

Maternal immunization downregulates offspring TCD4 regulatory cells (Tregs) thymic maturation without implications for allergy inhibition

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Abstract

The regulation of offspring allergy development mediated by maternal immunization was evidenced by several groups, and this mechanism seems to involve the induction of regulatory T cells (Tregs) on offspring. Here, we aimed to evaluate whether the effect of maternal immunization on offspring Tregs occurs as a result of peripheral or central modulation. Briefly, C57BL/6 female mice were immunized with OVA in Alum or Alum alone and boosted with OVA in saline or saline only after 10 and 20 days. Non-immunized offspring serum, thymus and spleen were evaluated at 3 or 20 days old, and some groups of pups were submitted to neonatal OVA-immunization protocol for the subsequent evaluation of antibody production and allergic response. Our experimental protocol could be validated because maternal OVA-immunization inhibited offspring allergic response as evidenced by the suppression of offspring IgE production and allergic lung inflammation. Interestingly, maternal immunization reduced the frequency of offspring thymic Tregs with an opposite effect on spleen Tregs. Furthermore, after neonatal immunization, the frequency of lung-infiltrated Tregs was also augmented on offspring from immunized mothers. In conclusion, maternal OVA-immunization can inhibit the thymic maturation of offspring Tregs without implications on peripheral Tregs induction and allergy inhibition.

1 | INTRODUCTION

The regulation of offspring allergy development mediated by maternal immunization with allergens was evidenced by several groups¹⁻⁴ and since 2003, it was suggested, in a murine model, that this regulation can be mediated by the induction of regulatory T cells (Tregs) on offspring.⁵

Tregs play a pivotal role on several immune-suppressive mechanisms including self-tolerance⁶ and allergy.⁷ Tregs can be classified in two main populations according to its origin, the thymus-derived naturally occurring Treg cells (nTregs) and the non-thymic-induced Treg cells (iTreg), and these populations can exert divergent functions in murine allergic response.⁸ Both Tregs populations can also acquire effectors functions and enhance lung allergic

response,⁹ and a similar mechanism was also suggested in asthmatic children.¹⁰

A recently published hypothesis named “MatIgG primary modulation theory” proposes that the maternal immunization with allergens can modulate offspring intrathymic maturation of T cells¹¹ including Tregs, this hypothesis had been experimentally evidenced for murine B cells^{12,13} but still has not been evidenced for murine Treg cells. Whereas the impact of maternal immunization on offspring Tregs maturation and peripheral Tregs is not elucidated, we aimed to elucidate this issue in a standardized murine protocol of offspring allergy inhibition mediated by maternal allergen immunization.¹⁴

2 | MATERIALS AND METHODS

Briefly, C57BL/6 wild-type female mice were immunized subcutaneously with 150 µg of OVA (Sigma, Saint Louis, MO, USA) in 6 mg of aluminium 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 100 µg of OVA in saline or with saline only (Alum-immunized animals). Females were mated 21 days post-immunization and bleed at term to obtain serum samples. To evaluate offspring IgE responses, pups 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 obtain serum samples. To evaluate offspring lung inflammation, pups from both groups were immunized at 25 d.o. with 150 µg of OVA (Sigma, USA) in 6 mg of Alum and boosted (i.p.) after 10 and 20 days with 100 µg of OVA in saline. These animals were nasally administered with 100 µg of OVA (InvivoGen, San Diego, CA, USA) at 55, 56, 57, 58 and 59 days of age in PBS. Bronchoalveolar lavage (BAL) and lung tissue were obtained at 60 days of age by washing the lungs and then surgically removing the tissue as previously described.¹³ A schematic representation of the experimental protocols used in this work is illustrated in Figure 1. To perform all experiments presented in this manuscript, we analyse a maximum of three pups from at least three mothers.

OVA-specific IgG and IgM serum levels were measured by ELISA as previously described.^{5,14} The anaphylactic anti-OVA IgE titre was measured through passive cutaneous anaphylaxis (PCA) as previously described.¹³

Cytometry analyses were performed on the thymus, spleen, BAL and dissociated lung tissue samples. 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, CD25, CCR3, MHC-II, CD3, CD19, CD45 or FoxP3 (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 described¹³; thymic populations were identified as TDP (CD4⁺CD8⁺), TCD4 (CD4⁺CD8⁻), TCD8 (CD4⁻CD8⁺) or Tregs (CD4⁺CD8⁻CD25^{high}) and peripheral (spleen and lung) Tregs were identified as CD4⁺CD25^{high} cells. FoxP3 staining was performed according to the BD Biosciences staining protocol.

