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IgG from atopic individuals can mediate non-atopic infant thymic and adult peripheral CD8+ TC2 skewing without influence on TC17 or TC22 cells

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Key words
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Summary
The potential of IgG antibodies as allergy regulators has been discussed for decades and was brought to light that anti-allergen IgG is related to allergy inhibition in children during the first years of life and that IgG repertoire can differ between atopic and non-atopic individuals. Here, we aimed to evaluate in vitro the differential effects of purified IgG from atopic and non-atopic individuals on the production of IL-4, IL-17, and IL-22 by human intra-thymic and mature peripheral CD8+ T cells respectively termed as TC2, TC17, and TC22 cells. We additionally evaluated the IFN-γ production by CD8+ T cells. Thereupon we used infants thymic tissues from non-atopic mothers and blood samples from individuals clinically classified as non-atopic. Thymocytes or PBMCs were cultured with IgG from atopic or non-atopic individuals. As controls, we used commercial IgG (Intravenous immunoglobulin - IVIg) or mock condition. The phenotype and intracellular cytokine production were evaluated using flow cytometry. IgG from atopic individuals could increase the frequency of TC2 cells in non-atopic infant thymic and adult peripheral cell cultures compared to all control conditions. Due to the TC2 cell's potential to collaborate with pathology and severity of asthma in humans, this evidence can cooperate with the understanding of the development of an atopic state.

Introduction

Our group has been discussing the potential of IgG molecules to modulate the functional activity of thymic T cells according to the individual's immune status (1, 2). The IgG repertoire can differ between atopic and non-atopic individuals (3, 4) and its reactivity to IgE can play a pivotal role in the mechanism by which non-atopic individuals produce IgE without a response to allergen exposure (5).

It was described that IgG molecules from atopic individuals modulate *in vitro* IFN-g production by human intra-thymic TCD4 and CD8+ T cells (6) and IgG from non-atopic individuals can induce *in vitro* IFN-g and IL-10 production by human intra-thymic gdT cells (7). In a recent translational approach, it was evidenced *in vitro* that IgG from allergy-tolerant mice and allergy-tolerant humans (non-atopic) can exert similar effects upon neonatal thymocytes inhibiting the maturation of IL-17-producing gdT cells (8).

In a similar but not translational context, it was demonstrated that IgG from atopic dermatitis (AD) patients could induce the *in vitro* production of IL-17 and IL-10 by infant intrathymic TCD4, CD8+ T, and iNKT cells (9, 10).

Together, these findings indicate a broad potential of these molecules to exert modulatory functions that can directly influence the peripheral atopic immune status of mice and humans.

$CD8^+$ T cells, unlike TCD4 cells, are refractory to corticosteroids (11) and have the capacity to produce IL-4 (TC2) inducing murine lung eosinophilia (12) and allergic asthma (13). In humans, TC2 cells are also related to the pathology and severity of asthma (14, 15) and increased in the airways of Chronic Obstructive Pulmonary Disease (COPD) patients (16). Indeed, human $CD8^+$ T cells produce more IL-4 than TCD4 cells (17, 18), which suggests that they can play a pivotal role in the development of allergic diseases.

This study aimed to evaluate *in vitro* the possible differential effects of purified IgG from atopic and non-atopic individuals on cytokine production by human intra-thymic $CD8^+$ T cells that can exert some modulatory influence on allergic responses, mainly TC2 cells. We also examined whether mature peripheral $CD8^+$ T cells exhibit a similar profile in response to atopic and non-atopic IgG.

Materials and methods

Thymic tissues were obtained from 13 infants who underwent corrective cardiac surgery at the Hospital do Coração (HCor), São Paulo, Brazil. The evaluated patients did not exhibit signs of immunodeficiency, genetic syndromes or allergic reactions, and patient age of fewer than 7 days was used as an inclusion criterion (patient age, mean ± standard error (SE): 3.3 ± 0.52 days). Parental allergic backgrounds were evaluated, and only children with non-atopic mothers were included in this study.

Atopic individuals were clinically classified (clinically allergic, reactive to at least two allergens in the Skin Prick Test - SPT), and blood samples were collected to confirm the atopic state by detectable titers of specific IgE to at least two allergens. Non-atopic individuals were classified in the same parameters but without any clinical allergy symptoms, not reactive to any tested allergen in the SPT and without detectable titers of specific IgE to any tested allergen. Each sample of thymic tissue or peripheral blood mononuclear cells (PBMCs) was obtained from a different donor and analyzed in three independent experiments. The ethics committees at the HCor and the School of Medicine at the University of São Paulo approved this study (CAAE: 15507613.4.0000.0060).

