Preconception immunization can modulate intracellular Th2 cytokine profile in offspring: \textit{in vivo} influence of interleukin 10 and B/T cell collaboration

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Abstract

Introduction: In the last few years our group has been studying the mechanisms involved in the inhibition of allergy in offspring mediated by preconception maternal immunization, but these mechanisms are not fully understood. Such mechanisms that we have studied aimed at the passive transfer of maternal antibodies and its influence on offspring immune status.

Aim of the study: To evaluate whether maternal immunization could modulate intracellular Th1/Th2 profiles in offspring.

Material and methods: C57Bl/6 female wild type mice (WT), interleukin (IL)-10$^{-/-}$ or CD28$^{-/-}$ mice were immunized or not with ovalbumin (OVA) and were mated with respective lineage males and offspring were evaluated at 3 days old (d.o.), 20 d.o., or 20 d.o. after neonatal immunization.

Results: Preconception OVA immunization induced a marked reduction in IL-4 secretion by TCD4$^+$ cells of WT offspring when compared with offspring from non-immunized mothers. The maternal immunization of IL-10$^{-/-}$ mice induced an increase in the TCD4$^+$IL-4$^+$ percentage in offspring and a reduction in TCD4$^+$IFN-$\gamma^+$ cells. The maternal immunization in CD28$^{-/-}$ mice induced augment IL-4 intensity in 3 and 20 d.o. offspring TCD4$^+$ cells.

Conclusions: Our results reveal that maternal immunization with OVA can down-regulate the Th2 pattern in offspring and this regulation is dependent on IL-10 and B/T cell collaboration.

Key words: allergy, OVA, maternal fetal-interface, IL-10, Th1/Th2 balance, B cells.

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Introduction

Allergic reactions are characterized by intense Th2 cytokine production profile and exacerbated immunoglobulin (Ig) E production. The balance between Th1 and Th2 cytokine profiles is regulated mainly by interferon (IFN)-$\gamma$ (Th1-related) and interleukin (IL)-4 (Th2-related) production. The main sources of these cytokines are TCD4 cells but both cytokines can also be produced or regulated by B cells [1].

The prevention strategies to regulate allergy development are classified into three levels, with the primary strategy aiming to prevent allergen sensitization, the secondary aiming at the prevention of allergy development in already sensitized individuals, and tertiary aiming at the treatment of the symptoms [2]. Primary strategies have been studied by our group in type I hypersensitivity murine models over the last 15 years [3-12]. These models consider three important factors: the maternal immunity during pregnancy, neonatal immunity, and the interaction between the two. The maternal and fetal immune system interaction occurs either through the placenta or through breast-feeding. Previously, we reported that maternal immunization with the dust mite \textit{Dermatophagoides pteronyssinus} (Dp) can inhibit offspring Th2 exacerbation [13] characteristics that was also observed after maternal immunization with OVA [14]. In the same work, we also reported that the passive transfer of maternal IgG can modulate offspring IL-10 pro-
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Material and methods

Mice

C57BL/6 inbred wild type mice (WT) or IL-10 and CD28 genetically deficient (IL-10<sup>−/−</sup> and CD28<sup>−/−</sup>) males and females were used at 8-10 weeks old. The animals were purchased through the Central Animal Facility of the School of Medicine and Institute of Biomedical Sciences – USP. The offspring (F1) of both sexes were used. All experiments described in this manuscript were approved by the University of São Paulo School of Medicine Animal Ethics Committee (CEP-FMUSP: 122/14 – São Paulo, SP, Brazil).

Murine immunization

Female WT mice were immunized subcutaneously with 1500 μg of OVA (Sigma, USA) in 6 mg of aluminum hydroxide (FURP, São Paulo) and boosted intraperitoneally (ip) after 10 and 20 days with 1000 μg of OVA in saline totaling 200 μl per immunization or booster. Females were mated 21 days after immunization. The pups of immunized and non-immune mothers were immunized with the same antigen used for maternal immunization. Three-day-old (3 d.o.) offspring were evaluated or treated ip with 100 μg of OVA in 0.6 mg of alum (totaling 20 μl), boosted after 10 days with OVA in saline (totaling 200 μl) and evaluated at 20 d.o. (20 (Im) d.o.). Some groups of animals were not immunized at 3 d.o. and were evaluated at 20 d.o. (20 d.o.). OVA immunization protocols were also performed using IL-10<sup>−/−</sup> or CD28<sup>−/−</sup> mice and are schematically represented in Figure 1.

