

## CORRESPONDENCE

# Induction of Treg cells after oral immunotherapy in hen's egg-allergic children

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To the Editor,

Food allergy affects a significant number of children and is the most frequent reason for anaphylactic reactions at this age. Specific oral tolerance induction (SOTI) is an increasingly attractive strategy, and success has been achieved in a majority of patients. However, the immunologic mechanisms underlying SOTI are still unclear and additional advances in such therapies will require a greater understanding of the mechanism of tolerance/desensitization induction. We have recently published that allergic children have increased frequencies and absolute counts of effector-memory  $CD4^+$  T cells ( $T_{EM}$ ) in comparison with non-allergic children (1) and tolerance achievement after SOTI normalized the levels of these  $T_{EM}$  (1).  $T_{EM}$  is a subset of cells with immediate effector function that can rapidly produce inflammatory mediators, playing a key role in food allergy (1, 2). In addition to the deletion of reactive lymphocytes, the generation of regulatory T cells (Treg) has been also postulated as a mechanism capable of preventing inappropriate reactivity against innocuous antigens. Treg cells are a subset of  $CD4^+$  T cells with suppressive function (3), which have demonstrated in animal models a crucial role in the oral tolerance to food antigens (4). However, whether Treg are implicated in the SOTI-mediated desensitization to food allergens has not been elucidated in humans.

Here, we performed an analysis of immune subsets in 18 egg-allergic children [mean (range) age = 9.26 (4–14) years], following a SOTI protocol with egg, comparing immune values before SOTI ( $T_0$ ) and when egg desensitization was achieved ( $T_{end}$ ). All subjects had histories of acute clinical reactions to egg, including immediate reactions (urticaria, vomiting, and/or anaphylaxis), a positive egg-white skin prick test (SPT), and a positive serum-specific IgE. Prior to being included to the desensitization protocol, the clinical history records and immunological analysis were reassessed and an oral challenge test with egg was carried out on those who had not had clinical episodes within the last 3 months. A SOTI protocol with powdered pasteurized egg mixed with juice or milkshakes was established enabling patients to continue treatment at home, with periodic weekly visits to the outpatient allergy department. On the first day, fractionated doses were administered until reaching 31 mg of egg. Subsequently, weekly increases were made in the clinic until 10 g of powdered egg, the equivalent of one egg, was reached.

All the children enrolled in this study achieved a complete desensitization to egg. A child was considered desensitized when he was capable of ingesting 10 gr of powdered egg without any adverse reactions. A normal diet including egg was recommended to patients after desensitization achievement. 22 age-matched healthy children were also studied as control group [mean (range) age = 7.20 (3–13) years]. The study was

conducted according to the Declaration of Helsinki, approved by the clinical ethics committee of the center, and written informed consent was obtained from all legal guardians. Percentage and absolute counts of Treg,  $CD4^+$  T-cell subsets, monocytes, basophils, and granulocytes were determined in fresh peripheral blood samples as previously described (5).

## SOTI induces an increase in the frequency and number of Treg cells

The results showed that SOTI did not modify the percentages or absolute counts of monocytes, basophils, neutrophils, or eosinophils (Table 1). Thus, the immune mechanism implicated in the SOTI-mediated desensitization seems not to be associated with significant changes in these populations. We observed that desensitization ( $T_{end}$ ) was related to a significant increase in the frequency and absolute counts of Treg (Table 1, Fig. 1a,b). Regarding the phenotype of these Treg, we did not observe significant changes in the proportion of naïve Treg (defined as  $CD4^+CD25^+Foxp3^+CD45RA^+$ ) or effector Treg (defined as  $CD4^+CD25^+Foxp3^+CD45RA^{neg}$ ) after SOTI. However, the increase in the Treg number was more significant for the effector-Treg subset (Table 1).

