



Tolerogenic microenvironment in neonatal period induced by maternal immunization with ovalbumin

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ABSTRACT

Maternal immunization with allergens, such as ovalbumin (OVA), can inhibit the development of an allergic response in offspring. The regulatory mechanisms seem to be mediated by maternal antibodies (MatAbs) and factors generated by the maternal–fetal interface. The aim of this study was to verify the pathways of inhibitory Ab transference after maternal immunization with OVA and the effect of the offspring's dendritic cells (DCs) on the generation of regulatory T (Treg) cells. We verified that preconceptional OVA immunization induces high levels of proinflammatory and regulatory cytokines in the amniotic fluid, allowing the transference of high levels of anti-OVA IgG1 Abs to the offspring. Using an adoptive nursing protocol, we verified that maternal immunization leads to MatAb transference by the placental route and by breastfeeding contribute to the inhibition of anaphylactic IgE and IgG1 Ab responses in immunized offspring. We observed that maternal immunization decreased eosinophil numbers in recovered bronchoalveolar lavage fluid and in the lung tissue, whereas with a lack of control of airway responsiveness to methacholine. Maternal immunization induced in young offspring a decreased percentage of CD11c+ DCs expressing MHC class II and CD40 molecules. Moreover, DCs from both groups of offspring when pulsed with OVA, were able to induce Treg cells *in vitro*. Similarly, OVA immunization at the neonatal stage increased the frequency of Treg cells, regardless of the mother's immunization status. These findings emphasize that maternal immunization leads to a complex interaction of regulatory factors, with MatAbs, DCs and Treg cells affecting the tolerance of offspring during an allergic response.

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Introduction

Early postnatal exposure to an allergen has been shown to enhance the risk of subsequent allergic reactivity to the same allergen in adult life (Warner, 2004). The hypothesis that early events occurring during fetal development and/or in childhood could determine allergic disease in adulthood is supported by clinical and epidemiological observations (Prescott et al., 2006). For these reason is relevant to investigate maternal vaccination strategies

to prevent early allergic responses due to post-natal allergen exposure.

Neonatal defense against bacterial, viral, protozoan and fungal infections in utero or after delivery is dependent on maternal immunity because neonates lack immunological memory and often have a slower capacity to develop immune responses (Adkins et al., 2004; Marodi, 2006; Levy, 2007; Futata et al., 2012). The neonatal dendritic cell (DC) system is often immature and exhibits decreased production of Th1-polarizing cytokines and is thus biased toward Th2-type responses (Adkins et al., 2004; Levy, 2007). While the neonatal immune system is immature, the system is highly plastic, and mature immune function can be achieved in certain specialized circumstances, e.g. BCG vaccination (Adkins et al., 2004). Moreover, maternal antibodies (MatAbs) can shape the B cell repertoire of the offspring long after the MatAbs themselves become undetectable (Fink et al., 2008).

Preconceptional immunization with allergens has shown an interesting ability to efficiently inhibit IgE development in offspring (Fusaro et al., 2002; Victor et al., 2003). A schedule of maternal

Abbreviations: MatAb, maternal antibodies; d-o, day old; OVA, ovalbumin; Treg, regulatory T cells; PCA, passive cutaneous anaphylaxis.

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immunization using low amounts of allergen did not induce allergen sensitization in offspring (Victor et al., 2003). Rat models have shown the suppression of the allergen-specific IgE response in offspring from both actively and passively immunized mothers (Jarrett and Hall 1983). The suppression of allergen-specific IgE antibody (Ab) production in offspring could not be induced by paternal immunization or genetic factors (Nygaard et al., 2013). Effective preconceptional immunization to control the offspring allergic response may also be verified even when mothers were subsequently exposed to high doses of allergen during pregnancy or breastfeeding (Fusaro et al., 2009). Maternal immunization upregulates the inhibitory IgG receptor FcγRIIb on the B cells of neonates at a very early age (3 days old (d-o)) and in young mice (Victor et al., 2010).

Several mechanisms that act synergistically, including MatAbs, regulatory T (Treg) lymphocytes and factors that are major components of maternal immunomodulation, are required to prevent allergic responses in offspring. The mechanisms underlying the IgE Ab response resulting from maternal immunization involve a complex interaction among inhibitory MatAbs, regulatory cytokines, including TGF-β (Fusaro et al., 2007) and Treg cells (Verhasselt et al., 2008) that remains incompletely understood.

Here, we investigated the influence of the MatAb source and the presence of cytokines in maternal compartments, such as the amniotic sac, and in milk from ovalbumin (OVA)-immunized mice. Therefore, the DC phenotype and the cells' ability to induce Treg cells were characterized as possible mechanisms of suppressing the allergic IgE response of offspring.

Materials and methods

Animals

BALB/c mice of both sexes (8–10 weeks old) were obtained from the animal facilities at the School of Medicine, University of São Paulo, and bred in our pathogen-free animal facility. Wistar Furth rats (3–4 months old) were used to evaluate the passive cutaneous anaphylaxis (PCA) reaction. All experiments were approved by the Ethics Committee for Animal Research of the Institute of Biomedical Sciences.

Protocols

Maternal immunization

Female BALB/c mice were subcutaneously (s.c.) immunized with 150 µg OVA (grade V; Sigma–Aldrich, St. Louis, MO, USA) in 6 mg Al(OH)₃ and i.p. boosted with 100 µg OVA without adjuvant on days 10 and 20 after immunization, as previously described (Fusaro et al., 2009). One day later, the females were mated with non-immunized male BALB/c mice.

Offspring immunization

Three-day-old mice of both sexes were i.p. immunized with 10 µg OVA in 0.6 mg Al(OH)₃ and then i.p. boosted 10 days later with 10 µg OVA in saline solution and bled after 7 days, as previously described (Victor et al., 2010). Other groups were immunized i.p. at an early stage (25 d-o) with an injection of 150 µg OVA and boosted 10 days later with 100 µg OVA.