Spleen cell suspensions

Spleens were collected, and their cells were isolated for culture or flow cytometry analyses. Single-cell suspensions were prepared with cell strainers (BD Biosciences, MA, USA) and placed in Petri dishes containing RPMI 1640 culture medium (Sigma). The cell suspension was treated with lysis buffer (Biosource – ACK Lysis Buffer, Rockville, MD, USA) for 2 minutes, and the cell suspension was washed twice with RPMI medium. The cells were subsequently resuspended in 1 ml of RPMI medium with 10% FBS (III HyClone, Logan, USA), and cellular viability was quantified with 0.5% Trypan blue in a Neubauer chamber.

Fig. 1. Schematic representation of experimental protocols used on WT, IL-10<sup>−/−</sup> and CD28<sup>−/−</sup> mice. WT, IL-10<sup>−/−</sup> or CD28<sup>−/−</sup> non-immunized females were mated with normal males and offspring were evaluated at 3 d.o., 20 d.o. or immunized with OVA in the neonatal period and evaluated at 20 d.o. (A). OVA immunized WT, IL-10<sup>−/−</sup> or CD28<sup>−/−</sup> females were mated with normal males and offspring were evaluated at 3 d.o., 20 d.o. or immunized with OVA in the neonatal period and evaluated at 20 d.o. (B)
Murine flow cytometry

For surface staining, single-cell suspensions were prepared in flow cytometry buffer (PBS, 1% BSA). Directly conjugated antibodies with Alexa Fluor 700, PE-Texas Red, FITC, APC or PE (BD Biosciences), anti-CD19 (1D3), anti-B220 (RA3-6B2), and anti-CD4 (RM4-5) were used at optimal concentrations after the titration experiments. Cell gating was based on the specific isotype control values as well as the fluorescence minus one (FMO) to determine intracellular cytokine levels. All analyses were performed using FlowJo software (Tree Star Inc). For intracellular cytokines, cells were cultured for 24 hours at 3 × 10^6 cells/ml in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated FCS (HyClone) without stimulus 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 IFN-γ (XMG1.2) or IL-4 (11B11) or matched isotypes using the production intensity (p = 0.0337; Fig. 5D) reveals enhanced IL-4 expression (p = 0.0043) as mediated by maternal immunization at 3 d.o. (Fig. 5B), which was maintained until 20 d.o. (p = 0.0341, Fig. 5D) when compared with offspring from non-immunized mothers. The percentages of IL-4+ and IFN-γ+ were not altered in TCD4+ (3 d.o. IL-4, p = 0.1953 and IFN-γ, p = 0.4371; 20 d.o. IL-4, p = 0.4336 and IFN-γ, p = 0.0671; 20 (Im) d.o. IL-4, p = 0.1255 and IFN-γ, p = 0.9167) and B (CD19+; 3 d.o. IL-4, p = 0.2255 and IFN-γ, p = 0.4300; 20 d.o. IL-4, p = 0.3327 and IFN-γ, p = 0.3391; 20 (Im) d.o. IL-4, p = 0.2752 and IFN-γ, p = 0.3040) cells at any evaluation period (Fig. 3A-C and 5A-C), as well as the evaluation of intensity of IL-4 and IFN-γ in immunized offspring (TCD4+ IL-4, p = 0.8064 and IFN-γ, p = 0.5606; CD19+ IL-4, p = 0.7640 and IFN-γ, p = 0.1302; Fig. 3E-F and 5E-F).

