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ORGINAL ARTICLE

Preconceptional immunization with an allergen inhibits offspring thymic Th17 cells maturation without influence on Th1 and Th2 cells

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ABSTRACT. The mechanisms through which maternal immunization can modulate offspring thymic maturation of lymphocytes are not fully understood. Here, we aimed to evaluate whether maternal OVA-immunization can inhibit the maturation of Th17 cells on offspring thymus. C57BL/6 females were immunized with OVA in Alum or Alum alone and mated with normal WT males. Offspring thymus was evaluated at three or 20 days of age. The demonstration that maternal OVA-immunization can inhibit offspring allergy development validated our experimental protocol. First, we observed that maternal OVA-immunization can inhibit the expression of RORγT and IL-17 molecules on immature T cells ($CD4^+CD8^+$) and TCD4 cells ($CD4^+CD8^-$) without interference on TCD8 cells ($CD4^-CD8^+$) on three-day-old offspring. A very similar effect could be observed on 20-day-old offspring. Additionally, a Th2 skewed profile could be found on the spleen of immunized pups from OVA-immunized mothers, but no influence was detected on offspring thymic Th1/Th2 profiles. Together, these data demonstrate that maternal immunization with an allergen can modulate offspring thymic maturation of Th17 cells without influencing Th1/Th2 patterns.

Key words: Th17, thymus, allergy, preconceptional immunization, offspring

INTRODUCTION

The regulation of offspring allergy development mediated by maternal immunization with allergens was evidenced by several groups [1-5], and the involvement of IL-17-producing TCD4 cells (Th17 cells) was observed in the main clinical manifestations of allergic diseases [6-8].

A recent hypothesis proposes that the maternal immunization with allergens can modulate offspring intrathymic maturation of T cells [9] and results in alterations cytokine production by TCD4 cells. Those alterations can exert a pivotal role in offspring immune regulation, and this hypothesis was evidenced experimentally for murine γδT, Treg, and Breg cells [10-12] but still has not been evidenced for murine Th17 cells.

The identification of Th17 cells in mice and humans provided new insights into our understanding of the development of allergy. Some pieces of evidence indicated that Th17 cells might contribute to the pathogenesis of classically recognized Th2-mediated allergy [13], and with the pathogenic potential in allergy [14, 15]. Elevated levels of IL-17 have been found in the lungs, bronchoalveolar lavage (BAL), and sera from asthmatic patients [16].

Although IFN-γ and IL-4 can negatively regulate Th17 cells [14], the differentiation of Th17 seems to occur from a distinct effector lineage compared to Th1 and Th2 precursor cells [15]. Owing to its importance in the development of allergies, a better understanding of possible regulatory mechanisms on its maturation in a primary lymphoid organ is of great importance. Whereas the impact of maternal immunization on offspring Th17 cells maturation is not elucidated, we aimed to clarify this issue in a standardized murine protocol of offspring allergy inhibition mediated by maternal allergen immunization [17].

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METHODS

Briefly, C57BL/6 wild-type female mice were immunized subcutaneously with 1500 μg of OVA (Sigma, USA) in 6 mg of aluminum hydroxide (Alum; FURP, Sao Paulo) or with 6 mg of Alum alone. After 10 and 20 days, these animals were boosted by intraperitoneal route (i.p.) with 1000 μg of OVA in saline or with saline only (Alum-immunized animals). Females were mated 21 days postimmunization. Offspring from Alum- and OVA-immunized mothers were immunized at 3 days old (d.o.) with 150 μg of OVA (Sigma, USA) in 6 mg of Alum, boosted i.p. after 10 days with 100 μg of OVA in saline, and bleed at 20 d.o. to evaluate serum levels of IgE IL-17, IFN-γ, and IL-4. Offspring lung inflammation was assessed on pups from both groups that were immunized at 25 d.o. with 150 μg of OVA (Sigma, USA) in 6 mg of Alum and boosted (ip) after 10 and 20 days with 100 μg of OVA in saline. These animals nasally received 100 μg of OVA (InvivoGen, San Diego, CA, USA) at 55, 56, 57, 58, and 59 days of age in PBS. Bronchoalveolar lavage (BAL) was obtained at 60 days of age by washing the lungs, as previously described [17].

