IgG₁ levels were significantly increased in the sera of pregnant mice and fetuses, in the amniotic fluid, and in the milk of mice that

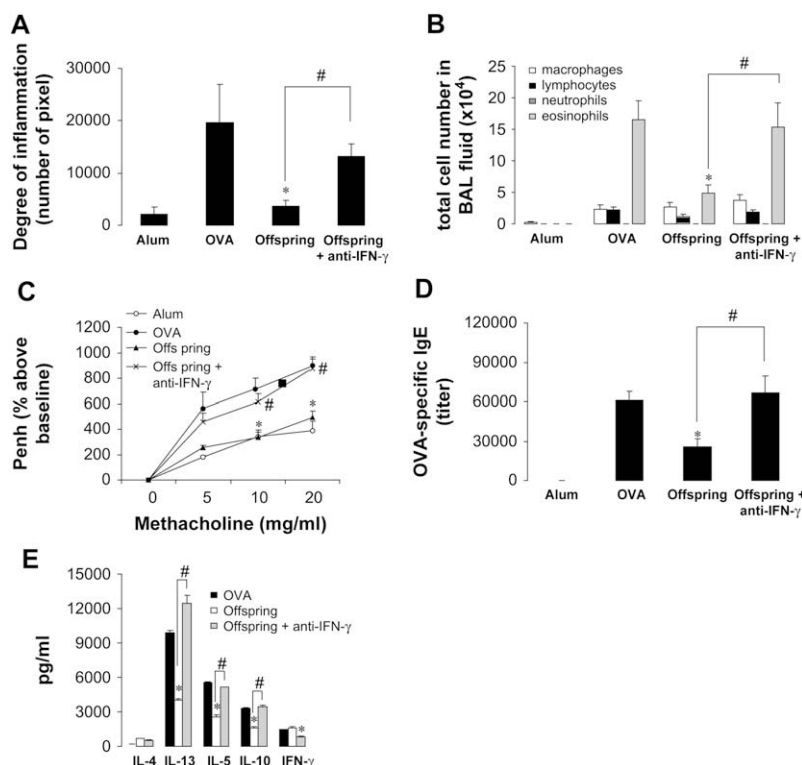


FIG 5. Maternofetal transfer of tolerance depends on IFN- γ . *In vivo* blocking of IFN- γ significantly increased the degree of inflammation in the lung (A), the number of eosinophils in the BAL fluid (B), the methacholine-induced AHR (C), the OVA-specific IgE levels (D), and T_H2 cytokine production (E) from spleen cell cultures, although it had no effect on the asthma phenotype of the OVA-immunized control group that was born of naive mothers. The control mAb had no effect on any of the tested groups (data not shown). Data are expressed as means $\pm$ SEMs ($n \geq 9$ animals per group). * $P < .05$, 1-way ANOVA and the Bonferroni multiple comparisons test. Penh, Enhanced pause.

had been tolerized before pregnancy (Fig 4, A). Similarly, OVA-specific IgG2a and IgG2b levels were increased (data not shown). However, IgG2a and IgG2b levels were lower and diminished faster than OVA-specific IgG1 levels, which remained increased in the offspring up to 12 weeks after tolerization (Fig 4, B). OVA-specific IgE was not detectable.

To directly analyze the role of antigen-specific immunoglobulins in the transfer of tolerance, we tolerized FcRn-deficient female mice, which cannot transfer IgG antibodies across the placenta, and mated them with FcRn-deficient male mice. The offspring of these animals were nursed either by their own tolerized mothers or by naive foster mice so that some pups received IgG not through the placenta but through breast milk and some received IgG neither through the placenta nor through breast milk (Fig 1). First we ensured that FcRn^{-/-} mice can be tolerized and immunized as well as wild-type animals (data not shown) and that IgG1 is indeed not transferred through the placenta in these animals: the offspring of FcRn^{-/-} mice that were tolerized with OVA before conception had no detectable OVA-specific IgG1 in sera (Fig 4, C), demonstrating that immunoglobulin is indeed not transferred through the placenta in significant amounts in these animals. In our model FcRn^{-/-} mice transferred tolerance to the offspring only when the missing diaplacental IgG transfer was compensated by IgG transfer through breast milk from tolerant mothers but not when the offspring were cross-fostered by naive mice. Complete interruption of IgG transfer in FcRn^{-/-}

mice that were cross-fostered by naive mice abrogated tolerance transfer and asthma protection (Fig 4, D-G). This demonstrates that the relatively low IgG1 titer in the neonates nursed by their own tolerant mothers was already sufficient to protect the offspring against an asthma-like phenotype.