Findings from animal models and human studies show that allergic diseases are due to an aberrant immune response mediated through the T-helper type-2 cells (Th2), and Treg cells have proved to be crucial in the control of allergic responses by suppressing allergen-specific effector T cells (6). Here, we show that desensitization achieved after SOTI was accompanied by an increase in the frequency and number of Treg. In agreement with these findings, successful venom and aeroallergen immunotherapy have shown to be associated with the induction of peripheral tolerance in T cells by generation of Treg that secrete the suppressive cytokines IL-10 and TGF- $\beta$  (6). Little is known about how these Treg are exactly generated. Treg can be generated in the thymus, but they can also be induced in the periphery (named iTreg) by differentiation from conventional T cells (7). We observed a slight increase in the number of naïve Treg, and thus, an increased thymic output cannot be excluded as the origin of the generated Treg. However, the Treg increase was more marked in the subset of effector Treg, pointing to a peripheral induction of Treg from naïve  $CD4^+$  T cells under subimmunogenic antigen presentation (7). In fact, studies in mice showed that administration of antigen chicken ovalbumin (OVA) by oral route induced the generation of OVA-specific iTreg cells, but concomitant effector T-cell generation was inefficient (8). Thus, SOTI could be favoring the generation of antigen-specific iTreg without increasing the differentiation or activation of effector  $CD4^+$  T cells.

**Table 1** Percentages and absolute counts of immune subsets in egg-allergic children before SOTI (T0) and after tolerance achievement (Tend). Values are given as median (25–75th percentiles)

|               | Percentage                     |                   |        | Cells/ $\mu$ L   |                  |        |
|---------------|--------------------------------|-------------------|--------|------------------|------------------|--------|
|               | T0                             | Tend              | p      | T0               | Tend             | p      |
| Treg Cells    | 7.89 (7.11–9.99) <sup>†</sup>  | 8.42 (7.41–9.81)  | 0.010* | 73 (49–93)       | 84 (61–105)      | 0.037* |
| Naïve Treg    | 48.35 (43.7–55.2) <sup>‡</sup> | 51.88 (44.5–55.6) | 0.948  | 34 (24–49)       | 39 (32–59)       | 0.064  |
| Effector Treg | 52.21 (45.4–56.5) <sup>‡</sup> | 48.64 (45.0–56.1) | 0.879  | 35 (26–48)       | 43 (31–55)       | 0.031* |
| Monocytes     | 5.63 (4.91–7.38) <sup>§</sup>  | 6.54 (4.95–7.93)  | 0.723  | 327 (263–475)    | 383 (286–506)    | 0.717  |
| Basophils     | 1.26 (0.91–1.87) <sup>§</sup>  | 1.18 (0.74–1.57)  | 0.985  | 71 (52–86)       | 75 (52–87)       | 0.655  |
| Granulocytes  | 49.40 (42.8–55.4) <sup>§</sup> | 48.31 (40.8–52.1) | 0.179  | 2624 (1772–3911) | 2499 (2096–3207) | 0.334  |
| Neutrophils   | 84.17 (74.3–87.7) <sup>¶</sup> | 84.06 (76.4–90.2) | 0.642  | 2084 (1530–3337) | 1982 (1744–2654) | 0.281  |
| Eosinophils   | 14.36 (10.9–23.6) <sup>¶</sup> | 14.25 (8.9–20.0)  | 0.796  | 339 (231–660)    | 460 (274–631)    | 0.427  |

<sup>†</sup>Percentage of Treg cells in the total of CD4<sup>+</sup> T cells.

<sup>‡</sup>Percentage of naïve or effector Treg in the total of Treg cells.

<sup>§</sup>Percentage of cells in the total of leukocytes.

<sup>¶</sup>Percentage of cells in the total of granulocytes.

\*p < 0.05 Wilcoxon test for paired samples.