Adoptive nursing mother protocol

Female BALB/c mice were immunized with OVA, boosted twice and mated with non-immunized male BALB/c mice at 21 days after immunization. Offspring were nursed by a foster mother soon after delivery; the group of offspring from immunized (Im) mothers was nursed by non-immunized (non-Im) mothers and vice versa.

Offspring were immunized with OVA at 25 d-o and boosted 10 days later.

Collection of amniotic fluid and milk

Pregnant mice were subjected to cesarean section at full term (21 days). During the cesarean procedure, samples of amniotic fluid were obtained and stored at –70 °C until use. The milk samples were harvested as previously described (Fusaro et al., 2002).

Passive cutaneous anaphylaxis (PCA) reaction

Mouse IgE antibody causes potent sensitization of rat skin by PCA. Therefore, IgE Ab levels were estimated based on PCA in rats according to the protocol of Mota and Wong (1969). Serum dilutions from immunized mice were inoculated intradermally (50 µL) on the shaved backs of rats. After 18 h, the rats received 0.5 mg OVA in 1.0 mL (0.5%) Evans Blue solution through tail vein injection. PCA titers were expressed as the reciprocal of the highest dilution that yielded a spot larger than 5 mm in diameter. The anaphylactic activity of IgG1 was evaluated based on the PCA reaction in mice, as described by Ovary, 1958. Previously shaved mice received intradermal injections of serial dilutions of pooled sera, which were previously treated for 1 h at 56 °C for heat inactivation of IgE. After 2 h, the mice were intravenously challenged with 0.25 mg OVA in 0.5 mL Evans Blue solution. All tests were conducted in triplicate. PCA titers were expressed as the reciprocal of the highest dilution resulting in a spot larger than 5 mm in diameter.

Determination of cytokines

Amniotic fluid was assayed for cytokines using a Mouse Inflammation Kit Cytometric Bead Array (CBA; BD Pharmingen, CA, USA) and analyzed by flow cytometry (FACSCalibur, BD, San Jose, CA, USA).

Determination of Ab levels

The anti-OVA IgA, IgG1, IgG2a and total IgG Ab levels were measured by ELISA. The results were expressed as titers with respect to serial dilutions of a titrated serum pool from immunized adult mice with high levels of anti-OVA Ab. Abs were purchased from Southern Biotech. Ass., Birmingham, AL, USA.

Assessment of airway responsiveness (AHR) to methacholine

Offspring immunized with OVA at 3 days-old, boosted 10 days later received intranasal challenges of 150 µg OVA at 43 d-o, comprising two doses at 7-day intervals and three doses over three sequential days. After 24 h AHR to methacholine was performed. The resistance of the respiratory system (Rrs) and elastance (Ers) were monitored in mice that were anesthetized, tracheostomized and mechanically ventilated by a flexiVent tube, as previously described in mice (Lima et al., 2002; Itami et al., 2003; Macedo-Soares et al., 2004; Enobe et al., 2006). Different doses of methacholine (3, 30, 300, 3000 and 9000 µg/mL) were aerosolized by an ultrasonic device (Respira Max, NS, São Paulo, Brazil), and data were obtained at 30 s and 1, 2 and 3 min. The Rrs and Ers are shown for each dose of the agonist as a percentage of the baseline level obtained before the next dose of agonist. The dose of methacholine required to reduce resistance and respiratory system compliance to 60% was estimated by a log dose–response curve.

Bronchoalveolar lavage fluid (BALF)

To obtain BALF, bronchoalveolar lavage (BAL) was performed by catheterizing the trachea using an 18-gauge needle in euthanized mice. Each mouse was submitted to three washes with 0.5 mL PBS. BALF samples were immediately placed on ice, the total cells were quantified, and smears were prepared by Wright-Giemsa staining for differential cells counts.

Histology of the lung

Lungs were inflated with 10% neutral buffered formalin before being dehydrated in 70% ethanol. Sections were cut at a thickness of 3 μ m, mounted on slides and staining with Luna was used to quantify tissue eosinophils.

Co-culture of DCs with OVA-specific CD4⁺ T cells

Spleens were obtained aseptically and mashed through cell strainers (BD Biosciences, San Diego, CA, USA). Mononuclear spleen cells (MSCs) were isolated after centrifugation in Ficoll-Hypaque solution (Sigma, St. Louis, MO, USA). After two washes, the cell pellet was diluted in RPMI 1640 containing 10% fetal calf serum (RPMI-S, HyClone III, Logan, UT, USA). DCs from the spleen were purified using anti-CD11c-coated magnetic beads (N418 clone; Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's procedure. CD4⁺ T cells were purified by negative selection from the spleen using magnetic beads. The purity of the CD11c⁺ and CD4⁺ T cells was greater than 90–95%.

Purified splenic DCs from the offspring of Im or non-Im mothers were co-cultured with purified CD4⁺ T cells from DO11.10 transgenic mice. DCs (1×10^5 /well) were incubated with OVA (400 μ g/mL) in a 96-well microplate (Corning-Costar) in RPMI-S for 24 h at 37 °C and 5% CO₂. Purified CD4⁺ T cells from DO11.10 mice were labeled with 5 μ M 5,6-carboxyfluorescein diacetate succinimidyl ester (CFDA, SE; Molecular Probes, Netherlands) for 15 min at room temperature in the dark. The cells were then washed with RPMI-S. Co-cultures with OVA-pulsed DCs (10^5 /well) and CFSE-labeled CD4⁺ T cells were performed at a 1:4 ratio of DCs/CD4⁺ T cells. After 72 h of incubation, CD4⁺CD25⁺Foxp3⁺ T cells were assessed by flow cytometry.

