

Ovalbumin-specific IgE modulates ovalbumin-specific T-cell response after repetitive oral antigen administration

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Background: Some patients outgrow their food allergies even though their serum antigen-specific IgE levels remain high. **Objective:** To elucidate the role of T cells in outgrowing food allergies in the presence of antigen-specific IgE, we tracked antigen-specific T-cell responses after oral antigen administration.

Methods: Ovalbumin (OVA)-specific T-cell receptor (TCR) and OVA-specific IgE transgenic (Tg) mice (OVA-TCR/IgE-Tg) and OVA-specific TCR Tg (OVA-TCR-Tg) mice were fed with high doses of OVA or PBS every other day. After 7 administrations, OVA-specific proliferation and cytokine production of mononuclear cells of the spleen, mesenteric lymph nodes, and Peyer's patches and the number of splenic CD4⁺CD25⁺ T cells were analyzed.

Results: Without OVA administration, the splenocytes from OVA-TCR/IgE-Tg mice exhibited a higher proliferative response and produced more IL-4 and IL-10 and less IFN- γ than those from OVA-TCR-Tg mice. The proliferative responses of the splenocytes from either OVA-TCR/IgE-Tg mice or OVA-TCR-Tg mice fed with OVA were significantly reduced compared with those from PBS-fed mice. The number of OVA-specific TCR⁺ T cells decreased in the spleen from OVA-fed mice, whereas the number of CD4⁺CD25⁺ T cells increased. The suppressed proliferation of splenocytes of OVA-fed mice was partially resumed by neutralization of TGF- β 1, but not of IL-10.

Conclusion: The presence of OVA-specific IgE modulated the OVA-specific responses of the splenocytes. Irrespective of the presence of OVA-specific IgE, repetitive oral administration of OVA induced tolerance, which seems to be composed of clonal deletion/anergy and TGF- β 1-mediated active suppression. (J Allergy Clin Immunol 2005;115:822-7.)

Key words: Food allergy, oral tolerance, IgE, ovalbumin, anergy, clonal deletion

Abbreviations used

OVA: Ovalbumin
TCR: T-cell receptor
Tg: Transgenic

Food allergies are not a single disease, nor are they caused by one pathophysiologic disturbance. Irrespective of the involvement of IgE, antigen-specific T cells play a crucial role in the pathogenesis of all forms of food allergies.^{1,2} Food allergies develop in genetically predisposed individuals, presumably when oral tolerance fails to develop normally or when it breaks down.¹ Of note, patients with IgE-mediated reactions may outgrow their food allergy even though they still have considerable levels of food allergen-specific IgE.

Engagement of antigen-specific IgE antibodies on Fc ϵ RI with antigens allows mast cells to produce various cytokines and Fc ϵ RI-expressing antigen-presenting cells to present food antigens efficiently to T cells.³⁻⁵ Moreover, monomeric IgE has also been reported to influence mast cell function via Fc ϵ RI and thereby facilitate mobilization of antigen-exposed dendritic cells.^{4,6} Therefore, it is conceivable that the presence of antigen-specific IgE affects the induction of oral tolerance through antigen-specific T-cell responses. Accumulating evidence indicates that T-cell anergy, clonal deletion, and active suppression by regulatory T cells play a central role in the development of oral tolerance.⁷ However, animal models have shown that feeding antigen after disease onset might not effectively induce tolerance leading to ameliorate disease.^{8,9} The underlying immunologic mechanisms involved in tolerance induction against food allergies in the presence of food antigen-specific IgE remains uncertain.

Recently, we established a unique transgenic (Tg) mouse that constitutively produces a secreted form of ovalbumin (OVA)-specific IgE.^{10,11} The Tg mouse exhibits high levels of serum OVA-specific IgE, large amounts of IgE immobilized on Fc ϵ RI expressed on mast cells, and the upregulated expression of IgE-loaded CD23 on B cells. To clarify the effects of the presence of antigen-specific IgE on the development of oral tolerance, we tracked antigen-specific T cells *in vivo* and assessed

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cytokine responses in OVA-IgE Tg mice. Because the precursor frequency of T cells present in normal mice is too low to detect antigen-specific T-cell responses, we used double Tg mice with OVA-specific IgE and T cells bearing OVA-specific T-cell receptors (TCRs; OVA-TCR/IgE-Tg). We found that oral tolerance develops irrespective of the presence of antigen-specific IgE and that clonal deletion/anergy and the induction of TGF- β 1-producing cells play crucial roles.

METHODS

Animals

DO11.10 mice with T cells bearing the Tg TCR that recognizes the 323-339 peptide fragment of OVA (OVA-TCR-Tg) were a generous gift from Dr S. Sakaguchi (Kyoto University, Kyoto, Japan). Both the OVA-TCR-Tg and OVA-specific IgE Tg mice (OVA-IgE-Tg) had a Balb/c background. Heterozygous OVA-TCR-Tg mice were crossed with heterozygous OVA-IgE-Tg mice. Mice with both heterozygous OVA-TCR-Tg and heterozygous OVA-IgE-Tg (OVA-TCR/IgE-Tg) and littermates with heterozygous OVA-TCR-Tg alone (OVA-TCR-Tg) were used in these studies. These mice were bred under standard pathogen-free conditions in the animal facility of University of Fukui. All animal experiments were performed in accordance with institutional guidelines as approved by the Animal Care Review Board of University of Fukui.