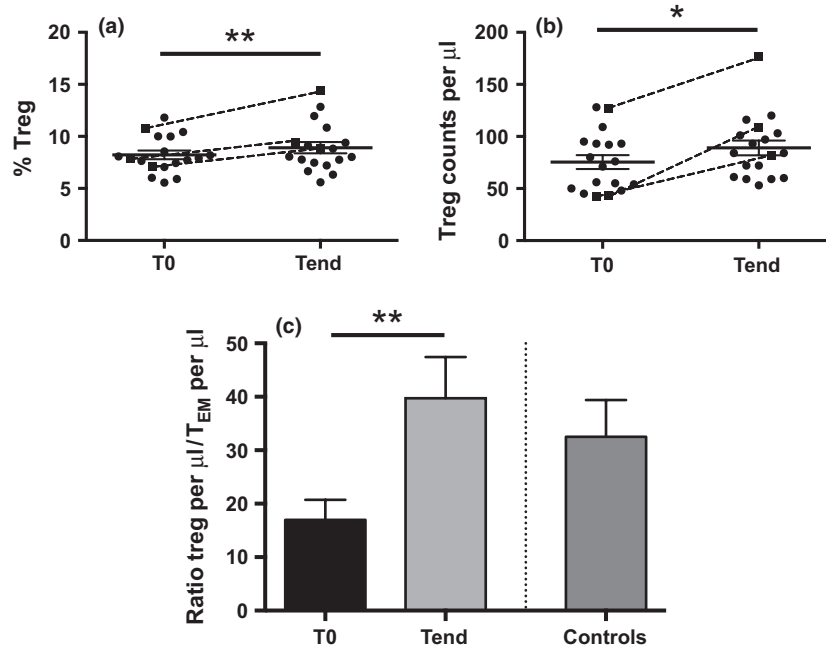


**Figure 1** Treg values and ratio between Treg and T<sub>EM</sub>. Percentage (a) and number of Treg cells per  $\mu$ L of peripheral blood (b) in egg-allergic children (n = 18) before SOTI (T0) and after tolerance achievement (Tend). Horizontal lines represent the mean  $\pm$  S.E.M. Dotted lines represent the variation from T0 to Tend in 3 representative children. (c) Mean  $\pm$  S.E.M. ratio between the number (per  $\mu$ L of peripheral blood) of Treg and effector-memory CD4<sup>+</sup> T cell (T<sub>EM</sub>) at T0, Tend and in a control group of age-matched healthy children (n = 22). \*p < 0.05; \*\*p < 0.01 using the Wilcoxon test for paired samples.

### Treg increase is associated with a decrease in effector immune cells implicated in the allergic process

Immune response to allergens in health and disease is the result of a balance between Treg cells and effector T cells, and a change in the dominant subset may lead to allergy development or tolerance achievement (6). We observed that the ratio between the number of Treg and T<sub>EM</sub> in periphery experimented a 2.3-fold increase after SOTI (p = 0.010), reaching a value comparable with the Treg/T<sub>EM</sub> ratio observed in healthy controls (p = 0.281) (Fig. 1c). As mentioned above, SOTI could induce an increase in the number of Treg without a

concomitant increase in effector cells. This fact joined to the probed effect of Treg suppressing (3) and inducing the senescence of effector T cells would explain the profound change in the Treg/T<sub>EM</sub> ratio.

Effector CD4<sup>+</sup> T cells are differentiated or expanded from naïve CD4<sup>+</sup> T cells in the presence of a stimulus. Therefore, the proportion of T<sub>EM</sub> in periphery could be a good marker of the 'reactivity' of T cells in the presence of the allergen. In allergic children, increased number of circulating Treg was correlated with lower frequencies of T<sub>EM</sub> (Fig. 2a). However, this correlation was not observed in the control group (Fig. 2b). Therefore, we observed that the normalization of