Immunophenotyping of DCs and CD4⁺CD25⁺FoxP3⁺ T cells

Cells were incubated with a rat anti-mouse CD16/32 mAb (2.4G2 clone, BD Biosciences, CA, USA) to avoid nonspecific Ab binding and were then labeled for 30 min with the following mAbs for CD11c, CD40, H-2^d or CD4 and CD25 (BD Biosciences, CA, USA) for 30 min at 4 °C. After washing, the cells were fixed with 4% paraformaldehyde for 10 min at 4 °C and with PBS-0.5% saponin (Sigma) and FITC-conjugated anti-FoxP3 (eBioscience, San Diego, CA) for the detection of Treg cells. After washing, the cells were analyzed by flow cytometry (BD FACSCalibur, CellQuest Pro). A minimum of 250,000 events were collected from the lymphocyte gate.

TGF- β measurement

TGF- β was measured in milk samples by ELISA following the manufacturer's recommendations (Promega, Madison, WI, USA).

Statistics

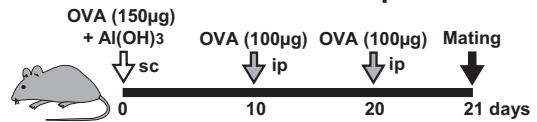
Values for all measurements are expressed as the mean $\pm$ SEM. A comparison between two groups was performed using the Mann–Whitney test; differences were considered significant when the *P* value was ≤ 0.05 . Three or more groups were analyzed using the Kruskal–Wallis test with Dunn's post-test. The data were analyzed using the GraphPad Prism 3.0 software package (GraphPad, San Diego, CA, USA).

Results

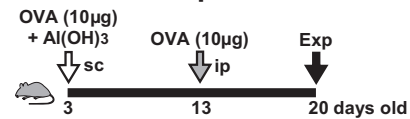
Preconceptional immunization with OVA induces cytokines in amniotic fluid and anti-OVA Ab transference to offspring

BALB/c mice were immunized with OVA as shown in Fig. 1A. To verify the changes in cytokine levels in the maternal–fetal microenvironment, we assessed cytokine levels in amniotic fluid obtained

A. maternal immunization protocol



B. immunization protocol at 3 days old



C. intranasal challenge protocol

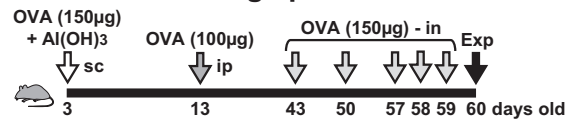


Fig. 1. Maternal and offspring immunization protocols. (A) Maternal immunization: female BALB/c mice were s.c. immunized with 150 μ g OVA in 6 mg Al(OH)₃ and i.p. boosted with 100 μ g OVA without adjuvant on days 10 and 20 after immunization. One day later, the females were mated. (B). Immunization at 3 d-o were performed with 10 μ g OVA in 0.6 mg Al(OH)₃ and i.p. boosted 10 days later with 10 μ g OVA without adjuvant and at 20 d-o were assessed for experiments (Exp). (C) Intranasal challenge protocol: offspring were immunized at 3 d-o and boosted 10 days later. At 43 d-o, offspring received five intranasal challenges of 150 μ g OVA, comprised by two doses at 7-day intervals and three doses over three sequential days. One day after the last intranasal challenge (60 d-o), AHR measurement, lung tissue analysis or BAL collection. s.c.: subcutaneous, i.p.: intraperitoneal, in: intranasal.

by cesarean section and in milk samples obtained from the stomachs of 10-day-old neonates.

Fig. 2A shows that maternal immunization induced increased levels of IL-12, IFN- γ , TNF- α and IL-10 in the amniotic fluid compared with the amniotic fluid of non-Im mothers. In particular, TNF- α and IL-10 reached 300 ± 55 pg/mL and 233 ± 30.5 pg/mL, respectively. The highest cytokine concentrations were those of IL-6 and MCP-1 in both groups of mothers. No detectable levels of cytokines were found in the milk samples. Moreover, the TGF- β levels in the milk samples were similar between the groups of mothers, whereas increased levels of anti-OVA IgA and IgG Abs were detected in the milk samples from Im mothers (Fig. 2B).

To verify the persistence of MatAbs in offspring, we evaluated non-immunized offspring at different ages, from 2 d-o to 80 d-o. Samples were pooled ($n = 10$) to assess anti-OVA IgG1 Ab levels by ELISA and to evaluate anaphylactic IgG1 Ab levels based on the PCA reaction in the mice. Fig. 2C shows that anti-OVA IgG1 Ab levels were found at high levels in 2 d-o offspring due to the placental transference of Abs. The Ab peak at 2 d-o may reflect transfer of antibodies due to breastfeeding, which decreased up to 50 d-o. In addition, anaphylactic anti-OVA IgG1 Abs were not transferred to offspring by the placental pathway, as verified in the 2 d-o mice, whereas the titers peaked at 20 d-o due to breastfeeding. These findings showed that breastfeeding contributes to the transference of both anaphylactic and non-anaphylactic Abs to offspring.

Effect of MatAb transference on IgE response of offspring

Usually, allergic disease development occurs early in life so the effect of maternal allergen exposure on the development of the IgE response was evaluated in the neonatal period (at 3 days). This period in mice have been described as reflecting, to a large extent, the human immune maturation process observed in neonates and infants (Adkins et al., 2004).