For intracellular cytokines staining, we adopted a previously standardized protocol for spontaneous cytokine production analyses.¹² Briefly, thymocytes were cultured for 24 hours at 3×10^6 cells/mL in RPMI (Gibco, Bleiswijk, Netherlands) supplemented with 10% heat-inactivated foetal clone sera without the stimulus and in the presence of 10 µg/mL Brefeldin A (Sigma-Aldrich). Cells were first stained for surface markers followed by fixation, permeabilization and intracellular staining with conjugated antibodies that recognize murine IL-10, IL-17, IFN-γ and IL-4.

Acquisition of 300 000 events per sample was performed in the lymphocyte quadrant (as determined by ratio size/granularity) on an LSRFortessa cytometer (BD Biosciences), and analysis was performed using FlowJo software 10.1 (Tree Star). All these 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 in vivo studies were taken from 3 to 5 separate experiments with 11 to 22 mice per group. Differences were considered significant at $P \leq 0.05$, as assessed by the Mann-Whitney *U* test.

3 | RESULTS

We evidenced that OVA-immunized females produce high serum levels of OVA-specific IgG compared to Alum-immunized group (Figure 2A). We also observed that non-immunized offspring derived from OVA-immunized females has high serum levels of OVA-specific IgG without specific IgM production at 20 d.o. (Figure 2B). In response to neonatal immunization, the total and anaphylactic IgE production of offspring from OVA-immunized females was inhibited compared to offspring from Alum-immunized females (Figure 2C). The evaluation of lung allergic inflammation induction on both groups of offspring that were immunized at 25 d.o., reveals a reduced number of total infiltrated cells with a marked reduction of neutrophils and eosinophils number on BAL of 60 d.o. offspring from OVA-immunized mothers compared to offspring from Alum-immunized mothers (Figure 2D).

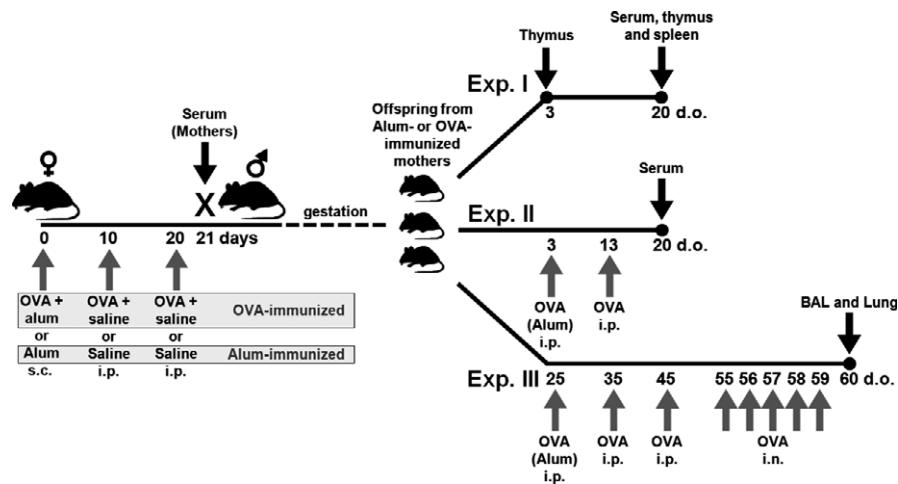


FIGURE 1 Schematic representation of the experimental protocols. C57BL/6 females were immunized by subcutaneous route (s.c.) with OVA in Alum or Alum alone and boosted intraperitoneal route (i.p.) after 10 and 20 d. Twenty-one days after immunization both females were bled and mated with normal males. Offspring from both groups were submitted to three different experiments (Exp.): Exp. I - non-immunized pups that were evaluated at 3 or 20 d.o.; Exp. II - pups that were immunized at neonatal age with OVA and evaluated at 20 d.o.; Exp. III - pups that were immunized with OVA at 25 d.o., boosted at 35 and 45 d.o. by i.p. route, nasally administered (i.n.) with OVA at 55, 56, 57, 58 and 59 d.o. and evaluated at 60 d.o.