Cytometry analyses were performed on thymus and BAL. For staining, single-cell suspensions were prepared in FACS buffer (PBS, 1% BSA). Conjugated antibodies (PE, APC, BD Horizon-V450, PE-Texas Red, PercP, or FITC) that recognize murine CD4, CD8, CCR3, MHC-II, CD3, and CD45 (all provided by BD Biosciences, USA) were used at optimal concentrations. Cell gating was based on specific isotype control values. The strategy to define BAL populations was previously described [17], those cells were identified as immature T ($CD4^+CD8^+$), mature TCD4 ($CD4^+CD8^-$), and mature TCD8 ($CD4^-CD8^+$).

For intracellular cytokine staining, we adopted a previously standardized protocol for spontaneous cytokine production analyses. Briefly, thymocytes were cultured for 24 hours at 3×10^6 cells/mL in RPMI (Gibco) supplemented with 10% heat-inactivated fetal clone (FCS-III, HyClone) sera without stimulus and in the presence of 10 μg/mL brefeldin A (Sigma-Aldrich). Cells were stained for surface markers followed by fixation, permeabilization, and intracellular staining with conjugated antibodies that recognize murine IL-17, RORγT, IFN-γ, and IL-4.

To measure IL-17, IFN-γ, and IL-4 in serum samples, we used a mouse CBA kit (Cytometric Bead Assay, BD Biosciences) according to the manufacturer's instructions. In brief, microspheres of different fluorophore intensities were sensitized with anti-IL-17 and incubated either with serum or supernatant to generate a standard curve or in the presence of capture antibodies against the same cytokine conjugated to PE for 2 hours at room temperature. After washing with buffer supplied by the manufacturer, the microspheres were analyzed by flow cytometry, and the levels were determined using CBA analysis software.

Acquisition of 300,000 events per sample was performed in the lymphocyte quadrant (as determined by ratio size/granularity) on the LSRFortessa cytometer (BD Biosciences). The analysis was performed using FlowJo software 10.1 (Tree Star). All experiments were approved by the local Animal Ethics Committee (CEUA-IMT: n-000359A- Sao Paulo, SP, Brazil). Statistical analysis was performed with GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA).

Data from 3 to 5 separate *in vivo* experiments with 11 to 22 mice per group were taken. Differences were considered significant at $P \leq 0.05$, as assessed by the Mann-Whitney U test.

RESULTS

Using our previously described maternal allergen immunization protocol [17-19], we validated the inhibition of the offspring allergic response in terms of the total serum IgE, allergic pulmonary inflammation, and serum levels of IL-17 produced in response to OVA (*figure 1A*).

Several mechanisms could contribute to the reduced production of IL-17 in offspring from OVA-immunized mothers compared to Alum-immunized mothers, but, Th17 cells represent a primary source of IL-17. First and due to its importance as a main peripheral lymphoid organ, we evaluated the frequency of Th17 cells on offspring spleen. As observed in *figure 1B*, maternal immunization could not influence the rate of Th17 cells on 3 and 20 d.o. offspring spleen when compared to offspring derived from control (Alum-immunized) mothers.

Next, we evaluated the thymuses of offspring at neonatal age and observed that maternal immunization did not influence the frequency of immature T ($CD8^+CD4^+$), mature TCD4 ($CD8^-CD4^+$), and mature TCD8 ($CD8^+CD4^-$) cells (*figure S1*) but, induced a decrease in offspring intrathymic immature T and mature TCD4 IL-17-producing T cells frequency compared to the control group (Alum-immunized mothers) without influence on mature TCD8 cells (*figure 1C*).

At the same age, we investigated whether this effect influences the intracellular frequency RORγT molecule in the same T cells maturation stages. A very similar profile could be observed with an increased expression on immature and mature TCD4 T cells compared to the control group and without influence on mature TCD8 (*figure 1C*). Such impairment of IL-17 production and RORγT expression on TCD4 cells was maintained until 20 d.o. offspring thymus compared to the control group (*figure 1D*).

To perform a more comprehensive assessment of the modulation of TCD4 cells in offspring, we additionally evaluate if maternal immunization could modulate Th1 and Th2 profiles on splenic and thymic TCD4 cells at the same age.