After OVA immunization at the neonatal stage (at 3 d-o) as shown in Fig. 1B, offspring from Im mothers showed significant inhibition of the IgE response compared with offspring from

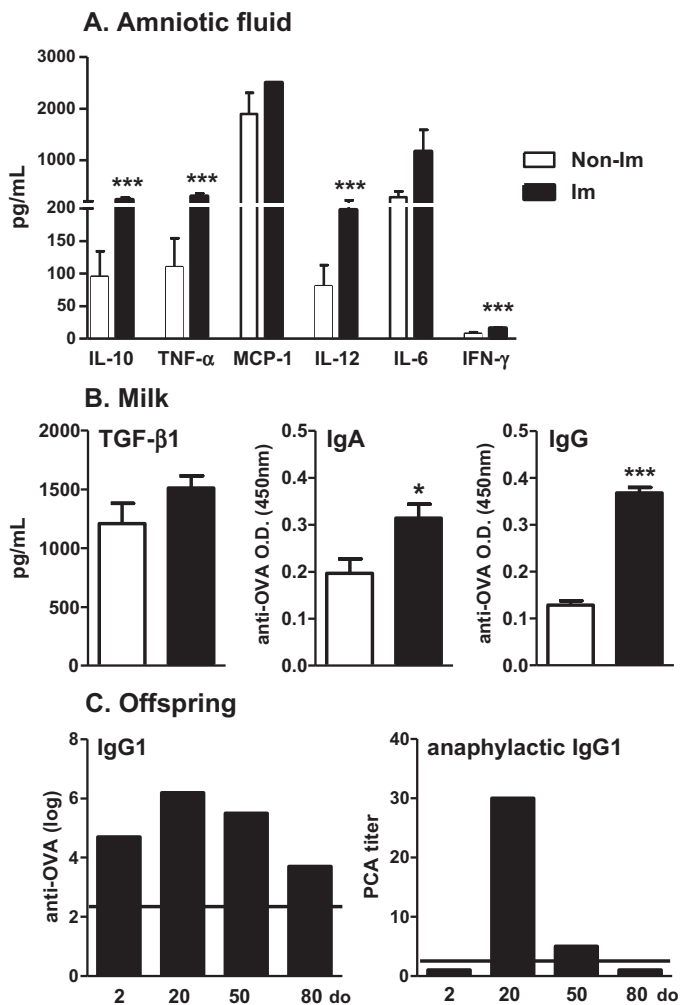


Fig. 2. Maternal immunization with OVA increases cytokine levels in the amniotic fluid and the passive transference of Abs to offspring. BALB/c mice were immunized (Im) or not immunized (Non-Im) with OVA and mated 21 days after immunization. (A) Amniotic fluid from mothers ($n=6$) was harvested by cesarean section, and cytokines were assessed by flow cytometry. (B) Milk samples were obtained from the stomachs of 10-day-old neonates to evaluate TGF-β1 and anti-OVA IgA and IgG Abs by ELISA. The bars represent the mean $\pm$ SEM. * $P < 0.05$, *** $P \leq 0.001$ compared with offspring from non-Im mothers. (C) Offspring from Im mothers were bled at 2, 20, 50 and 80 d-o, and the samples were pooled ($n=10$) to assess anti-OVA IgG1 Ab levels by ELISA and to evaluate anaphylactic IgG1 Ab levels based on the PCA reaction in the mice.

non-Im mothers (Fig. 3A). This finding confirms the results of previous studies (Fusaro et al., 2009; Victor et al., 2010). Anaphylactic anti-OVA IgG1 Ab levels were also significantly decreased in Im offspring from Im mothers compared to Non-Im offspring from Im mother (Fig. 3B).

Anti-OVA IgG1 or IgG2a Ab levels were intensively transferred to offspring, as shown in the non-Im mice from Im mother. The extent of MatAb transference of IgG1 (Fig. 3C) and IgG2a (Fig. 3D) levels, as shown in non-Im offspring, were higher than those levels induced by neonatal immunization. Ab levels of both IgG subclasses were also decreased in Im offspring from Im mothers compared with offspring from non-Im mothers.

We utilized adoptive nursing to verify the contribution of the different routes of Ab transference to the modulation of the IgE response in offspring (Fig. 4A). Offspring from Im mothers were nursed by non-Im mothers to verify Ab transference by the placental route, whereas offspring from non-Im mothers were nursed by Im mothers to assess Ab transference by milk (Fig. 4A). Groups were compared with Im offspring naturally nursed by non-Im or Im