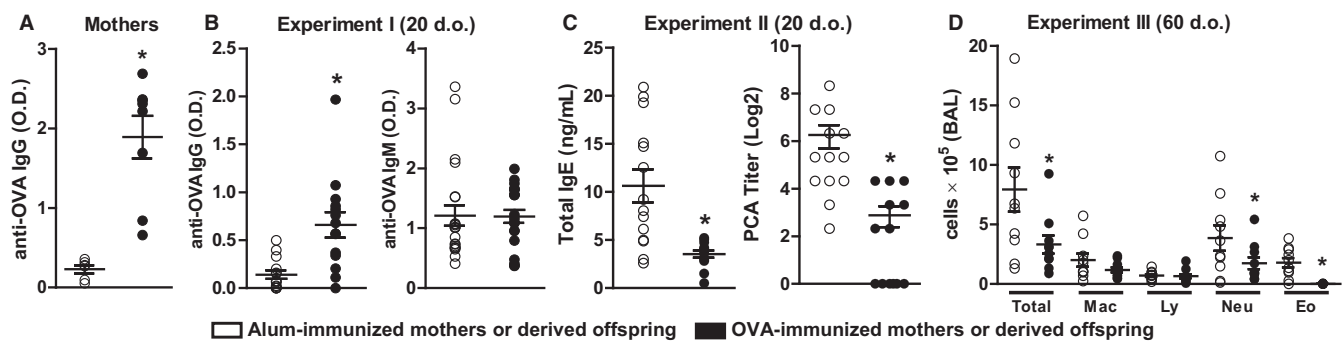
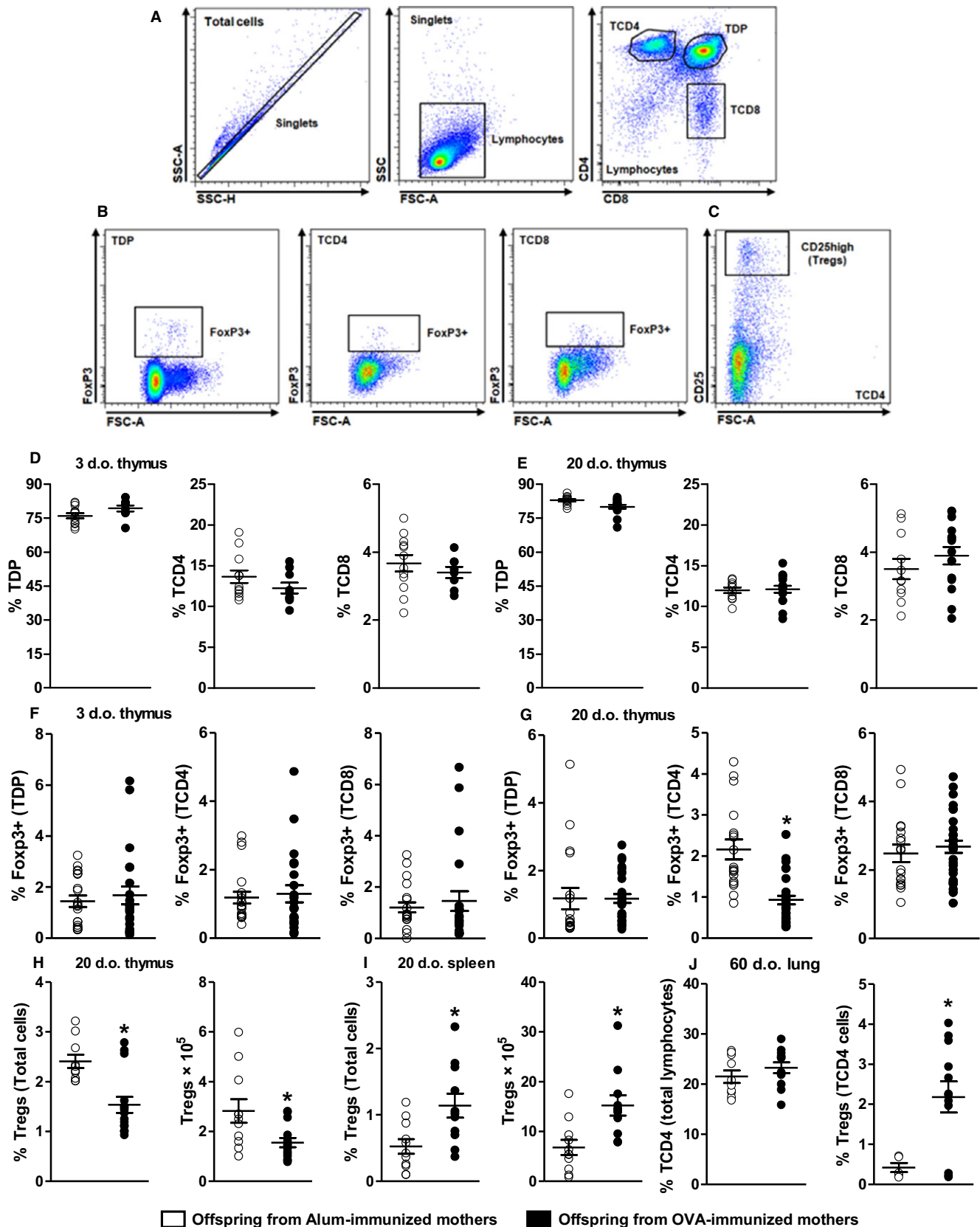