Interleukin 10 production and B/T cell cooperation are necessary for Th2 regulation induced by maternal immunization

Based on our previous publication about B10 cell involvement [19], we sought to investigate the IL-10 involvement by subjecting IL-10−/− mice to a maternal/neonatal immunization protocol and evaluating the offspring with the same parameters as WT mice. The maternal immunization of IL-10−/− mice induced an increase in the TCD4+IL-4+ (p = 0.0111) percentage in 3 d.o. offspring without influencing the production intensity (p = 0.5783; Fig. 6A). During the same period, no influences on offspring B cells were observed (IL-4+, p = 0.7491; IL-4 MFI, p = 0.1436; Fig. 6B).

After neonatal immunization, offspring from immunized mothers, compared with offspring from non-immunized mothers, showed a reduction in TCD4+IFN-γ+ cells (p = 0.0405) and a reduction in the IL-4 intensity of TCD4+ cells (p = 0.0053, Fig. 6C). Furthermore, maternal immunization augmented IFN-γ+ (p = 0.0419) and reduced IL-4+ (p = 0.0155) B cells of immunized offspring (Fig. 6D). No differences were observed in the intensity of IL-4 and IFN-γ production (IL-4, p = 0.3143 and IFN-γ, p = 0.8273; Fig. 6D).

CD28− mice are unable to produce IgE in response to allergen sensitization, as shown by the Th1-driven immunity with the predominance of IFN-γ production, and because its B/T cell cooperation is compromised [20]. Thus, we evaluated the effects of maternal immunization in CD28− mice and observed that maternal immunization induced augmented IL-4 intensity in TCD4+ cells (p = 0.0276, Fig. 7A).

Results

Maternal immunization regulates offspring Th2 and B cell cytokine production profiles

The evaluation of the percentage and intensity of cytokine production by offspring TCD4+ cells (Fig. 2) reveals that maternal immunization induced a marked reduction in IL-4 (p = 0.0337) and IFN-γ (p = 0.0447) intensity at 3 and 20 d.o. offspring from immunized mothers when compared with offspring from non-immunized mothers (Fig. 3B-D). By contrast, the evaluation of the percentage and intensity of cytokine production by offspring B cells (Fig. 4) reveals enhanced IL-4 expression (p = 0.0043) as mediated by maternal immunization at 3 d.o. (Fig. 5B), which was maintained until 20 d.o. (p = 0.0341, Fig. 5D) when compared with offspring from non-immunized mothers. The percentages of IL-4+ and IFN-γ+ were not altered in TCD4+ (3 d.o. IL-4, p = 0.1953 and IFN-γ, p = 0.4371; 20 d.o. IL-4, p = 0.4336 and IFN-γ, p = 0.0671; 20 (Im) d.o. IL-4, p = 0.1255 and IFN-γ, p = 0.9167) and B (CD19+; 3 d.o. IL-4, p = 0.2255 and IFN-γ, p = 0.4300; 20 d.o. IL-4, p = 0.3327 and IFN-γ, p = 0.3391; 20 (Im) d.o. IL-4, p = 0.2752 and IFN-γ, p = 0.3040) cells at any evaluation period (Fig. 3A-C and 5A-C), as well as the evaluation of intensity of IL-4 and IFN-γ in immunized offspring (TCD4+ IL-4, p = 0.8064 and IFN-γ, p = 0.5606; CD19+ IL-4, p = 0.7640 and IFN-γ, p = 0.1302; Fig. 3E-F and 5E-F).
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This effect was maintained after neonatal immunization ($p = 0.0370$, Fig. 7C), and we observed a simultaneous increase in the IFN-γ percentage ($p = 0.0051$) and intensity ($p = 0.0005$) in TCD4+ (Fig. 7C) and IFN-γ intensity ($p = 0.0055$) in B cells of 3 d.o. offspring derived from immunized mothers (Fig. 7D).