This finding demonstrates that (1) IgG transfer is crucial for tolerance transfer and (2) one route of IgG transfer, either diaplacental or through breast milk, is sufficient for tolerance transfer. The important role of IgG was further supported by data showing a decreased asthma-like phenotype of pups born to naive mothers but nursed by tolerized mothers (see Fig E3 in this article's Online Repository at www.jacionline.org) that were also protected and pups born to OVA-tolerized FcRn^{-/-} mothers but foster nursed by OVA-tolerized wild-type mothers (see Fig E4 in this article's Online Repository at www.jacionline.org).

Maternofetal transfer of tolerance depends on IFN- γ

The long-lasting suppression of the asthma-like phenotype in the F1 generation cannot be explained by the transferred antibodies alone because they are no longer detectable in the offspring 16 weeks after tolerization of the mothers (Fig 4, B). Interestingly, splenocytes isolated from up to 4-month-old naive offspring of OVA-tolerized mothers, which had not directly been in contact with OVA before, showed a significantly increased

production of IFN- γ in response to OVA stimulation, suggesting a role of these cells for the development of tolerance in the F1 generation (see Fig E5, A, in this article's Online Repository at www.jacionline.org). No other cytokines, such as IL-4, IL-13, or IL-10, were detectable. *In vitro* stimulation of MACS-separated splenocytes with OVA (data not shown), as well as FACS staining, revealed CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells as sources for IFN- γ (see Fig E5, B), as well as in an early (4 weeks) and a late stage (4 months) after birth. Additionally, we found IFN- γ -producing CD3⁺CD4⁺CD8⁺ double-negative T cells. However, the natural killer T-cell marker CD49b was not expressed. There were no other sources for IFN- γ production, such as B cells, dendritic cells, or macrophages, detectable. The IFN- γ -producing T cells had the typical phenotype of activated T_H1 memory T cells and expressed CD44⁺TCR α / β ⁺CD62L⁺CD69⁺CD25⁺GITR⁺CD11c⁺ on their surfaces, whereas costimulatory markers (CD154, CD152, CD137, CD134, CD70, and CD30) were not expressed (data not shown).

When IFN- γ was inhibited *in vivo* in the offspring by means of repeated application of anti-IFN- γ mAb during the sensitization period (days 0, 6, 13, and 20), the offspring of female mice that had been tolerized before conception were no longer protected from an asthma-like phenotype (Fig 5), whereas the anti-IFN- γ mAb did not modify the immune response of the control animals (inflammation of anti-IFN- γ mAb-treated control mice: $21,542 \pm 1624$ pixels; number of eosinophils of anti-IFN- γ mAb-treated control mice: $7.97 \pm 1.32 \times 10^4/\text{mL}$; IgE levels of anti-IFN- γ mAb-treated control mice, $71,235 \pm 14,325$ [titer]).

DISCUSSION

In the present study we demonstrate in a murine asthma model that immunologic, antigen-specific tolerance induced by oral antigen application before pregnancy can be transferred from the mother to the offspring and protects against the development of an asthma-like phenotype in the F1 generation. Importantly, the offspring of tolerized mothers were protected, even when immunized as late as 8 months after birth. Our data show that maternal immunologic memory has a significant and persistent effect on the immune response of the offspring, indicating that, for example, allergy prevention strategies might be effective for more than 1 generation.

Our findings even surpass recent evidence from epidemiologic studies that trace the temporal sequence of events leading to the development of allergies back to prenatal exposures, namely exposures of the mother during pregnancy. For instance, prenatal exposure to a farming environment rich in microbial compounds through the mother during pregnancy leads to upregulation of receptors of the innate immune system and is inversely correlated with the incidence of allergic rhinoconjunctivitis and asthma in the children.⁵ Exposure of the pregnant mother to allergens^{13,14} correlates with sensitization of the child to these allergens, supporting the notion that when assessing risk factors of allergies in an infant's medical history, attention must be paid to environmental exposures affecting the mother.