FIGURE 2 Maternal immunization protocol can induce specific IgG and inhibit offspring allergy development. WT female mice were immunized with OVA in Alum or Alum only, boosted 10 and 20 d later and mated at day 21 with WT males. Serum levels of anti-OVA IgG were evaluated on mothers at term (A). Serum levels of anti-OVA IgG, anti-OVA IgM were evaluated on 20 d.o. non-immunized offspring derived from Experiment I (B). Total IgE and anti-OVA anaphylactic IgE were evaluated on 20 d.o. OVA-immunized offspring derived from Experiment II (C). The frequency of total cells, macrophages (Mac), lymphocytes (Ly), neutrophils (Neu) and eosinophils (Eo) was evaluated on 60 d.o. OVA-immunized offspring BAL derived from Experiment III (C). Data are presented as individual values, means and SEM. * $P \leq 0.05$, compared with Alum-immunized mothers or offspring from Alum-immunized mothers

These initial results validate the maternal immunization protocol as an inhibitor of offspring allergy development. Thenceforth, we evaluated the influence of maternal OVA-immunization on offspring T cells maturation analysing the expression of Foxp3 on offspring intrathymic T cells precursors ($CD4^+CD8^+$ immature T cells; TDP), TCD4 mature cells ($CD4^+CD8^-$; TCD4) and TCD8 mature cells ($CD4^-CD8^+$; TCD8). The gating strategy to identify T cells populations is illustrated in Figures 3A-C. The frequency of thymic TDP, TCD4 and TCD8 cells was equally between offspring groups at 3 d.o. (Figure 3D) and at 20 d.o. (Figure 3E). On 3 d.o. pups (Figure 3F) no effect

of maternal OVA-immunization could be observed, at 20 d.o. a reduced expression of Foxp3 was detected on TCD4 cells of non-immunized offspring from OVA-immunized compared to those from Alum-immunized mothers without influence on precursors (TDP) or TCD8 cells (Figure 3G).

Since Foxp3 molecule is strongly expressed in TCD4 regulatory cells (Tregs), we perform a phenotypic evaluation on 20 d.o. offspring to confirm the reduction of Tregs. With this propose, we evaluate the frequency of $CD4^+CD25^{\text{high}}$ cells, the main phenotype of murine intrathymic Tregs and a lower frequency and an absolute



number of this population could be detected on offspring from OVA-immunized mothers (Figure 3H) corroborating the findings with the evaluation of a Foxp3 expression. To

evaluate peripheral Tregs, spleens of offspring at 20 d.o. were analysed and an opposite effect could be observed with a higher frequency and number of Tregs on offspring