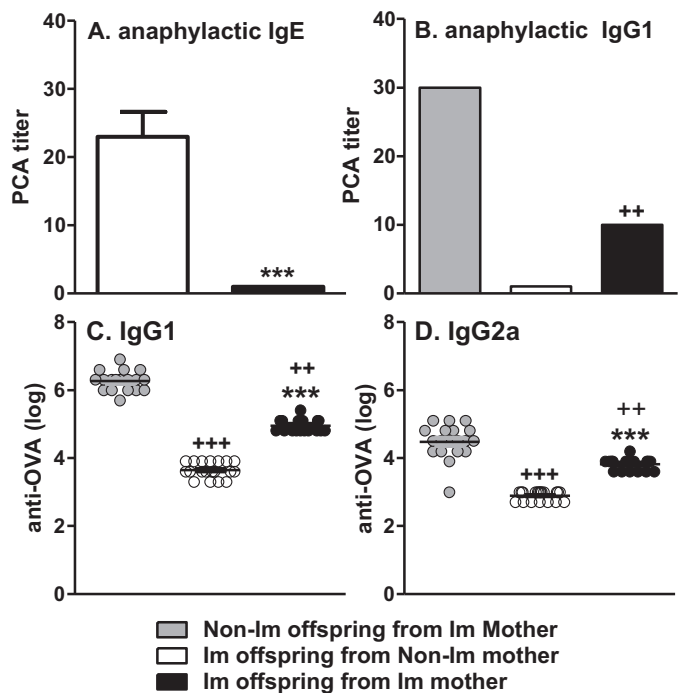


Fig. 3. Maternal immunization with OVA decreases the anti-OVA Ab response of offspring. Female BALB/c mice were immunized (Im mother) or not immunized (Non-Im) with OVA, boosted twice and mated 21 days after immunization. Neonates were immunized (Im offspring) or not (Non-Im offspring) with OVA at 3 d-o and boosted after 10 days. Samples obtained at 20 d-o were (A) analyzed for a PCA reaction to evaluate anaphylactic IgE levels and (B) pooled to assess anaphylactic anti-OVA IgG1 Ab levels. Additionally, (C) anti-OVA IgG1 and (D) IgG2a Ab levels were assessed by ELISA. The results are expressed as the mean $\pm$ SEM. ** $P < 0.01$, *** $P \leq 0.001$ compared with offspring from non-Im mothers; ** $P \leq 0.01$ compared with non-Im offspring.

mothers. Offspring were immunized at 25 d-o to verify the effect on the IgE response.

Fig. 4B shows marked inhibition of the IgE response in offspring from Im mothers and in offspring from Im mothers when nursed by either non-Im or Im mothers. These results showed that both pathways (prenatal, by the placental route, or postnatal, by breastfeeding) provide MatAbs that contributes to the inhibition of the IgE response.

Maternal immunization decreases influx of eosinophils into airways and airway responsiveness to methacholine

To verify the influence of maternal immunization on the inflammatory pulmonary response of offspring after neonatal immunization, we analyzed offspring immunized during the neonatal period (3 d-o) with five intranasal challenges of 150 μ g OVA at 43 d-o. This regimen comprised two doses at 7-day intervals and three doses over three sequential days (Fig. 1C). After 24 h, BAL was performed in mice up to 60 d-o, given the influence of age on the pulmonary inflammatory response.

Fig. 5A shows BAL cellularity was predominantly composed of eosinophils (80%) in both offspring groups, whereas the total eosinophil number significantly decreased in offspring from Im mothers. Moreover, lungs from OVA-immunized mice in the neonatal period from Im mother showed decreased number of eosinophils around the small bronchi and blood vessels, compared to offspring from non-immunized mother (Fig. 5B).

We also assessed AHR in response to increasing aerosol doses of methacholine and measured Rrs and Ers, as described previously (Lima et al., 2002; Itami et al., 2003; Macedo-Soares et al., 2004;

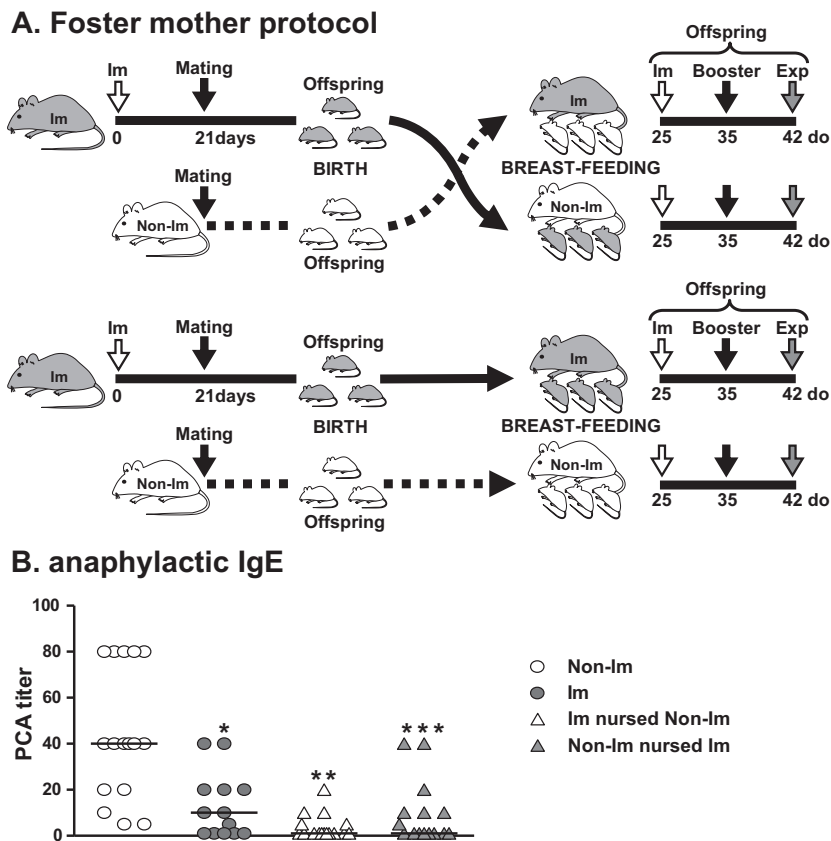


Fig. 4. Effect of MatAb transference by the placental route or by breast milk on the IgE response of offspring. After delivery, offspring were submitted to an adoptive nursing protocol. (A) A schematic diagram of foster mother protocol is shown. Female BALB/c mice were immunized (Im, gray) or not immunized (Non-Im, white) with OVA, boosted twice and mated 21 days after immunization. Offspring from Im mothers (gray) were nursed by non-Im mothers (Im nursed non-Im) and offspring from non-Im mothers (white) were nursed by Im mothers (non-Im nursed Im). Other groups of offspring were breastfed by their respective mother. All offspring were immunized with OVA at 25 d-o and boosted after 10 days. (B). Samples obtained at 42 d-o were analyzed for a PCA reaction to evaluate anaphylactic IgE levels. The results are expressed as the mean $\pm$ SEM. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ compared with offspring from non-Im mothers.

