

Report

IgG from atopic dermatitis patients induces IL-17 and IL-10 production in infant intrathymic TCD4 and TCD8 cells

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Abstract

Introduction Our group recently demonstrated that IgG modulates $\alpha\beta$ T cell cytokine production during the maturation process in the human thymus. The effects of this modulation are IgG repertoire dependent and can exert a systemic and long-term impact.

Objective To investigate whether IgG from atopic dermatitis (AD) patients can modulate cytokine production of infant intrathymic TCD4 and TCD8 cells *in vitro*.

Methods Thymic tissues were obtained from newborn children from nonatopic mothers, and thymocytes were cultured for 6 days with purified IgG from AD patients or with intravenous immunoglobulin (IVIG) or mock conditions as controls. Cells were gated as double positive T cells (TDP⁺CD4⁺CD8⁺), TCD4 cells (CD4⁺CD8⁻), or TCD8 cells (CD4⁻CD8⁺), and intracellular levels of IL-17A, IFN- γ , TNF- α , IL-4, IL-10, and TGF- β were evaluated by flow cytometry.

Results Compared to mock and IVIG culture conditions, IgG of AD individuals induced *in vitro* intracellular production of IL-17 and IL-10 by intrathymic TDP, TCD4, and TCD8 cells of infants. TGF- β was also detected at a higher frequency in response to AD IgG in TDP and TCD8 cells compared to mock and IVIG cultured conditions. An opposite effect was detected upon IFN- γ production in TCD4 cells, such that AD IgG reduced IFN- γ production compared to production under mock conditions but not under IVIG conditions.

Conclusion IgG of AD patients can stimulate cytokine production in infant thymocytes and thus resembles the peripheral profile observed in adults. These findings suggest a novel mechanism that can contribute to AD pathogenesis.

Introduction

In the last 15 years, our group has investigated adoptive maternal-fetal immune interactions in the context of allergy regulation.^{1–11} In 2014, the authors suggested that maternal immunization could influence intrathymic maturation of lymphocytes on offspring.¹² Two years later, we proposed a hypothesis named “MatlgG primary modulation theory” that questioned whether IgG could directly influence the maturation of lymphocytes on primary organs such as thymus and bone marrow.¹³

The potential of IgG antibodies as regulators of the immune response has been discussed for decades. More than 20 years ago, evidence that maternal IgG suppresses offspring IgE production was obtained in a murine model.¹⁴ In the following decades, further proof connecting maternal IgG to children allergy inhibition was published,^{15,16} including evidence that murine passive transfer of IgG can modulate the offspring B-cell phenotype.¹⁷

Recently, our group demonstrated that human purified IgG from atopic individuals influences the cytokine production profile of human intrathymic $\alpha\beta$ T cells *in vitro*;¹⁸ moreover, such an influence of IgG upon lymphocytes during the maturation process was not entirely dependent on the maternal origin of IgG. Our next step was to evaluate whether the IgG repertoire from AD patients¹⁹ correlates with cytokine modulation, thus favoring eczematous lesions.

Recently, Batista and colleagues demonstrated that the serum levels of inflammatory cytokines (e.g., IL-17 and IL-10) in adults with AD are higher than the levels in normal individuals,²⁰ suggesting a systemic lymphoid activation. However, this polarization pattern of a prominent inflammatory cytokine was not detected in infants, suggesting that this pattern is acquired over time.²¹

The role of cytokines in AD patients is still a topic of discussion. However, the frequency of Th17 cells, which are TCD4 cells that produce IL-17, positively correlates with AD severity

score,²² and some evidence suggests that IL-10 can play a suppressive role in the chronic inflammation of AD patients.²³

Since the thymus can mature diverse populations of lymphocytes including TCD4 and TCD8 cells, which are the main producers of cytokines in the human periphery, the aim of this study was to evaluate the potential effects of purified IgG from adults with AD in modulating infant intrathymic T cells.