In our study we could show 2 different factors that are crucially involved in maternofetal transfer of tolerance: transfer of antigen-specific IgG through the placenta or mother's milk and IFN- γ produced by CD4⁺ and CD8⁺ T cells in the protected offspring. Interruption of IgG transfer from mother to pup and inhibition of

IFN- γ in the offspring of tolerized mothers both abrogated the protective effect of maternal tolerance in the F1 generation.

It is known that maternal antibodies, in particular IgG antibodies, are actively transferred through the placenta to the fetus and through maternal milk to the neonate. They mediate maternal immunologic memory that is essential for the survival of the fetus and newborn.¹⁵ In the present study we measured significantly increased antigen-specific IgG serum levels in tolerized mothers, their fetuses, and their newborns and in the breast milk in contrast to those seen in naive control animals and their offspring. This indirectly indicated that IgG transfer might be involved in maternofetal tolerance transfer. An important role of IgG in tolerance has also been shown in other mouse studies in which allergen-specific IgG antibodies either induced in female rats by means of immunization¹⁶ or injected into pregnant mice as preformed mAbs¹⁷ were transferred to the offspring and reversed a seemingly genetically based IgE high-responder phenotype.

To directly investigate the role of IgG in the maternofetal tolerance transfer, we investigated FcRn^{-/-} mice in our model. Recent studies have shown that FcRn, a nonclassical MHC-I protein, is responsible for an efficient transfer of IgG antibodies across the placenta¹⁸ during pregnancy. Therefore in FcRn^{-/-} female mice IgG is not transported through the placenta.^{18,19} In our model FcRn^{-/-} mice transferred tolerance to the offspring only when the missing diaplacental IgG transfer was compensated by IgG transfer through breast milk from tolerant mothers but not when the offspring were cross-fostered by naive mice. Complete interruption of IgG transfer in FcRn^{-/-} mice that were cross-fostered by naive mice abrogated tolerance transfer and asthma protection. This finding demonstrates that (1) IgG transfer is crucial for tolerance transfer and (2) one route of IgG transfer, either diaplacental or through breast milk, is sufficient for tolerance transfer. This was supported by results showing a decreased asthma-like phenotype after nursing of pups from naive or tolerized FcRn^{-/-} mice by OVA-tolerized wild-type mice. Nevertheless, it is possible that other factors than IgG1 in the milk, such as regulatory T cells, as shown by Verhasselt,²⁰ might be partly responsible for the observed protective effects. However, in our study we could not find regulatory T cells or specific cytokines (IL-10 and IFN- γ) in the milk of tolerized female mice (data not shown).

Because tolerance in the offspring lasted longer than antigen-specific IgG antibodies were detectable, the maintenance of tolerance seems to be mediated by the IFN- γ -producing memory T cells that can be detected in the offspring of tolerized mice during the whole observation period. The most likely hypothesis for generation of antigen-specific memory in the offspring is the transfer of OVA-anti-OVA immunocomplexes through the placenta or breast milk, their uptake by fetal dendritic cells, and generation of OVA-specific T-cell memory in the fetus.^{21,22} The detection of IFN- γ production of memory T cells in response to OVA stimulation in the offspring that did not receive any OVA after birth clearly supports this hypothesis, although we were not able to detect OVA-anti-OVA complexes in the newborns with currently available detection methods. Several studies have shown the regulatory potential of IFN- γ , which is able to counter-regulate T_H2-dominated immune responses, such as allergic asthma.²³⁻²⁷ Moreover, it has been shown that the induction of maternal T_H1 immunity²⁸ or the administration of IFN- γ during pregnancy²⁹ protects the offspring against the development of

allergic asthma. However, in our study maternal tolerization did not induce T_H1 immunity or IFN- γ production. In contrast, we could find $CD3^+CD4^+$ and $CD3^+CD8^+$ IFN- γ -producing memory T cells only in the naive offspring after OVA stimulation, and we could show that inhibition of IFN- γ in the pups of tolerant mothers reversed the protective effect of maternal tolerance. These findings support the concept that IFN- γ is crucially involved in the maintenance of tolerance in our study. The $CD3^+CD4^+$ and $CD3^+CD8^+$ IFN- γ -producing cells expressed GTR and CD25, both markers for highly activated and regulatory T-cell types.^{30,31} However, they did not coexpress IL-10 and FoxP3, as described for regulatory T_H1 cells.³¹⁻³³ No other OVA-specific T-cell subtypes, such as T_H2 cells or naturally occurring regulatory T cells, could be detected in the tolerant offspring.