Suspensions of thymocytes or freshly thawed separated PBMCs were washed and resuspended in RPMI 1640 medium containing 10% FCS (HyClone - Fetal Clone III, USA).

An aliquot of cell suspensions diluted in trypan blue (Sigma, USA) was evaluated to access cell viability. Thus, 1×10^6 viable cells were cultured in 96-well plate (CoStar, USA) with 100µg/mL of IgG from atopic or non-atopic individuals or in control conditions (100µg/mL of commercially used IgG or without IgG, the mock condition), all in RPMI 1640 medium containing 10% FCS.

The concentration of 100 µg/mL of IgG was previously determined as optimal to modulate the cytokine production by lymphocytes (7-9, 19) and can be considered low when compared to biological conditions (6). The culture plates were incubated for 2 days. Subsequently, 1 µg/mL Brefeldin A (Sigma, Israel) was added to each well of the culture plates, and after 24 hours, cell staining was performed to evaluate cell labeling via flow cytometry.

For extracellular staining, 0.5×10^6 cells/mL thymocytes or PBMCs were transferred to test tubes, and 1 µg of each antibody was added to the cells (except for the unlabelled tubes). Then, the samples were incubated, washed, and fixed with formaldehyde. Thymocytes or PBMCs were stained with mouse anti-human CD4 and CD8 or isotype control antibodies (BD Pharmingen, NJ, USA).

For intracellular labeling, 1 µg of each antibody was added to the cells in 100 µL of PBS containing 0.05% saponin. Thymocytes and PBMCs were stained with anti-human IL-17A, IL-22, and IL-4 or isotype control conjugated with fluorochromes (BD Pharmingen).

Detailed method describing SPT, serum anti-allergen IgE determination, collection of blood samples, separation of human PBMC and thymocytes suspensions, determination of human total IgG subclasses, IgG purification, and additional cell culture and flow cytometry methods aspects can be accessed on the **online supplements** (**Methods** and **References**).

Results

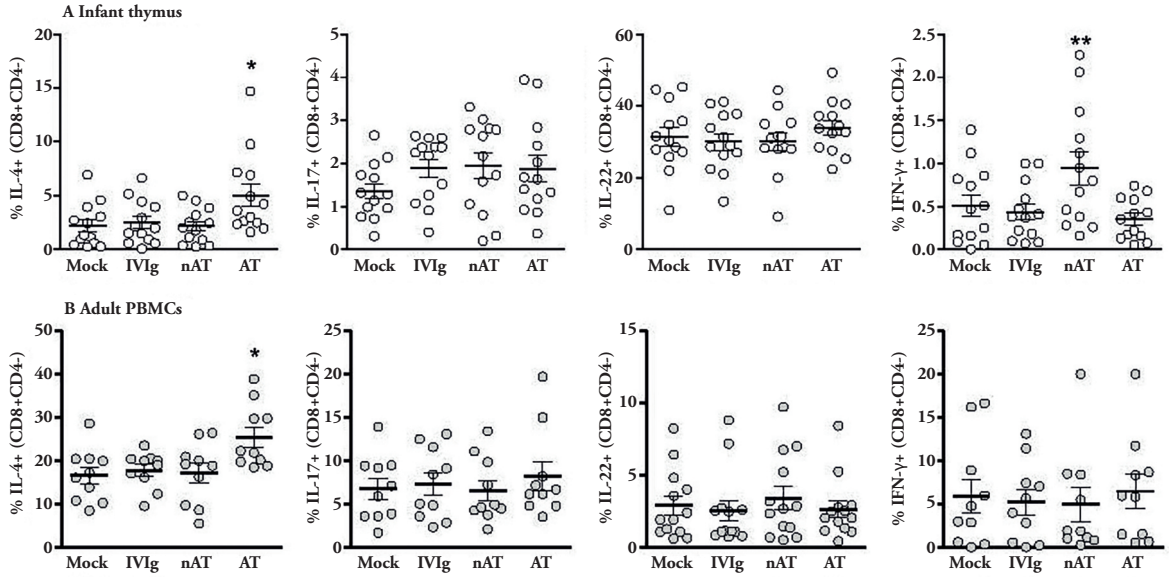
First, we evaluated atopic and non-atopic IgG subclasses and could observe that they can be found at similar levels between purified IgG samples (**online supplements figure 1S**). Next, Thymocytes or PBMCs were cultured in the presence of purified IgG for three days, and, after this period, we found that the culture conditions used in this study did not influence the frequency and viability of thymic nor peripheral $CD8^+$ T cells (**online supplements figure 2S**).