Enobe et al., 2006). Doses of methacholine that caused 50% maximal changes in the Rrs and Ers were calculated for each animal (Hulbert et al., 1985). Fig. 5C shows that the values of Rrs and Ers were similar between the groups. Offspring from Im mothers had Rrs and Ers curves that were similar to the curves for non-Im mothers.

Immunologic profile of offspring from Im mothers

Following the investigation of the regulatory mechanism mediated by maternal immunization, we verified the influence of maternal OVA immunization on tolerance in terms of DC immaturity, the influence of DCs to generate regulatory T cells (Treg), and the frequency of Treg in the offspring groups. Thus, first we analyzed the expression of costimulatory molecules on splenic DCs from 20 d-o non-immunized offspring. Fig. 6A shows that offspring from Im mothers showed a decreased percentage of DCs expressing MHC class II or CD40 molecules.

To verify the influence of DCs on the generation of antigen (Ag)-specific Treg cells, we co-cultured DCs pulsed with OVA with CD4+ splenic T cells from DO11.10 mice (Fig. 6B). Splenic CD11c+ cells from the offspring of Im or non-Im mothers were incubated at a ratio of 1:4 with CD4+ T cells from DO11.10 mice. The generation of CD4+ Foxp3+ T cells at the co-culture baseline levels and in the presence of OVA are shown in a dot plot (Fig. 6B). These results showed that there is a similar induction of Treg cells in co-cultures with the DCs of offspring from Im or non-Im mothers. Three independent experiments showed a similar profile of Treg induction

(data not shown). These results provide evidence that DCs from Im mothers cannot differentially induce Ag-specific Treg cells.

Next, we evaluated the frequency of Treg cells after neonatal OVA immunization. Neonatal immunization with OVA induced an increased percentage of CD4+CD25+Foxp3+ T cells in 25 d-o offspring compared with non-Im mice (Fig. 5C). Nevertheless, Treg numbers were unchanged in Im offspring from Im mothers.

Discussion

Maternal immunization with allergens, such as OVA, can inhibit the development of the allergic response of neonatal and young offspring. Among the mechanisms that mediate this regulation, MatAbs and factors generated by the tolerogenic maternal–fetal interface are prominent components. We previously observed that MatAbs induces an upregulation of inhibitory receptor FcγRIIb expression on B cells from the offspring of Im mothers which can contribute to inhibition of the offspring's allergic response. In this study, we evaluated MatAbs and the profile of DC and Treg cell generation as mechanisms induced by preconceptional OVA immunization.

Maternal immunization with OVA prior to gestation increased the levels of proinflammatory cytokines, such as TNF- α and IL-12, and of regulatory cytokines, such as IL-10, in amniotic fluid, by two- to three-fold, respectively, compared with the levels in non-Im mothers. Other factors, such as MCP-1 and IL-6, reached high levels during full-term gestation in both types of mothers.

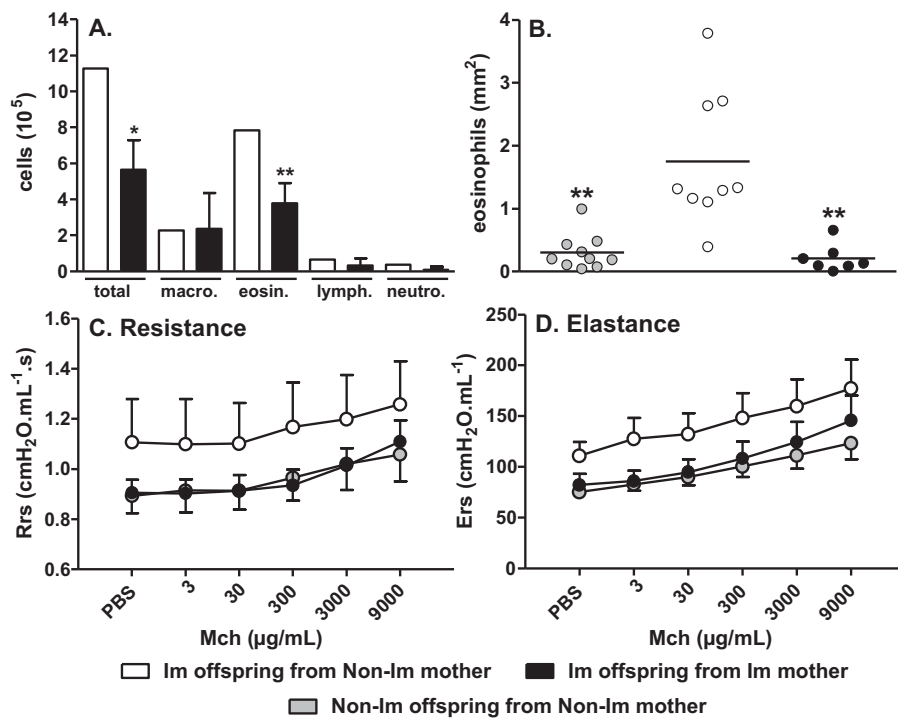


Fig. 5. Effect of maternal immunization with OVA on the airway inflammatory response of offspring. Offspring from mothers immunized (Im mother) or not immunized (Non-Im mother) with OVA were immunized or not at 3 d-o and boosted 10 days later. Beginning at 43 d-o, the offspring received with five intranasal challenges of 150 μ g OVA at 43 d-o. This regimen comprised two doses at 7-day intervals and three doses over three sequential days. (A) After 24 h, BAL was assessed, (B) eosinophil counts in Luna-stained lung sections; dose–response curves for methacholine as (C) Resistance (Rrs) and (D) elastance. The data are presented as the mean $\pm$ SEM. * $P \leq 0.05$, ** $P \leq 0.01$ compared with offspring from non-Im mothers.