Materials and methods

Patient samples

Thymic tissues were obtained from 14 patients who underwent corrective cardiac surgery at the Hospital do Coração (HCor), São Paulo, Brazil. Exclusion criteria were as follows: suffering from immunodeficiency, genetic syndromes, or allergic reactions and using a systemic immunosuppressant. A patient aged <7 days was utilized as an inclusion criterion (patient age, mean $\pm$ standard error [SE]: 3.4 ± 0.54 days). Parental allergic background was evaluated, and only children from nonatopic mothers were included in this study. Additionally, blood samples were collected from 10 subjects who were previously clinically classified as having moderate or severe AD (patient age, mean $\pm$ SE: 31.30 ± 3.29 years old) according to the EASI (Eczema Area and Severity Index).²⁴

The ethics committees at HCor and the University of São Paulo Medical School approved this study. Informed consent to use biological material for research was obtained from the parents of all children and from AD patients.

Thymic tissue dissociation, cell isolation, and storage

Thymocytes were released from the tissue samples using enzymatic dissociation, as recently described by our group.²⁵ Briefly, the thymus was cut into small fragments and transferred to propylene conical centrifuge tubes with an enzymatic solution (RPMI medium, 0.5 mg/ml collagenase A, 0.02 mg/ml DNase I – Roche Diagnostics, Mannheim, Germany). The digested fragments were gently homogenized and filtered through a plastic sieve to remove aggregates and stromal material.

The resulting cell suspensions were washed. Next, the pelleted cells were resuspended, and the low-density fraction was collected following centrifugation through a Ficoll-Paque gradient (GE Healthcare Bio Science, Uppsala, Sweden). The thymic cells were frozen and stored at -80°C until use.

Blood sample collection

Blood samples were obtained from each AD patient via venipuncture, transferred to tubes without anticoagulants, and centrifuged at 940 g for 10 minutes; sera were then fractionated, pooled, and stored at -80°C .

IgG purification

IgG was purified from pooled serum according to the specifications of the Melon Gel IgG Spin Purification Kit

(Thermo, Waltham, USA) and as recently described.¹⁸ Briefly, a purification gel was transferred to a column coupled to a polypropylene conical tube and was then centrifuged. The supernatant was discarded, and the purification gel was resuspended in a mild purification buffer at physiological pH. The supernatant was discarded, and the purification gel was again suspended in the same purification buffer. A pooled serum sample was added to the gel, and the mixture was homogenized. The supernatant (purified IgG) was collected, sterilized, and stored at -80°C for subsequent use in cell culture experiments. IgG concentration was determined using Coomassie Protein Assay Reagent (Pierce, Waltham, USA) according to the manufacturer's instructions. The purity of IgG, evaluated by SDS-PAGE, was above 90%.

Cell culture and flow cytometry

The methods for cell culture and flow cytometry have been previously described.^{18,25} Briefly, thymocyte viability was evaluated using a Neubauer chamber under an optical microscope (Laboroptik, Hessen, Germany). Next, 1×10^6 viable thymocytes were distributed to each well of a 96-well culture plate (CoStar, Kennebunk, USA) and cultured in $100\text{ }\mu\text{g/ml}$ of IgG purified from the pooled serum. As controls, we cultured thymocytes in mock conditions or with $100\text{ }\mu\text{g/ml}$ of IVIg. The culture plate was incubated for 6 days. Subsequently, Brefeldin A (Sigma, Jerusalem, Israel) was added to each well of the culture plate, and after 12 hours, cell staining was performed to evaluate cell labeling via flow cytometry.

To perform extracellular staining, thymocytes were transferred to test tubes, and antibody was added to the cells. The samples were then incubated, and the supernatant was discarded. Thymocytes were fixed with formaldehyde and stained with mouse anti-human CD4-V605, CD8-APC-Cy7, or isotype control antibodies (BD Pharmingen, New Jersey, USA) to identify populations of TDP cells ($\text{CD4}^+\text{CD8}^+$), TCD8 cells ($\text{CD4}^-\text{CD8}^+$), and TCD4 cells ($\text{CD4}^+\text{CD8}^-$).

To perform intracellular labeling, the samples were incubated with saponin. Afterward, the supernatant was discarded, and the cells were stained with mouse anti-human IFN- γ -V450, IL-17A-Alexa700, TNF- α -PE-Cy7, TGF- β -PE, IL-10-TexasRed, IL-4-APC, or isotype control conjugated with the corresponding fluorochromes (BD Pharmingen).

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

Using an LSRII Fortessa flow cytometer (BD Biosciences), 500,000 events per sample were acquired in the quadrant of lymphocytes. Compensation was performed using adsorbed microspheres (CompBeads – BD Biosciences). Data analysis was performed using FlowJo software (Tree Star, Ashland, USA).