After evaluating cell frequency and viability, we analyzed cytokine production in $CD8^+$ T cells. We found that treatment with IgG from atopic individuals induced elevated production of IL-4 in infant thymic and adult peripheral $CD8^+$ T cells compared to non-treatment and treatment with IVIg or IgG from non-atopic individuals (**figure 1 A, B**). IL-17 and IL-22 production on infant thymic and adult peripheral $CD8^+$ T cells did not appear to be influenced by any of the evaluated IgG. We also assessed IFN-γ production, and we could observe that IgG from non-atopic individuals induced an augmented production of IFN-γ in infant thymic $CD8^+$ T cells when compared to non-treatment and treatment with IVIg or IgG from atopic individuals. No effect could be observed on IFN-γ production by adult peripheral $CD8^+$ T cells.

Discussion

Although $CD8^+$ T cells are essential compounds of adaptive immunity contributing to clearance of intracellular pathogens and long-term protection, there is growing evidence suggesting that

Figure 1 *- Effect of purified IgG on non-atopic infant intrathymic and non-atopic adult peripheral CD8⁺ T cells.*



Thymocytes from infants less than 7 days old (n = 13) or PBMCs from adults (n = 10) were cultured for 3 days in RPMI medium supplemented with FCS. Those cells were cultured in the absence of IgG (mock), in the presence of 100 µg/mL of commercial IgG (IVIg), 100 µg/mL of IgG from non-atopic individuals (nAT) or 100 µg/mL of IgG from atopic individuals (AT). The frequency of cultured thymic ($CD8^+CD4^-$) $CD8^+$ T cells (A) and peripheral ($CD8^+$) $CD8^+$ T cells (B) that produces IL-4, IL-17, IL-22 and IFN-γ were evaluated. The results demonstrate individual values, and the lines represent the means with standard error. *P ≤ 0.05 compared with the mock, IVIg and nAT conditions. **P ≤ 0.05 compared with the mock, IVIg and AT conditions.

IL-4-producing $CD8^+$ T (TC2) cells can exert a pivotal role in the regulation of allergy development (20).

The possibility of IgG as allergy regulators has been discussed for decades, and anti-allergen IgG shown to be related to allergy inhibition in children during the first years of life (21-23). Here we demonstrate that IgG from atopic individuals can induce the thymic maturation of TC2 cells *in vitro*. In humans, TC2 cells are related to the pathology and severity of asthma (14, 15) and increased in the airways of Chronic Obstructive Pulmonary Disease (COPD) patients (16).

Human $CD8^+$ T cells were already described significant producers of IL-4 when compared to TCD4 cells (17, 18), although this is not consensual in the literature, our observations corroborate with the emerging evidence suggesting a pivotal role of TC2 cells in allergic diseases as cited above.

To elucidate if the observed results could be better related to allergy development, we also observed if IgG from atopic individuals could modulate the maturation of IL-17-producing $CD8^+$ T cells (TC17) that are related to the initiation and maintenance of autoimmunity diseases (24-26). Our findings indicated that IgG from atopic individuals could not influence the maturation of autoimmune-related TC17 cells.

We also evaluated if IgG from atopic individuals could modulate the maturation of IL-22-producing $CD8^+$ T cells (TC22), a $CD8^+$ T subset related to the development of a non-related atopic disease characterized by chronic skin inflammation, the Atopic Dermatitis (27). Since we did not observe any influence in the frequency of TC22, these results corroborate with the hypothesis that IgG from atopic individuals can mediate a mechanism restricted to allergy development.

Due to the significant importance of IFN-γ in allergy development, we also evaluated the production of this cytokine. Interestingly, IgG from non-Atopic individuals could induce augmented levels of this cytokine in infant thymic but not adult peripheral $CD8^+$ T cells. A similar effect mediated by non-Atopic IgG on thymic $CD8^+$ T cells was already described in the literature and can corroborate with the development of a non-Atopic as held by IgG donors (6). The IgG-mediated IFN-γ modulation on thymic $CD8^+$ T cells was also described using IgG from HIV infected individuals (28).