Because maternal tolerance induction with OVA had a marked effect on the development of an OVA-induced asthma-like phenotype in the offspring, we assessed further whether the maternofetal transfer of tolerance is allergen specific by sensitizing the offspring of mothers that were tolerized to OVA with BLG, a heterologous antigen. Our results show that the offspring of OVA-tolerized mothers produced BLG-specific serum IgE levels comparable with those of the offspring of naive mothers in response to sensitization with BLG, implicating an allergen-specific effect of maternofetal tolerance transfer.

Several studies demonstrate a critical role of breast-feeding for the maternal immunologic "priming" of the offspring.³⁴⁻³⁶ However, epidemiologic studies of breast-feeding have provided conflicting results about whether it is protective^{35,36} or even a risk factor^{37,38} for the development of asthma and atopy in children. In our study we could show that breast-feeding is not mandatory but is sufficient for the maternofetal tolerance transfer: on the one hand, in absence of antibody transfer through breast milk because of cross-fostering, placental antibody transfer alone mediated tolerance transfer from mother to offspring, and on the other hand, in the absence of a functional antibody transfer through the placenta, breast milk alone mediated the tolerance transfer from mother to offspring. Therefore our finding emphasizes the important role for breast milk as a carrier of immunologic memory and might implicate opportunities for prevention strategies for children that are at high allergy risk.

However, because the immunologic meaning of breast milk seems to depend on the immunologic state of the mother, it might also be a risk factor for the offspring that is dependent on the maternal situation. In a recent study Leme et al³⁹ showed the important role of breast milk in the transmission of the asthma risk in a murine model and demonstrated a sufficient, but not necessary, role of breast milk to transfer increased asthma susceptibility to the offspring.

Our results demonstrate that immunologic tolerance can be transferred from the mother to the offspring when the mother is tolerized before pregnancy. We could show here that the mother plays a special role not only because the uterine environment influences the development of the fetus but also because the immune status of the mother before pregnancy is critical for the immune response of the offspring to antigens like allergens. This effect persists far beyond the perinatal period.

Our data show that maternal immunologic memory has a significant and persistent effect on the immune responses of the offspring.

We thank Jana Bergmann, Marita Reiprich, and Bjoern Zweigait for their excellent technical assistance and Burkhard Tümmler for critical reading of the manuscript.

Clinical implications: Primary allergy prevention strategies might start before pregnancy.

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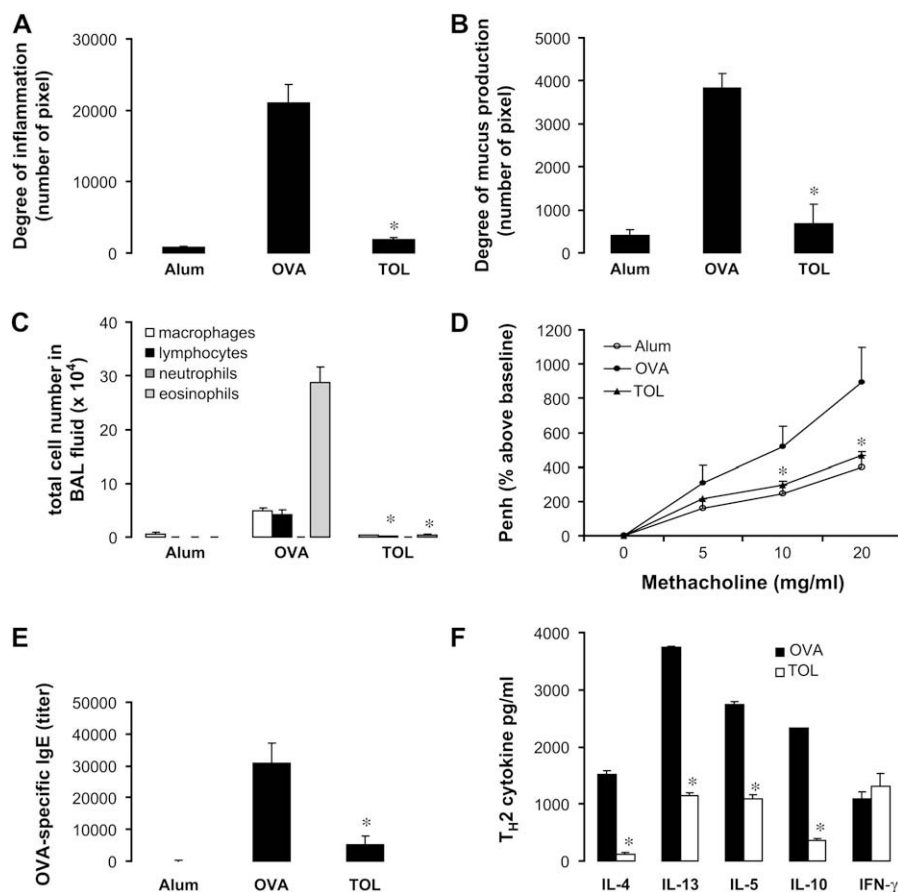