As observed in *figure 2*, no difference could be found on the frequency of IFN-γ- and IL-4-producing TCD4 cells on the spleen of 3 d.o. offspring (*figure 2A*), but at 20 d.o. we could observe that the maternal immunization induces a reduction in the frequency of splenic IL-4-producing TCD4 cells compared to offspring from the control group (*figure 2B-C*). At 20 d.o. mice no influence was observed on IFN-γ production (*figure 2B-C*).

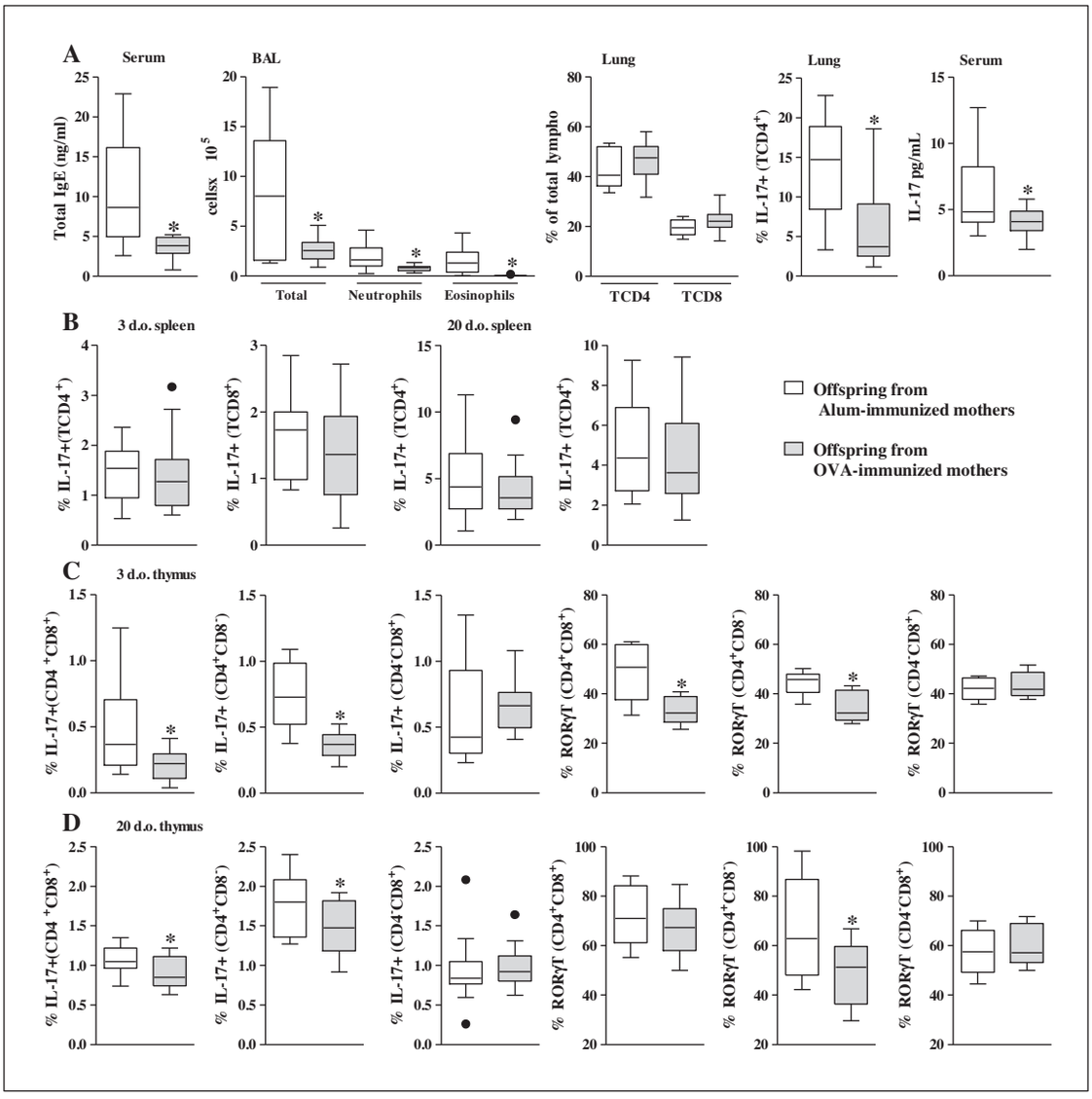

Figure 1

Effects of maternal immunization on offspring antibody production, lung inflammation, and Th17 cells. (A) Total IgE levels were determined by ELISA, and BAL preparation and dissociation of offspring lungs were performed in 43 d.o. offspring after five intranasal challenges. The differential cell counts in BAL were evaluated by flow cytometry. The dissociated lungs were assessed by flow cytometry, and Cytometric Bead Assay was used to determine serum levels of IL-17. **(B)** The frequency of TCD4 and TCD8 IL-17-producing cells was evaluated in 3 and 20 d.o. offspring spleen by flow cytometry. **(C)** The expression of IL-17 and RORγT molecules were evaluated in thymic TDP ($CD4^+CD8^+$), TCD4 ($CD4^+CD8^-$), and TCD8 ($CD4^-CD8^+$) cells of 3 d.o. offspring. **(D)** The expression of IL-17 and RORγT molecules were evaluated in thymic TDP ($CD4^+CD8^+$), TCD4 ($CD4^+CD8^-$), and TCD8 ($CD4^-CD8^+$) cells of 20 d.o. offspring by flow cytometry. The results are illustrated by box and whiskers graphs with 25th percentiles, and the Tukey method was used to plot outliers; $^*P \leq 0.05$ compared with the alum-immunized groups.

We also evaluated the frequency of IFN-γ- and IL-4-producing cells on offspring thymus, and we could observe that the maternal immunization could not influence the rate of immature T and mature TCD4 cells that produce IFN-γ and IL-4 at 3 and 20 d.o. pups (*figure 2D-E*).

DISCUSSION

These results indicated that maternal OVA immunization cannot influence the frequency of offspring splenic Th17 cells but can impair IL-17-production by the offspring's thymic TCD4 cells. The implication of Th17 in murine models of allergic lung inflammation is described in the literature [20, 21], and our observation suggests that the impairment of Th17 cells maturation on offspring from OVA-immunized mothers can be related to the tolerant immune status observed in these animals. Furthermore, the source of IL-17 can differentially modulate the murine allergic airway inflammatory response [22], an observation that reinforces the importance of evaluating different cells that produce IL-17 and can be matured in the thymus as we performed here with Th17 cells.

Here, we did not evaluate the mechanism that mediated Th17 impairment on offspring thymic maturation process. Still, some recent pieces of evidence have indicated that the thymic maturation of T and B cells can be influenced by the murine and human repertoire of IgG. In humans, this mechanism was demonstrated in the regulation of IL-17 production by TCD4 and iNKT cells [23, 24]. The IgG-