FIGURE 3 Maternal immunization downregulates the maturation of offspring Tregs with an opposite effect on peripheral Tregs. Illustrative dot plots of the gating strategy used to identify thymic TDP, TCD4 and TCD8 cells (A), to evaluate the expression of FoxP3 molecule in these cells (B) and to identify Tregs (C) are generically demonstrated in upper panels. WT female mice were immunized with OVA in Alum or Alum only, boosted 10 and 20 d later and mated at Day 21 with WT males. Non-immunized offspring from OVA-immunized or Alum-immunized mothers were evaluated at 3 and 20 d.o. (Experiment I). The frequency of TDP, TCD4 and TCD8 cells was demonstrated for 3 (D) and 20 d.o. (E) pups. The foxp3 expression on intrathymic offspring TDP, TCD4 and TCD8 cells was evaluated at 3 (F) and 20 (G) d.o. pups. The frequency and number of Tregs (TCD4 + CD25^{high}) were evaluated on 20 d.o. offspring thymus (H) and spleen (I) or on 60 d.o. immunized offspring lungs derived from Experiment III (J). Data are presented as individual values, means and SEM. * $P \leq 0.05$, compared with offspring from Alum-immunized mothers

from OVA-immunized compared to Alum-immunized mothers (Figure 3I). Moreover, we evaluated the frequency of Tregs on offspring lung tissue after immunization and pulmonary challenge with OVA, and we could observe that maternal OVA-immunization is related to an augmented infiltration of Tregs on offspring lung (Figure 3J). To further elucidate the modulation TCD4 cells maturation in offspring thymus, we additionally evaluate if maternal immunization could modulate thymic TCD4 cells cytokine production. As observed in Figure 4, no difference could be observed on the frequency of IL-10, IL-17, IFN- γ and IL-4 production at 3 (Figure 4A) and 20 (Figure 4B) d.o. pups derived from Alum- and OVA-immunized mothers.

4 | DISCUSSION

Since the suggestion of offspring Tregs induction mediated by maternal immunization with OVA,⁵ this population was evaluated only in peripheral organs and inflammation site. In the last years, some discussions about the effect of maternal immunization on offspring lymphocytes maturation, including Tregs, have emerged in the literature,^{11,15} but there is still no evidence in a standardized murine protocol of maternal allergen immunization to elucidate this issue.

The results obtained from our initial assessment reveals that immunized females produced a strong OVA-specific IgG response with a similar profile on non-immunized offspring from OVA-immunized mothers. Moreover, after offspring immunization with OVA, the main parameters of allergy development, the IgE production and the pulmonary infiltration of eosinophils were inhibited on the group derived from OVA-immunized mothers. Together, these observations corroborate with the literature^{5,16-20} thereafter validating our murine model of offspring allergy inhibition.

In the following step, we analyse the frequency of Tregs on offspring thymic tissue and we could observe that maternal OVA-immunization is related to a lower frequency of these cells on offspring thymus. At this point, we could not assert the mechanism by which maternal immunization could mediate negative effect on Tregs