FIG E1. Tolerization of BALB/c mice prevents the development of an asthma-like phenotype in response to the same antigen. BALB/c mice were tolerized to OVA orally (100 mg) on days -6 and -3 and immunized and challenged with OVA, as described in the Methods section. Lung inflammation (**A**), mucus production (**B**), cell numbers in the BAL fluid (**C**), AHR (**D**), OVA-specific IgE serum levels (**E**), and T_H2 cytokine production (**F**) of splenocytes from the OVA-immunized tolerized mice were significantly reduced compared with values seen in the OVA-immunized control mice. Data are expressed as means $\pm$ SEMs ($n \geq 9$ animals per group). * $P < .05$, 1-way ANOVA and the Bonferroni multiple comparisons test. TOL, Tolerized; Penh, enhanced pause.

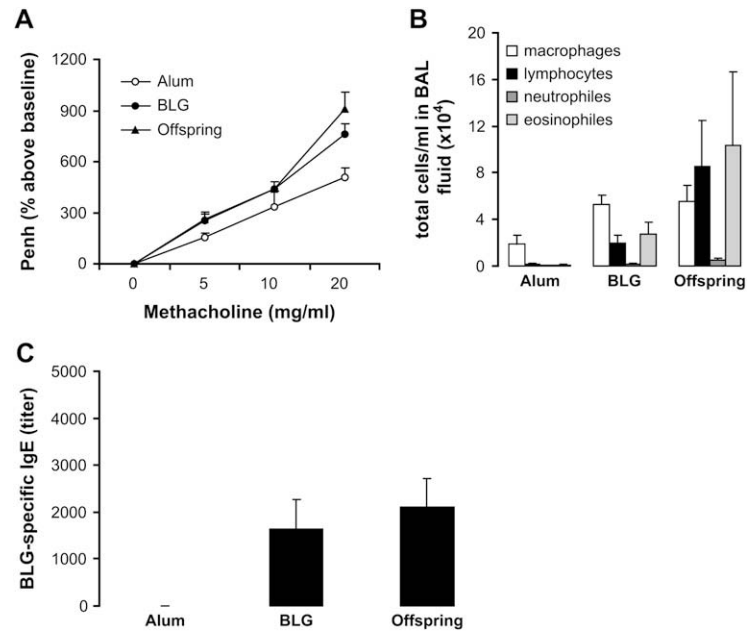


FIG E2. Transfer of tolerance is allergen specific. BALB/c mice were tolerized to OVA before conception, and the offspring were immunized with BLG at the age of 6 to 10 weeks. AHR (**A**), total cell numbers in the BAL fluid (**B**), and BLG-specific IgE levels (**C**) were not affected in the offspring of OVA-tolerized mothers. Data are expressed as means $\pm$ SEMs ($n \geq 9$ animals per group). * $P < .05$, 1-way ANOVA and the Bonferroni multiple comparisons test.