Statistical analysis

Comparisons among the evaluated groups were performed using the nonparametric Mann-Whitney test (to compare selected pairs of columns). Statistical significance was defined as a $P < 0.05$ and was determined using GraphPad Prism software (GraphPad software, La Jolla, USA).

Results

Purified IgG from AD patients modulates cytokine production by infant intrathymic TDP, TCD4, and TCD8 cells

The gating strategy to identify TDP, TCD4, and TCD8, and its viability are shown in Figure 1a. Figure 1b depicts TDP cells cultured with purified IgG from AD patients, and TDP cells were found to produce higher levels of IL-17A, IL-10, and TGF- β compared to the levels in both mock and IVIG-treated cells.

TCD4 cells, similar to TDP cells, were also influenced by IgG from AD patients. The production of IL-17A and IL-10 was detected at higher levels after culture with AD IgG compared to mock and IVIG-treated conditions (Fig. 2a). In contrast, IFN- γ production in TCD4 cells was inhibited by treatment with AD

IgG, but only when compared to production in mock condition (Fig. 2a).

TCD8 cells were also influenced by AD IgG. Elevated IL-17A, TNF- α , IL-10, and TGF- β were observed at higher levels in this cell population, compared to the mock and IVIG-treated conditions (Fig. 2b).

Discussion

Thymic tissue dissociation and culture with IgG model have been previously established¹⁸ and were valuable tools for elucidating the direct effect of interactions between soluble IgG and cell populations in the human thymus. As discussed by our group on the recently published "MatIgG primary modulation theory,"¹³ the effects of IgG in this model are possibly mediated by direct idiotype recognition of membrane clonal receptors or cytoplasmic molecules. However, the mechanisms by which IgG modulates T cells to produce cytokines still need to be elucidated.

In the present work, we used IVIG as an additional control beyond mock conditions. IVIG is a pool of purified human IgG antibodies obtained from thousands of healthy individuals and is