Discussion

Our group has already reported that the maternal immunization protocol with OVA in WT mice could down-regulate the intracellular IL-4 production on offspring, suggesting a Th2 inhibition [14], and four years later, we also observed that maternal immunization with
Fig. 3. Intracellular cytokine production in offspring T cells. WT female mice were immunized with OVA, boosted 10 and 20 days later and mated at day 21 with WT males. Offspring from immunized (n = 12) or non-immunized mothers (n = 12) were evaluated at 3 (3 d.o., A and B) and 20 d.o. (20 d.o., C and D) or immunized with OVA at 3 d.o., boosted at 13 d.o., and evaluated at 20 d.o. (20 [Im] d.o., E and F). Intracellular IFN-γ and IL-4 percentage and intensity (MFI) were evaluated in offspring TCD4+ T cells. The data are presented as the means ±SEM of each group. *p < 0.05, compared with the control group.
Fig. 4. Illustrative dot plots of the effect of maternal immunization on offspring cytokine production by B cells. Each sample was acquired using the singlet cell gate (determined by FSC-A/FSC-H parameters) and then the lymphocyte gate (determined by their relative size/granularity); samples were then acquired gated as CD19+ cells (left panels) and isotype control based cytokine gating to evaluate percentage and intensity in each evaluation period and experimental group was performed accordingly, as indicated in the right panels.

a dust mite extract (Dermatophagoides pteronyssinus) could not induce the same cytokine and phenotype profile in offspring cells [7].

Recently, using the same experimental protocol adopted in the present work, we observed that WT mice derived from OVA-immunized mothers cannot develop pulmonary allergy inflammation in response to the same allergen [19]. This work reveals that maternal immunization can suppress WT offspring IgE production and pulmonary inflammation, due to regulatory B10 cell induction in offspring.

Together, our previous observations raised some new questions, and it became necessary to evaluate the in vivo contribution of IL-10 and B/T cell collaboration to offspring allergy inhibition.

To assess the in vivo role of IL-10 in this mechanism, IL-10−/− mice were subjected to the same maternal and neo-
Fig. 5. Intracellular cytokine production in offspring B cells. WT female mice were immunized with OVA, boosted 10 and 20 days later and mated at day 21 with WT males. Offspring from immunized (n = 11) or non-immunized mothers (n = 11) were evaluated at 3 (3 d.o., A-B) and 20 d.o. (20 d.o., C-D) or immunized with OVA at 3 d.o., boosted at 13 d.o., and evaluated at 20 d.o. [20 (Im) d.o., E-F]. Intracellular IFN-γ and IL-4 percentage and intensity (MFI) were evaluated in offspring CD19+ B cells. The data are presented as the means ±SEM of each group. *p < 0.05, compared with the control group.
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Fig. 6. Effect of preconception immunization on IL-10⁻/⁻ offspring cytokine production. IL-10⁻/⁻ female mice were immunized with OVA, boosted 10 and 20 days later and mated at day 21 with IL-10⁻/⁻ male mice. Offspring from immunized (n = 9) or non-immunized mothers (n = 10) were evaluated at 3 d.o. (3 d.o.) or immunized with OVA at 3 d.o., boosted at 13 d.o., and evaluated at 20 d.o. [20 (Im) d.o.]. The intracellular IFN-γ and IL-4 percentage and intensity (MFI) were evaluated in the offspring TCD4⁺ T cells and CD19⁺ B cells at 3 (A-B) and after neonatal immunization at 20 d.o. (C-D). The data are presented as the means ±SEM of each group. *p < 0.05, compared with the control group.
Fig. 7. Effect of preconception immunization on CD28⁻/⁻ offspring cytokine production. CD28⁻/⁻ female mice were immunized with OVA, boosted 10 and 20 days later and mated at day 21 with CD28⁻/⁻ male mice. Offspring from immunized (n = 11) or non-immunized mothers (n = 9) were evaluated at 3 d.o. (3 d.o.) or immunized with OVA at 3 d.o., boosted at 13 d.o., and evaluated at 20 d.o. [20 (Im) d.o.]. Offspring intracellular IFN-γ and IL-4 percentage and intensity (MFI) were evaluated in terms of offspring TCD4⁺ T cells and CD19⁺ B cells (A-B) and after neonatal immunization at 20 d.o. (C-D). The data are presented as the means ±SEM of each group. *p < 0.05, compared with the control group.
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In conclusion, our results reveal, complementarily to previous observations of our group, that offspring allergy and Th2 cytokine profile inhibition mediated by maternal immunization with OVA seems to be dependent on IL-10 and efficient B/T cell collaboration in vivo.

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The authors declare no conflict of interest.

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