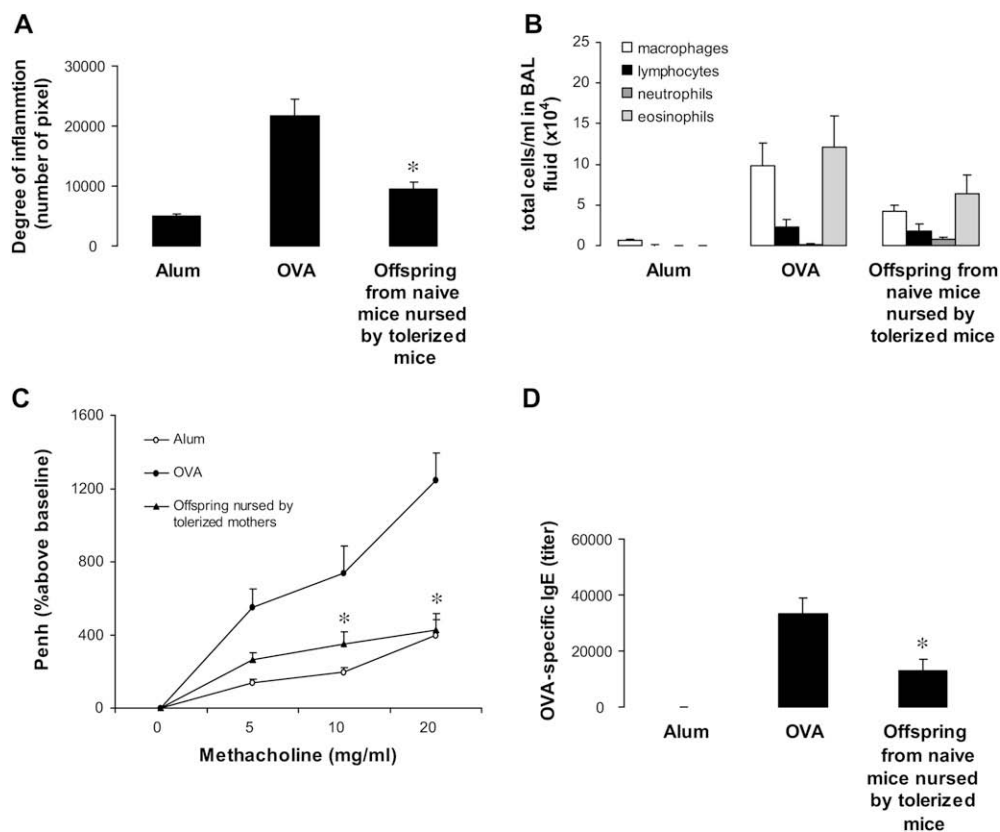


FIG E3. Offspring of naive BALB/c mice foster nursed by mice tolerized before conception are prevented from the development of an asthma-like phenotype. Neonates from naive mice were given to tolerized wet nurses immediately after birth. The offspring were immunized with OVA at the age of 6 to 10 weeks. Inflammation (**A**), total cell numbers in the BAL fluid (**B**), AHR (**C**), and OVA-specific IgE levels in the serum (**D**) were significantly reduced. Data are expressed as means $\pm$ SEMs ($n \geq 7$ animals per group). * $P < .05$, 1-way ANOVA and the Bonferroni multiple comparisons test. *Penh*, Enhanced pause.