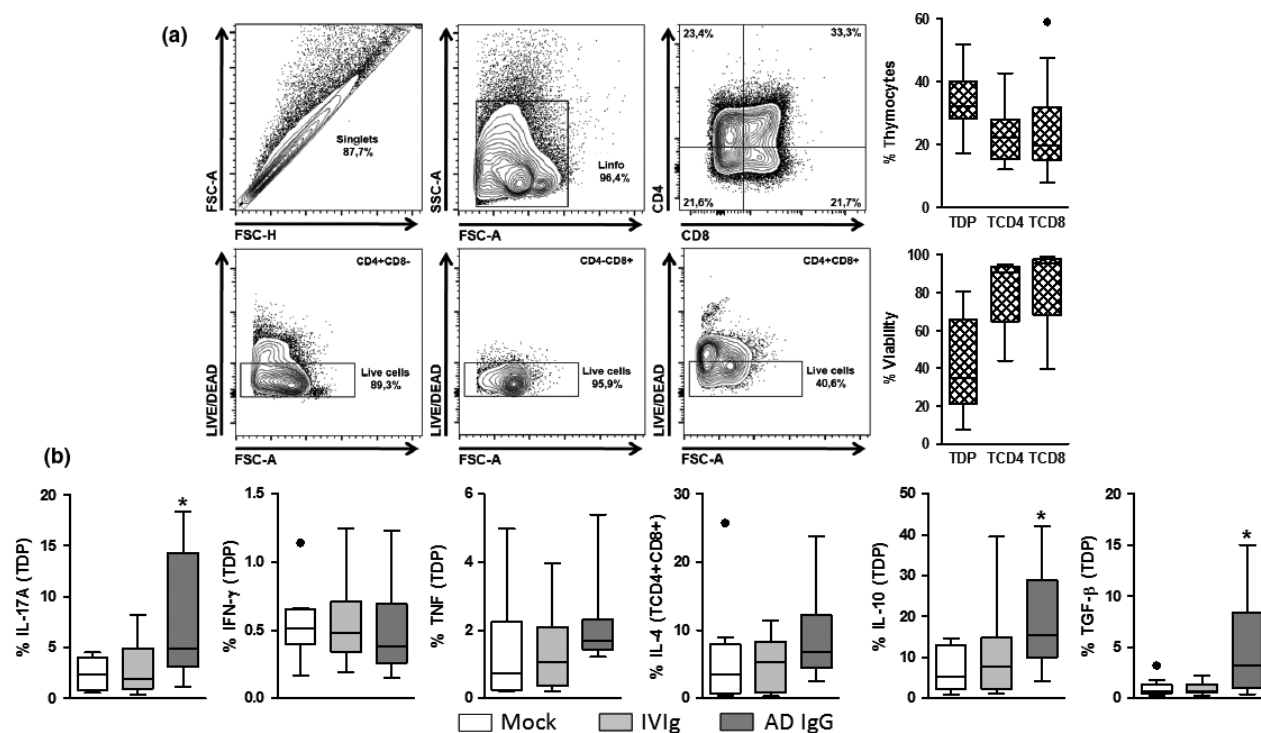


Figure 1 Frequency, viability, and the effect of purified AD IgG on infant intrathymic $\alpha\beta$ T cells. Thymocytes from children less than 7 days old ($n = 14$) were evaluated after 7 days in culture in RPMI medium supplemented with FBS. The gate strategy to identify TDP, TCD8, and TCD4 $\alpha\beta$ T cell populations and viability are demonstrated (a). Thymocytes were also evaluated after 7 days in culture in RPMI medium supplemented with FBS in the absence (mock) or presence of 100 μ g/ml of IVIG or IgG purified from AD patients. The frequencies of TDP cells (b) displaying intracellular IL-17A, IFN- γ , TNF- α , IL-4, IL-10, and TGF- β production were evaluated by flow cytometry. The results are illustrated by box-and-whisker plots with 25th percentiles, and the Tukey's method was used to plot outliers; $*P \leq 0.05$ when compared to mock and IVIG conditions

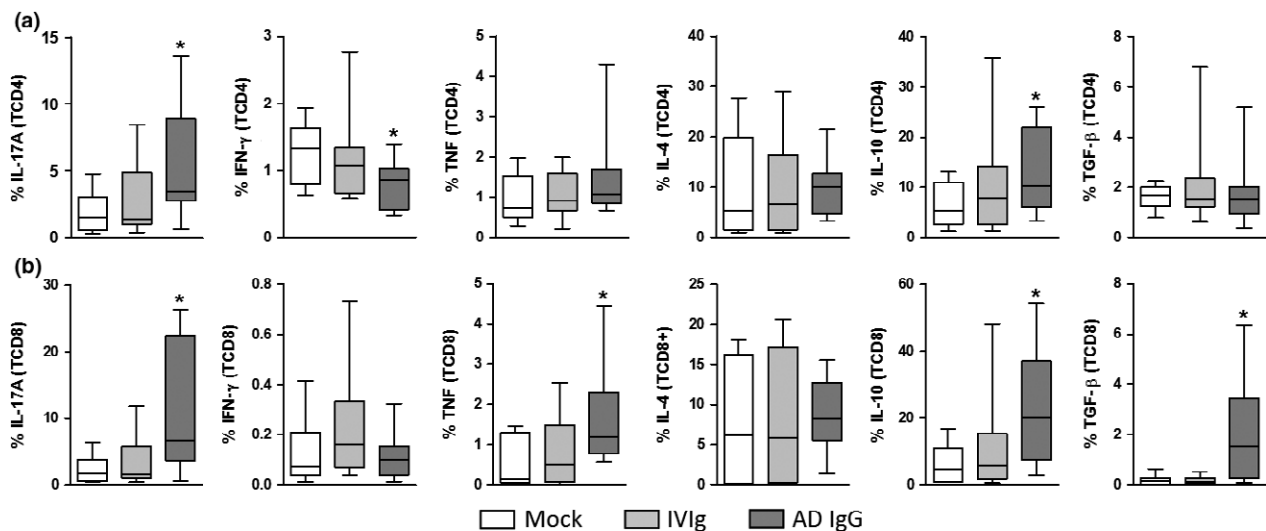


Figure 2 Effect of purified IgG on cytokine production by infant intrathymic TCD4 and TCD8 cells. Thymocytes from children less than 7 days old ($n = 14$) were evaluated after 7 days in culture in RPMI medium supplemented with FBS in the absence (mock) or presence of 100 μ g/ml of IVIG or IgG purified from AD patients. The frequencies of TCD4 (a) or TCD8 (b) cells displaying intracellular IL-17A, IFN- γ , TNF- α , IL-4, IL-10, and TGF- β production were evaluated by flow cytometry. The results are illustrated by box-and-whisker plots with 25th percentiles, and Tukey's method was used to plot outliers; * $P \leq 0.05$ when compared to mock and IVIG conditions