Taken together, the effects of purified atopic and non-atopic IgG, suggests that IgG from atopic individuals induces IL-4-producing thymic CD8⁺ T cells. In contrast, IgG from non-atopic individuals induces IFN-γ-producing CD8⁺ T cells. These observations corroborate with the higher frequency of IL-4-producing, and the lower frequency of IFN-γ-producing CD8⁺ T cells demonstrated in patients with allergic asthma when compared to healthy controls (29).

In the present paper, we did not demonstrate the effect of IgG from atopic and non-atopic individuals on the modulation of TCD4 cells. Still, it was shown in a very similar *in vitro* approach that IgG from atopic individuals can also modulate thymic TCD4 cells inhibiting IFN-γ and inducing IL-17 production (6).

If transposed to *in vivo* conditions, our findings can collaborate with the elucidation of allergy development in atopic individuals. This mechanism can involve the event of an atopic-prone IgG repertoire that can, since the neonatal period, favor the maturation of TC2 cells that reaches the peripheral tissues and collaborates with allergy development.

In this context, we can also suggest a role for maternal immunity since maternal IgG modulates children immune status by the passive transference during the gestational period (3, 21, 30-34). As theoretically discussed in 2017 with the "MatIgG primary modulation theory" (35), the passive transference of maternal IgG can modulate offspring immunity when maternal IgG reaches offspring primary organs modulating the immunity development according to maternal atopic state. This hypothesis has been confirmed with pieces of evidence obtained in murine (36, 37) and translational experimental approaches (38, 39).

Conclusions

Our observations are unprecedented in the literature and corroborate with studies that had demonstrated IgG as a molecule that modulates the thymic maturation of lymphocytes favoring the development of allergy. Furthermore, it yields novel but still exploratory observation, which can collaborate with the future elucidation of atopic state development in humans.

Acknowledgments

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Conflict of interests

The authors declare that they have no conflict of interests.

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Methods

SPT, serum anti-allergen IgE determination and collection of blood samples

The SPTs were performed in accordance with European standards (1) with an adapted panel of allergens that included the profile of Brazilian allergens (*i.e., Dermatophagoide farinae, Dermatophagoides Pteronyssimus, Blomia tropicalis, Gallus gallus domesticus, Litopenaeus vannamei, Farfanepenaeus subtilis*, *Farfanepenaeus paulensis, Sus scrofa domesticus, Bertholletia excels, Arachis hypogaea, Aspergillus fulmigatus, Penicillium notatum, Alternaria Alternata, Cladosporium herbarum, Blatella germânica, Periplaneta Americana, Avena nativa, Hordeum vulgare, Tricicum aestivum, Gallus gallus domesticus, Solenopsisi invicta, Culex pipiens, Canis familiaris Felis domesticus* and *Bos Taurus*) as described previously by our group (2). We excluded patients who used antihistamines, glucocorticosteroids or certain other systemic drugs that can influence the SPT results within 15 days before the test. We also excluded volunteers with severe eczema or dermographism.
Serum-specific IgE antibodies have been measured with a multiplex immunoblot assay (EUROLINE Inhalation 2 - EUROIMMUN AG, Lubek, Germany). The tested extracts were: *Dermatophagoide farinae, Dermatophagoides Pteronyssimus, Aspergillus fulmigatus, Penicillium notatum, Alternaria Alternata, Cladosporium herbarum,* cat, dog, horse, guinea pig, rabbit, hamster, timothy grass, cultivated rye, alder, birch, hazel, mugwort, english plantain and cage bird mix (budgerigar, chicken, canary bird and goose).
IgE determination was performed according to the specifications of the EUROLINE Inhalation 2 Kit (EUROIMMUN, Lubek, Germany) to obtain semi-quantitative results.
After confirmation of the individual atopic state, blood samples were obtained from each individual via venepuncture.

Separation of human PBMC and thymocyte suspensions

PBMC and thymocyte separations were performed using Ficoll-Paque Plus (GE Healthcare, Sweden) after centrifugation, as previously described by our group (2). Cell viability was assessed by flow cytometry.