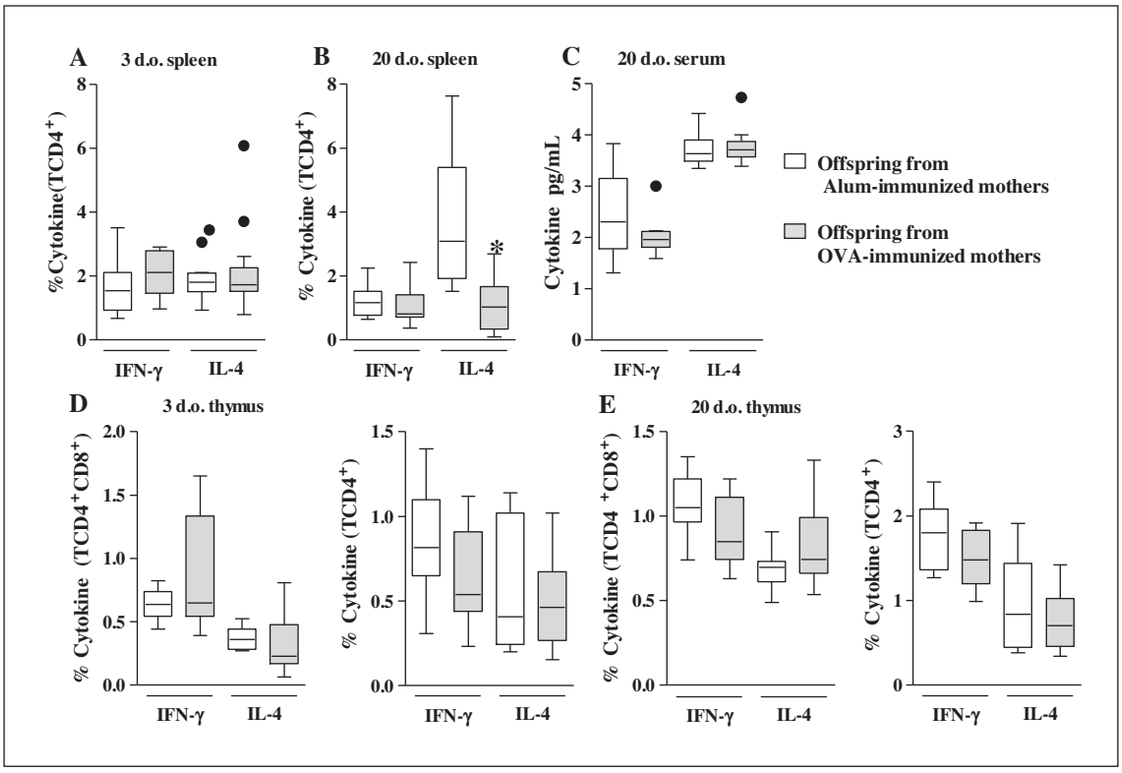

Figure 2

Effects of maternal immunization on offspring splenic and thymic Th1 and Th2 cells. The frequency of IFN-γ- and IL-4-producing TCD4 cells was evaluated in 3 (A) and 20 d.o. (B) offspring spleen by flow cytometry, and Cytometric Bead Assay was used to determine serum levels of IFN-γ and IL-4 (C). The frequency of IFN-γ- and IL-4-producing TDP (CD4 + CD8 +), TCD4 (CD4 + CD8-) cells was evaluated in 3 (D) and 20 d.o. (E) offspring thymus by flow cytometry. The results are illustrated by box and whiskers graphs with 25th percentiles, and the Tukey method was used to plot outliers; $*P \leq 0.05$ compared with the alum-immunized groups.

mediated mechanism was found in the downregulation of IL-17 production by murine γδT and B cells [18, 19] but not Th17 cells. Furthermore, this mechanism was observed in the modulation of other cytokines, including IFN-γ [25, 26].

Considering some evidence in the literature that suggests an additional modulatory effect of maternal OVA-immunization in the offspring Th1/Th2 balance, we additionally perform a brief approach in this context. We evaluated offspring splenic and thymic TCD4 cells production of the central cytokines related to the Th1 (IFN-γ) and Th2 (IL-4) profiles. Our results indicate that maternal immunization can be associated with a peripheral reduction of Th2 cell frequency in offspring but only in response to immunization with the same allergen. This peripheral Th2 inhibition corroborates with the allergy inhibition status observed in our results and with several previous observations in similar murine models of maternal immunization [5, 12, 17, 27-32].

Corroborating with the observation that Th17 cells are developed from a distinct effector lineage compared to Th1 and Th2 cells [15], our results demonstrated that maternal immunization could not influence offspring thymic Th1/Th2 balance.

In 2006, it was demonstrated that a Th17 skewed profile could be observed due to the presence of IL-6 and TGFβ1, which can subvert Th1 and Th2 differentiation generating Th17 cells [33]. Because our results demonstrate no effect on Th1 and Th2 maturation, it is possible that IL-6 and TGF-β do not mediate the mechanism induced by maternal immunization but future approaches using mice genetically deficient of these cytokines will better elucidate this mechanism.

In conclusion, our results demonstrate that maternal immunization with OVA can inhibit offspring thymic maturation of Th17 without the influence of Th1/Th2 profiles and suggest that this mechanism had some implications in the mechanism of offspring allergy inhibition as a consequence of maternal allergen-immunization.

CONFLICT OF INTEREST

The authors declared no conflicts of interest.

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