routinely used in the treatment of patients with primary immunodeficiencies.²⁶ Among the exclusion criteria of donors, several pathologies are considered, including AD. Therefore, IVIG represents a wide sampling of IgG purified from non-AD donors.

Our results revealed that when compared to mock and IVIG conditions, AD IgG exhibits a distinct cytokine-inducing potential. AD IgG could induce higher levels of IL-17 in all evaluated populations. These results corroborate with the evidence that AD patients demonstrate higher levels of IL-17 in serum.²⁰ TCD4 cells that produce IL-17 are known as Th17 cells; these cells are increased in the peripheral blood and on acute lesional skin of AD patients.²⁷ It has also been described that AD patients have higher levels of Th17 cells in peripheral blood when compared to that of allergic contact dermatitis patients, and the Th17 cell frequency positively correlates with the AD severity score.²² The induction of a Th17 pattern early in life is important in pediatric AD; some recent evidence about the mechanism permissive for AD initiation in children skin is characterized by decreased Th1 response and upregulated Th17-associated mediators compared to adult AD skin.²⁸ Other groups also observed that Th17 cells can reach skin of patients with AD amplifying local inflammatory response that can mediate systemic AD-related immune dysregulation.²⁹ Moreover, the Th17 upregulation can induce IL-19,^{30,31} suggesting a link between Th17 and Th2 axes since concomitant high levels of IL-19 and Th17 activation were described on Asian AD patients' skin that holds a strong Th2 component.³² This Th17/Th2 link on AD still needs to be elucidated, and it is very important since enhanced Th2 profile is a major immune characteristic of pediatric AD patients.³³

Together with our evidence, we hypothesize that the IgG repertoire of individuals who are predisposed to develop AD can contribute to the induction of Th17 cells in the initial stages of TCD4 development and that this can possibly occur at an early age. Additionally, it is also possible that these IgG-induced Th17 cells that reach peripheral circulation and lesional skin contributing to the development and exacerbation on AD. However, further investigations are needed to elucidate this hypothesis.

Unlike IL-17, the role of IL-10 in AD patients is not well defined. In the literature, we found evidence that describes decreased levels of IL-10 production in the peripheral blood cells of neonates after stimulation with mixed TLR agonists,³⁴ and a similar profile was also described in peripheral blood cells of severe AD patients, but not mild AD patients, after stimulation with phorbol myristate acetate (PMA) and ionomycin.³⁵ In contrast, another group described that peripheral cells from AD patients produce higher levels of IL-10 in response to LPS,³⁶ revealing that AD patients seem to produce different IL-10 levels in response to different intensive polyclonal stimulation that did not reflect a physiological condition. Recently, two independent groups evaluated serum cytokine levels in AD patients without stimulation, and both described high serum levels of IL-10 in AD patients compared to the levels in healthy individuals,^{20,37} suggesting that high IL-10 production is a physiological characteristic of adult AD patients. In a murine model of AD, it was observed that IL-10 production by dendritic and Tr1 cells can alleviate AD inflammation.³⁸ Moreover, the impaired function of B10 cells (regulatory B cells that produce IL-10) are related to an impaired regulation upon IgE production on a

murine model of AD.³⁹ The immune-regulatory properties of IL-10 has been described years ago,^{40–42} but its role on human AD is still unclear and thus prevents us from long discussion; however, some evidence suggests that the high IL-10 production described above can play a suppressive effect upon human AD chronic inflammation.²³ This suppressive effect can result from a feedback mechanism as a consequence of a chronic inflammatory state but, according to our data, this elevated IL-10 production can be induced early in life and seems to be mediated by IgG/intrathymic T cells interactions. Based on these evidences, we suggest that IL-10 may play a role from the very early stages of AD development, but further evidences need to be obtained to elucidate this issue.