Determination of human total IgG subclasses

Total IgG subclasses were measured according to the specifications of the BINDARID Radial Immunodiffusion Kit (RID - Binding Site, UK). Briefly, 5 mL of each purified IgG pool was applied to a subclass-specific RID plate and incubated at room temperature for 72 hours. Ring diameters were measured, and the concentrations were determined using a reference table provided in the kit. Calibrators supplied with the kit were used as controls.

IgG purification

IgG was purified from pooled serum samples according to the specifications of the Melon Gel IgG Spin Purification Kit (Thermo, USA) and as previously described by our group (3, 4). All samples were collected, sterilized, and stored at - 80°C for subsequent use in cell culture experiments. The IgG concentration was determined using Coomassie Protein Assay Reagent (Pierce, USA) according to the manufacturer's instructions. The purity of IgG, evaluated by SDS-PAGE, was above 95%. IgA, IgE, and IgM were undetectable on samples after purification.

Additional Cell culture methods aspects

For cell viability analysis, extracellular staining was performed as described above, and cells were incubated with Live/Dead (PE-Texas red) fluorescent reagent (ThermoFisher, USA). All extracellular and intracellular analyses were performed using viable cells.

Additional flow cytometry methods aspects

To standardize intracellular cytokine detection, we applied stimulation with PMA and ionomycin for 6 hours as a positive control (5). As a negative control, we used *ex vivo*-stained cells. Positive controls induced 4- to 6-fold percentages of all evaluated cytokines on TCD8 cells with similar mean fluorescence intensity (MFI) levels. In contrast, in the negative controls, the rates of all evaluated cytokines on TCD8 cells were 0.5% or smaller.
Compensation for the instrument was performed using microbeads adsorbed with anti-mouse antibodies (CompBeads, BD Biosciences) and their conjugated antibodies. Acquisition of 300,000 events per sample was conducted in the lymphocyte quadrant (as determined by ratio size/granularity) on an LSR-Fortessa cytometer (BD Biosciences, USA), and analysis was performed using FlowJo software 10.1 (Tree Star).
All antibodies were titrated to determine the optimal concentration for specific staining. Cell gating was based on the isotype control values and the FMO setting.

Statistical analysis

Comparisons among the evaluated groups were performed ANOVA method to compare all pairs of columns. Statistical significance was defined as a p-value < 0.05 determined using GraphPad Prism software (CA, USA).

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Figure 1S *- IgG subclasses of purified IgG. IgG from atopic or non-atopic adults was pooled, and radial Immunodiffusion evaluated IgG subclasses. Data are presented as means ± SEM.*

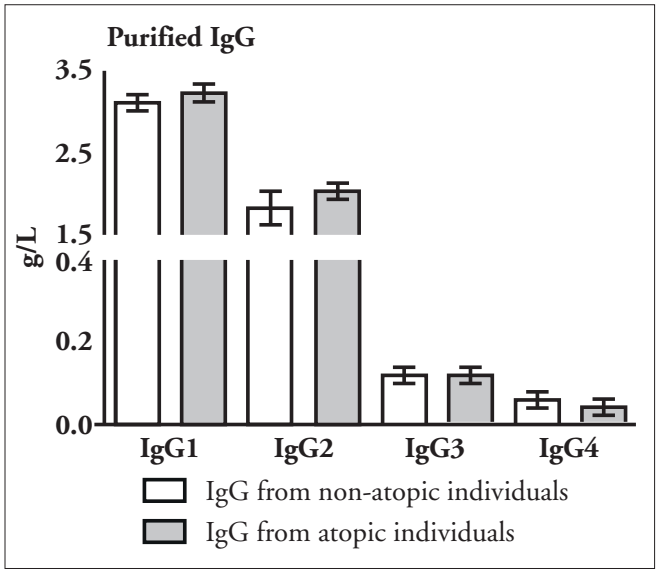

Figure 2S *- Effect of purified IgG on non-atopic infant intrathymic and non-atopic adult peripheral TCD8 cells. Thymocytes from infants less than 7 days old (n = 13) or PBMCs from adults (n = 10) were cultured for 3 days in RPMI medium supplemented with FCS. Those cells were cultured in the absence of IgG (mock), in the presence of 100 µg/mL of commercial IgG (IVIg), 100 µg/mL of IgG from non-atopic individuals (nAT) or 100 µg/mL of IgG from atopic individuals (AT). The frequency and viability of cultured TCD8 cells were evaluated on thymocytes (A) and PBMCs (B). The results demonstrate individual values, and the lines represent the means with standard error.*