Immunization with OVA was performed prior to conception to avoid inoculations during pregnancy. Moreover, we selected the subcutaneous route for Ag priming of the mothers. One hypothesis that may explain the increased levels of cytokines in the amniotic fluid after maternal immunization is that Abs form complexes with cytokines, which reach the amniotic fluid. In this context, it has been described the occurrence of natural antibodies against a large panel of antigens including a highly conserved antigens as actin, tubulin, myosin, cytochrome c as well as cytokines and growth factors in naïve mice and healthy untreated individuals (Bendtsen et al., 1989; Fomsgaard et al., 1989; Avrameas, 1991). Furthermore, we verified the presence of Ag-specific Abs, either free or complexed with the Ag, in the amniotic fluid of mothers immunized with OVA (Fusaro et al., 2009).

During full-term gestation, the fetus may be exposed to high levels of cytokines in the amniotic fluid, either through the mucosa, due to ingestion of amniotic fluid, or through the skin, allowing mucosal immune maturation of the offspring. However, the reason for the upregulation of proinflammatory cytokines and regulation in this compartment in Im mothers requires further investigation.

At the postnatal stage, high levels of TGF- β were found and did not differ between the groups of mothers, whereas high anti-OVA IgA and IgG Ab levels were transferred to offspring. It is possible that TGF- β may increase, depending on the route of exposure of the mother to the Ag, as low-dose, oral Ag delivery during pregnancy increased the amount of TGF- β via breastfeeding (Fusaro et al., 2009).

For the kinetics of MatAbs, we detected peak Ab levels in 20 d-o offspring due to breastfeeding, transferred into the circulation via FcRn on the intestinal mucosa. Similarly, anaphylactic Abs (anti-OVA IgG1) from milk were also transferred to offspring. In humans and rodents, FcRn mediates the transport of maternally derived IgG in ingested milk across the epithelial cell layer of the proximal

small intestine (Borvak et al., 1998; Kim et al., 2009) and newborn FcRn $^{-/-}$ mice are IgG deficient (Israel et al., 1996).

We detected that maternal immunization transferred intensively IgG1 Abs to offspring, including anti-OVA anaphylactic IgG1 Abs. The anaphylactic activity of IgG1 Abs is closely related to their structural conformation and proper N-glycosylation, favoring binding to the mast cell surface (Faquim-Mauro et al., 2003). FcRn can mediate the intestinal absorption of IgG anti-IgE/IgE immune complexes, implying that FcRn possibly mediates the transplacental passage of maternal IgE via IgG anti-IgE/IgE immune complexes (Paveglione et al., 2012).

Nevertheless, the strategy of immunizing mothers can significantly decrease both IgE and IgG1 anaphylactic Abs in the offspring. This finding reinforces previous data showing that maternal immunization with OVA prevents the development of an IgE response in offspring (Fusaro et al., 2007; Victor et al., 2010). Moreover, maternal immunization inhibits anti-OVA IgG1 and IgG2a from Im offspring despite high Ab levels produced upon offspring immunization. MatAbs can form complexes with the OVA provided in a neonatal immunization, preventing offspring exposure to the allergen.

We utilized an adoptive nursing protocol to verify the contribution of different routes to the prenatal or postnatal transference of Abs or other factors to offspring. Offspring were immunized at 25 d-o, allowing Abs transference during the period of breastfeeding. It was clear that both placental and milk-transferred Abs or factors could significantly inhibit the IgE response of offspring. The postnatal period is an important period for Ab transference in mice, in contrast to humans. We previously verified that IgG transference from mice immunized with OVA during pregnancy or breastfeeding caused an increase in regulatory cytokines, such as IL-10, at the prenatal stage; yet, only postnatal treatment prevented neonatal sensitization (Victor et al., 2010). Inhibition via breast milk has