TGF- β production was also augmented by AD IgG but only on TDP and TCD8 cells. Few references can be found in the literature evaluating TGF- β production in AD patients; however, the production of this cytokine seems to alleviate AD.^{43,44} TGF- β production by TCD8 cells was described as a TCD8 lymphocyte signature in pediatric type 1 diabetes.⁴⁵ In this work, the authors revealed that TCD8 cells simultaneously produce TGF- β , IL-10, granzyme B, and perforin, and suggested that this population is an attempt to counteract an especially aggressive inflammatory disease; however, this population was not described in AD patients.

Our results also reveal the reduction of IFN- γ production by TCD4 in response to AD IgG. In contrast to our results, some authors have described higher levels of IFN- γ in AD serum.²⁰ This high IFN- γ production on skin-derived TCD4 cells seems to be induced by IL-33 secreted by keratinocytes.⁴⁶ However, other authors have reported that skin-derived TCD4 cells produce reduced levels of IFN- γ when compared to the levels produced by psoriasis skin-derived TCD4 cells,⁴⁷ and that pediatric AD²⁷ and Asian AD patients³² skin are characterized by decreased Th1 markers including IFN- γ gene expression. Therefore, our results suggest another route for IFN- γ induction in the skin lesions of TCD4 AD patient.

Taken together, these results suggest that the repertoire of IgG antibodies from AD patients can differentially influence cytokine production by $\alpha\beta$ T cells during their maturation in humans. Considering that an IgG repertoire differs between groups of humans, mainly in idiotypic diversity, this evidence reinforces our group hypothesis that the IgG repertoire is responsible for the diverse effects upon T cells during its maturation.

Furthermore, it is important to note that we used a dose that is 10- to 40-fold lower than the dose typically used in *in vitro* experiments described in the literature that is approximately 6.0 mg/ml,⁴⁸ a concentration that was determined based on the physiological concentration of IgG. We used this low dose to approximate our findings of a physiological condition because the normal range of IgG in the umbilical cord serum is approximately 0.8 g/l,⁴⁹ representing a 10- to 20-fold lower concentration compared to serum from a normal adult.

Therefore, it is interesting to discuss that our observations can exert some impact *in vivo*. It is possible that an infant prone to develop AD produces an IgG repertoire that reaches the thymus; as this can still occur during its gestation, this IgG repertoire can modulate the cytokine production by self $\alpha\beta$ T cells and favor the progression of these infants into AD patients after birth.

Even if it does not occur during gestation period, we suggest that self-IgG could modulate peripheral $\alpha\beta$ T cells cytokine production after birth and contribute to AD development later in life. Recently, our group evaluated the effect of purified IgG from atopic or nonatopic individuals (classified by skin prick test combined with clinical symptoms), and we observed that the effect mediated by IgG upon thymic $\alpha\beta$ T cells was also observed on adult peripheral $\alpha\beta$ T cells.¹⁸

A recent study evaluating the serum of infants with AD revealed that the levels of certain cytokines, including IFN- γ , do not corroborate with our results;²¹ on the other hand, the evaluation in adults revealed augmented levels of IL-17 and IL-10 without influencing TNF- α and IL-4, which was very similar to the profile of cytokine modulation that we observed.²⁰

Together, these results suggest that the effects induced by AD IgG on thymocytes can be possibly observed in the periphery of adults; however, we did not evaluate the direct effect of IgG on peripheral cells of AD patients to ensure this hypothesis. We also cannot ascertain the duration of the intrathymic impact on cytokine production in AD patients. However, the work cited above revealing a similar profile of cytokines in adults included AD patients who ranged in age from 18 to 48 years old²⁰ and was consistent with our hypothesis of a long-term effect of IgG.

Another potential concern of IgG modulation in children born from AD mothers is that IgG could be modulated by passively transferring maternal IgG during gestational and/or breastfeeding periods, which is a possibility that is closely related to our group hypothesis and was recently suggested in a murine model of allergy regulation.⁵⁰ However, at this point, we do not have strong experimental evidence to claim this mechanism in humans. We believe that further evidence can emerge from the literature to elucidate these *in vivo* effects in the next few years.

In conclusion, we suggest a novel mechanism of immune modulation on AD patients that consists of an IgG repertoire that influences intrathymic maturation of TDP, TCD4, and TCD8 cells in a manner that favors the development of AD.

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