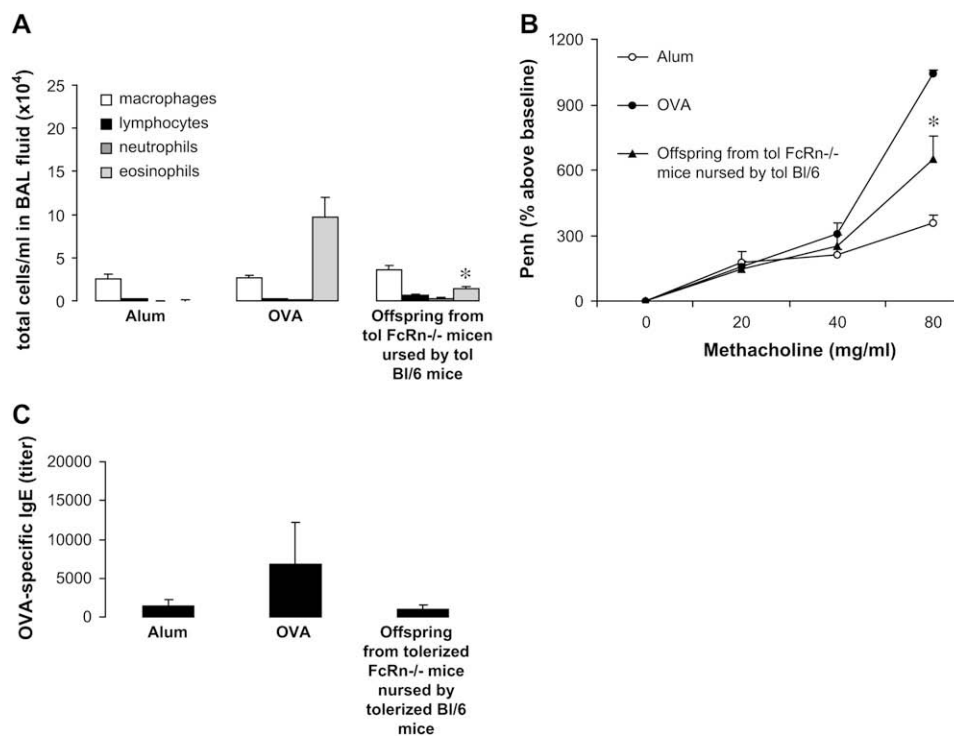


FIG E4. Offspring of tolerized FcRn^{-/-} mice foster nursed by mice tolerized before conception are prevented from development of an asthma-like phenotype. Total cell numbers in the BAL fluid (**A**), AHR (**B**), and OVA-specific IgE serum levels (**C**) were significantly reduced. Data are expressed as means $\pm$ SEMs ($n \geq 7$ animals per group). * $P < .05$, 1-way ANOVA and the Bonferroni multiple comparisons test.

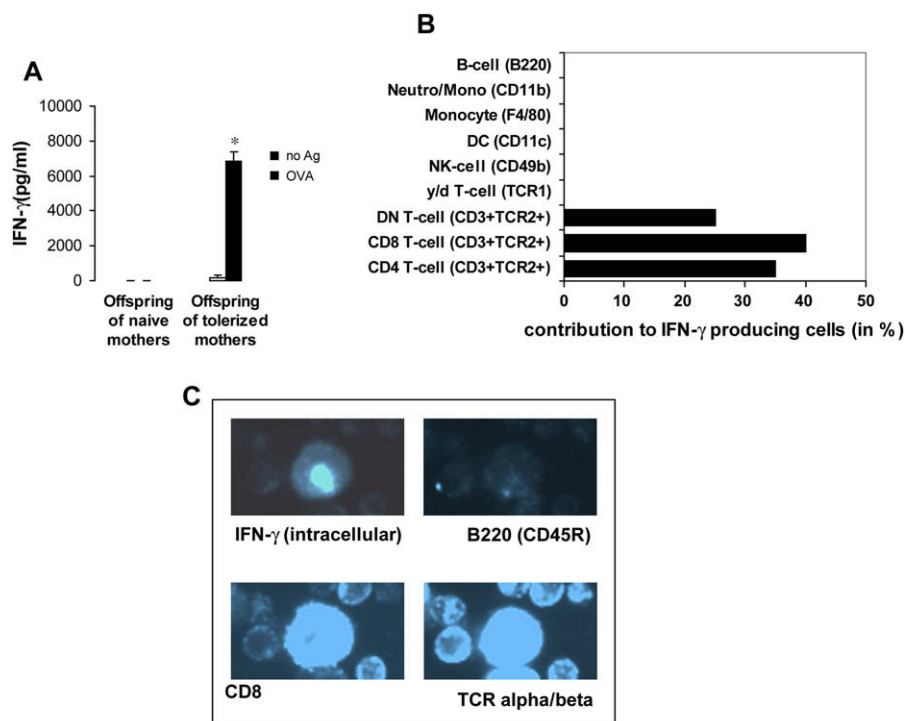


FIG E5. Antigen stimulation of splenocytes induces IFN- γ -producing memory T cells in naive mice of tolerized mothers. Splenocytes were isolated from the offspring of BALB/c mice (4 months old) that had been tolerized to OVA before conception. OVA stimulation induced IFN- γ production in spleen cells, although the offspring had not directly been in contact with OVA before (**A**). Phenotype of OVA-specific IFN- γ -producing splenocytes from offspring of tolerized mothers (**B**). Expression of lineage markers (DN, CD4 and CD8 double-negative T cells). Exemplary picture of an IFN- γ -producing CD8 T cell: the cell shows an activated, blastic phenotype (**C**). $n \geq 3$ animals per group. $*P < .05$. Fig E5, A, Data are expressed as means $\pm$ SEMs from 3 independent experiments. Fig E5, B and C, Representative results from 1 of 3 experiments are shown. NK, Natural killer.