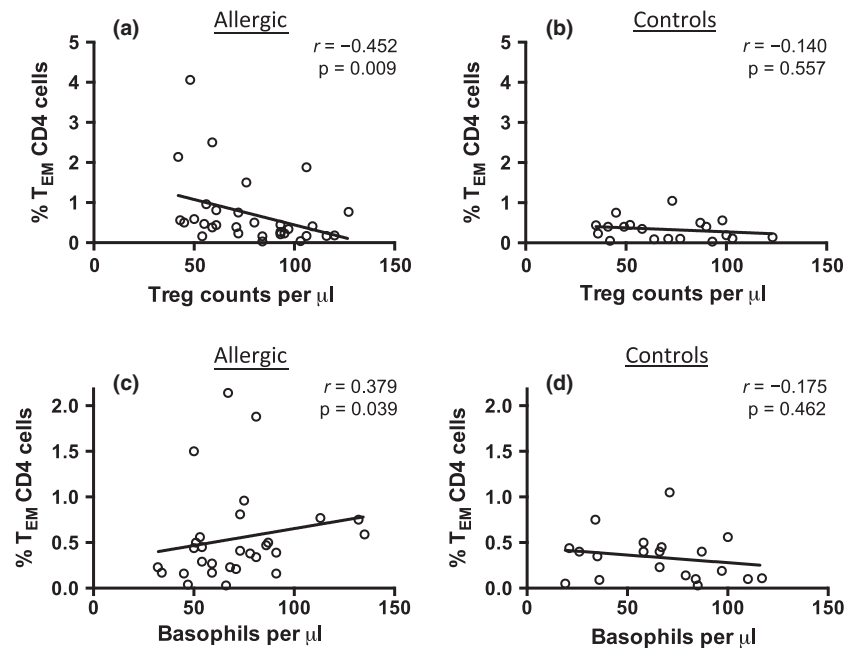


**Figure 2** Effect of Treg/ $T_{EM}$  balance in effector cells. Correlation between the number of circulating Treg and the percentage of  $T_{EM}$  in allergic (a) and healthy donors (b). Correlation between the percentage of  $T_{EM}$  and the number of basophils per  $\mu\text{L}$  of peripheral blood in allergic (c) and healthy donors (d). Correlation was determined using the Spearman's correlation test.

the  $T_{EM}$  frequency previously described after SOTI (1) would be associated with the increase in the number of circulating Treg. This fact is supported by studies demonstrating that depletion of the Treg cells before *in vitro* culture significantly enhanced the expansion of antigen-specific effector T cells from allergic children (9).

Basophils produce cytokines such as Interleukin-4 (IL-4) and IL-13, which are central for the manifestations of allergic disease, and have showed to participate in the tolerance to food allergens (10). Inflammatory cytokines produced by effector CD4 T cells are essential in the expansion and activation of basophils and mast cells. Therefore, we analyzed how the balance between Treg and effector T cells could affect to basophils and granulocytes. We did not find any significant correlation between  $T_{EM}$  and eosinophils or neutrophils ( $p > 0.05$ ). However, in allergic children, we observed a direct correlation between the frequency of  $T_{EM}$  and the number of circulating basophils ( $p = 0.039$ ; Fig. 2c). Interestingly, this correlation between  $T_{EM}$  and basophils was not observed in the control group ( $p = 0.462$ ; Fig. 2d). Comparing values at T0 and Tend, we did not observed significant changes in the proportion or in the number of basophils after SOTI (Table 1). However, our results suggest that in allergic children, basophils and  $T_{EM}$  are correlated, and the recovery of the Treg/ $T_{EM}$  balance after SOTI could contribute to the normalization of the basophils values and the disappearance of symptoms.

Summing up, Treg cells are responsible for the control of effector immune responses against harmless environmental antigens, and their ratio determines the development of a healthy or an allergic immune response (6). Here, we show that desensitization induction after SOTI was associated with an increase in the frequency and number of Treg that participate in the control of the effectors cells (notably  $T_{EM}$ ) implicated in

the allergic process. We have not determined whether the Treg generated are allergen specific, due to the very low proportion of these cells in peripheral blood. However, changes in immune homeostasis after SOTI, which probably are more pronounced in the allergen-specific subsets (8), are also reflected in the peripheral values of total Treg. Due to the high rate of success with SOTI protocols, we have no data about children who do not acquire tolerance, and we cannot determine whether Treg values could constitute a reliable predictive marker of tolerance. However, our results provide an interesting basis for the use of this subset as a predictive marker of clinical outcome and as a tool to follow the efficacy of immunotherapy easily measurable in peripheral blood.

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Victoria Fuentes-Aparicio<sup>1</sup>; Elena Alonso-Lebrero<sup>1</sup>; Lidia Zapatero<sup>1</sup>; Sonsoles Infante<sup>1</sup>; Raquel Lorente<sup>2,3</sup>; M. Ángeles Muñoz-Fernández<sup>2,3</sup> & Rafael Correa-Rocha<sup>2</sup>  
<sup>1</sup>Sección de Alergia Infantil, Hospital Materno Infantil 'Gregorio Marañón', Madrid, Spain; <sup>2</sup>Laboratorio de

Immunobiología Molecular, Instituto de Investigación Sanitaria Gregorio Marañón (IISGM), Madrid, Spain; <sup>3</sup>Networking Research Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Madrid, Spain  
 E-mail: rafael.correa@iisgm.com

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