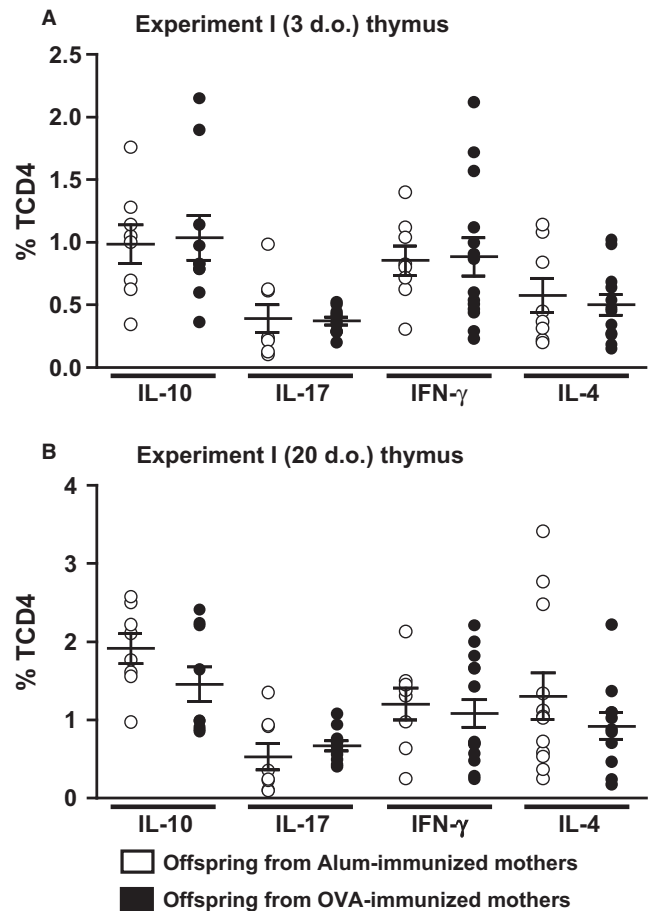


FIGURE 4 Intracellular cytokine production in offspring thymic TCD4 cells. WT female mice were immunized with OVA in Alum or Alum only, boosted 10 and 20 d later and mated at day 21 with WT males. Non-immunized offspring from OVA-immunized or Alum-immunized mothers were evaluated at 3 and 20 d.o. (Experiment I). Intracellular IL-10, IL-17, IFN- γ and IL-4 percentage were evaluated in offspring TCD4 cells. Data are presented as individual values, means and SEM

maturation, but we suggest that offspring B10 cells can be involved because it was recently demonstrated, in the same maternal immunization protocol, the induction of offspring B10 cells.¹³ B10 cells compose the main population of B cells with regulatory functions mediated by the production of IL-10 and that can regulate allergy development.²¹

These cells participate in Tregs maturation process,²² and its induction is related to a negative regulation of Tregs maturation.²³⁻²⁵

Furthermore, the reduced levels of offspring Tregs could be observed only at the weaning period; this suggests the involvement of passively transmitted factors after birth. In this context, maternal antibodies may exert a function, but only a few evidences about this hypothesis can be found in the literature. Between then, antibodies inducing clonal deletion of thymocytes was demonstrated years ago²⁶ and, more recently, a modulatory effect on T cells cytokine production was evidenced in human thymocytes.^{27,28} Until the present moment, no direct evidence of modulatory effects of antibodies on Tregs maturation is described.

To evaluate if the reduced maturation of offspring Tregs can represent a counterpoint compared to the literature, where Tregs is described as a key regulator of peripheral allergy inhibition on several murine models,²⁹⁻³² we also evaluated the frequency of Tregs on offspring spleen. Controversially to intrathymic observations, our results indicate that maternal OVA-immunization induces an augment of offspring peripheral Tregs what corroborates with literature observations.

Whereas Tregs has been shown to exert a pivotal role on allergic lung inflammation inhibition site, we also evaluated the frequency of this population on offspring lung after allergenic challenge. These analyses reveal, in accordance with the literature, that the allergic lung inflammation inhibition is related to a higher frequency of infiltrated Tregs.⁹ With our observations, we cannot assert that the offspring undergo an inhibition of nTregs with a stimulation of iTregs according to the literature classification for Tregs,⁸ but this possibility needs to be considered for future studies with similar approaches.

From its characterization, Tregs has been described as autoimmune diseases regulators,³³ and its thymic maturation is related to self-tolerance maintenance.³⁴ In the present work, we did not evaluate the possible implication of thymic Tregs reduction induced by maternal immunization on offspring autoimmune diseases development. However, our investigation suggests that any implication can occur due to the B6 genetic background of the mice strain is related to an inefficient induction of autoimmune disease³⁵ and to the opposite effect that was observed on the peripheral frequency of Tregs. Some recent discussion has suggested that the pathogenic mechanisms of autoimmunity can contribute to the severity of asthma,³⁶ but there are still scarce evidence in the literature so that we can advance our findings aiming the elucidation of this hypothesis.

Considering some evidence in the literature that suggest a modulatory role of Tregs in the development of others TCD4 cells subpopulations, we additionally perform a brief approach in this context. We evaluated offspring thymic

TCD4 cells production of the main cytokines that can be related to TCD4 subpopulations, IL-10 (Tr1), IL-17 (Th17), IFN- γ (Th1) and IL-4 (Th2), and these results indicate any influence of maternal immunization in the primary site of TCD4 cells maturation. In humans, it was described that Tregs can be responsible for the control of Th2 cells and allergy induction,³⁷ and in murine models, it was observed that Tregs can influence the development of Th17 cells.^{38,39} Those results were obtained from experiments with peripheral Tregs from both species, and this could be the reason that they do not corroborate with our observations; further investigations need to be undertaken to elucidate this issue.

5 | CONCLUSIONS

Together, our evidence suggests, in an unprecedented way, that maternal allergen immunization can negatively impact offspring thymic maturation of Tregs without implications on offspring allergy inhibition and peripheral frequency of this cells.

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CONFLICT OF INTEREST

The authors declare that they have no relevant conflict of interest.

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