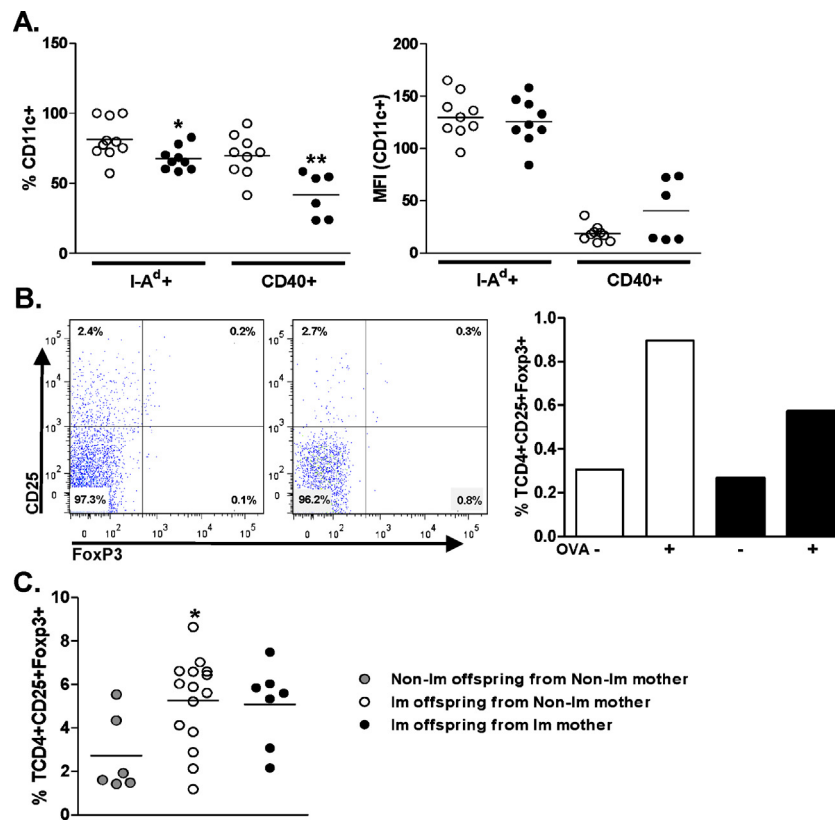


Fig. 6. Effect of DCs on the induction of Ag-specific Treg cells. (A) Splenic CD11c⁺ cells from the offspring of mothers immunized (Im mother) or not immunized (Non-Im mother) with OVA at 20 d-o were analyzed by flow cytometry for the percentage of I-A^d and CD40 and the mean fluorescence intensity (MFI) of I-A^d and CD40. The results are expressed as the means. (B) Purified splenic CD11c⁺ DCs from 25 d-o BALB/c offspring were pulsed with OVA and co-cultured at a 1:4 ratio with purified splenic CD4⁺ T cells from DO11.10 transgenic mice. After a 72 h co-culture, the frequency of CD4⁺CD25⁺Foxp3⁺ T cells was assessed. A histogram shows CD4⁺CD25⁺Foxp3⁺ T cells in the co-cultures that were unstimulated (left side) or stimulated with OVA (right side) using DCs from the offspring of non-Im mother (light bar) or from Im mother (dark bar). One of three independent experiments is shown. (C) Offspring from Im mothers or Non-Im mothers with OVA were immunized (Im) or not immunized (Non-Im) at 3 d-o and boosted 10 days later. The percentage of CD4⁺CD25⁺Foxp3⁺ T cells was assessed at 25 d-o. **P* ≤ 0.05, ***P* ≤ 0.01 compared with offspring from non-Im mothers.

already been described in rat models, indicating the importance of this pathway in the transference of factors modulating the allergic response of offspring (Jarrett et al., 1979; Jarrett and Hall, 1983). Moreover, OVA aerosols were able to induce tolerance in exposed mothers and protection from allergic asthma that did not require the transfer of immunoglobulins. This effect was mediated by regulatory CD4⁺ T lymphocytes and depended on TGF- β signaling in T cells (Verhasselt et al., 2008).

Our model of preconceptional immunization demonstrates the important role of MatAbs in the inhibition of the allergic response of offspring. The duration of the inhibitory effect on the allergic response was paralleled by the presence of MatAbs in the offspring's circulation (Victor et al., 2010). Anti-OVA IgG1 Abs were detectable in offspring up to 50 d-o, emphasizing that the period for protection is mainly the young stage of life. This finding could possibly explain the failure of maternal immunization to control AHR, even though 60 d-o offspring from Im mothers had similar Rrs and Ers curves as non-Im animals. Offspring were analyzed at this age because the development of the pulmonary inflammatory response is age dependent (Hamada et al., 2000). It is possible that an adjuvant is necessary to develop airway inflammation at a very young age, as the repeated exposure of very young mice (16 d-o) to OVA or a pollutant alone as leachate of residual oil fly ash (ROFA-s) had no effect on AHR (Hamada et al., 2000). In contrast, very young mice exposed to both OVA and ROFA-s showed significantly increased AHR. It is possible that exposure to pollutant aerosols can disrupt the normal resistance to sensitization to inhaled allergens, promoting the development of airway hypersensitivity.

We verified a lack of control of offspring AHR by maternal immunization, despite the decreased eosinophils influx to airway. Probably it is due to the different route/dose of maternal exposure with OVA. Our maternal preconceptional immunization protocol with OVA performed with low doses of OVA, was clearly able to induce Ab transference to offspring, by both placental and breast-milk. This maternal protocol seems to be protective in the early phase of life during the Ab permanence, not interfering in the generation of offspring regulatory T cells. Other maternal OVA exposure protocol during pregnancy, as by oral administration were able to protect asthma-like phenotype offspring, by decreasing AHR, mucus hypersecretion and eosinophils influx to airway, even when immunized as late as 8 months after birth (Polte et al., 2008; Polte and Hansen, 2008). Maternal transfer of tolerance seems to involve transfer of antigen-specific IgG through the placenta or milk and IFN- γ produced by CD4 and CD8T cells (Polte et al., 2008; Polte and Hansen, 2008). Moreover, aerosolized OVA during pregnancy protocol were efficient to induce offspring T regulatory cells (Gerhold et al., 2012). The generation of regulatory or memory T cells may explain the long-lasting effect of tolerance status of offspring.

Although we have observed a decreased inflammatory infiltration of eosinophils in BAL as well as in lung tissue of offspring from Im mothers, this finding does not seem to have influenced in the AHR to metacholine. In asthmatic patients, treatment with anti-IL-5 inhibits eosinophilia, but not AHR (Leckie et al., 2000). In mice, eosinophils are related with Th2-allergic lung inflammation and IFN- γ production by eosinophils is essential for lung inflammation

(Kanda et al., 2009). Otherwise eosinophilia in IL-5 transgenic mice prevents AHR development (Kobayashi et al., 2003).

Neonatal immunization induces Treg cells, regardless of the maternal immunization status. This induction may be one mechanism of preventing an inflammatory response in the early stages of life. Moreover, we verified a reduction in DC CD11c⁺ cells expressing MHC class II or CD40 molecules in offspring from Im mothers. Decreased MHC class II expression could be indicative of the immaturity of the DCs.

DCs are a key cell type that initiates and coordinates Treg division. We observed that DCs from the offspring of Im mothers generated the same frequency of Treg cells as those DCs from the offspring of non-Im mothers. DCs may contribute to Treg division *in vivo*, but this finding requires further investigation to understand how MatAbs or other maternal factors affect tolerogenic DCs.

Overall, the data highlight the role of MatAbs, together with DCs and Treg cells, in tolerance during the allergic response of offspring due to maternal immunization.

Conflict of interest

The authors have no financial conflict of interest.

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