Oral antigen administration and preparation of mononuclear cells from the spleen, Peyer's patches, and mesenteric lymph nodes

Female OVA-TCR/IgE-Tg mice or OVA-TCR-Tg littermates at 6 to 9 weeks of age were fed with either 250 mg OVA (Sigma-Aldrich Co, St Louis, Mo) dissolved in 0.5 mL PBS or PBS alone (control) every other day for as many as 7 doses by intragastric intubation. Two days after the last feeding, spleens, Peyer's patches, and mesenteric lymph nodes, which were aseptically removed, were gently crushed and filtered through a 40- μ m nylon mesh (Falcon, Bedford, Mass). Spleen cells were incubated for 5 minutes in TRIS-buffered NH₄Cl to remove red blood cells. Cells from the Peyer's patches were previously prepared after organ digestion in RPMI 1640 (Nissui Co, Tokyo, Japan) supplemented with collagenase (Roche, Indianapolis, Ind) and DNase I (Sigma-Aldrich Co) for 60 minutes at 37°C. The mononuclear cell suspensions were prepared in cell culture medium consisting of RPMI 1640 supplemented with 50 mmol/L HEPES, 100 U/mL penicillin, 100 μ g/mL streptomycin, 2×10^{-5} mol/L 2-mercaptoethanol, and 10% heat-inactivated FCS (Sigma-Aldrich Co).

Antigen-specific cell proliferation and cytokine production

The mononuclear cells (1×10^5 /well) were cultured for 92 hours in a 96-well round bottom plate (Falcon) with various concentrations of OVA. In some experiments, CD4⁺ T cells were purified from splenocytes by using the MACS CD4⁺ T-cell Isolation Kit (Milteny Biotec GmbH, Bergisch Gladbach, Germany). The purity of CD4⁺ T cells in the separated cells was >95%. The CD4⁺ T cells were stimulated with various concentrations of OVA and irradiated (30 Gy) splenocytes (2×10^5 /well) from wild-type female Balb/c mice. Cell proliferation was assessed by adding 1 μ Ci/well of [methyl-³H] thymidine (10 Ci/mmol; Amersham Bioscience Co, Piscataway, NJ) during the last 16 hours of culture. Incorporation of [methyl-³H] thymidine was measured by liquid scintillation counting. All

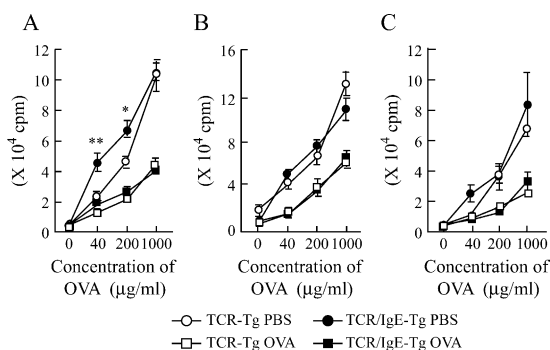


FIG 1. Effects of oral OVA administration on OVA-specific cell proliferation. Mononuclear cells purified from the spleen (A), Peyer's patches (B), and mesenteric lymph nodes (C) of OVA-fed or PBS-fed OVA-TCR/IgE-Tg mice and OVA-TCR-Tg littermates were restimulated with the indicated concentrations of OVA. Shown are the means $\pm$ SDs of 12 independent experiments. * $P < .05$; ** $P < .01$.

determinants were performed in triplicate and measured as the mean counts per minute $\pm$ SD. Before the thymidine pulse, supernatants were collected, and cytokine content was determined by using ELISA. In some experiments, the cells were cultured in the presence of 10 IU/mL recombinant IL-2 (PeproTech Inc, Rocky Hill, NJ), 10 μ g/mL anti-IL-10 mAb (BD Biosciences, San Jose, Calif), 10 μ g/mL anti-TGF- β 1 mAb (R&D Systems, Inc, Minneapolis, Minn), or a combination of mAbs or control antibodies (BD Biosciences).

Cytokine measurements

IL-4, IL-10, and IFN- γ were measured by a 2-site sandwich ELISA. Antibody pairs and standard recombinant mouse cytokines for the ELISA were purchased from BD Biosciences and PeproTech Inc, respectively. For measurements of TGF- β 1, cells were cultured in serum-free medium consisting of RPMI 1640 supplemented with Nutridoma SP (1% vol/vol; Roche) for 72 hours. TGF- β 1 in the supernatants was measured by the TGF- β 1 Emax ImmunoAssay System (Promega, Madison, Wis).

mAbs and flow cytometry

Antimurine CD3, CD4, B220, CD11c, and CD25 mAbs were purchased from BD Biosciences. KJ1-26, recognizing the TCR specific to OVA, was purchased from Caltag (Burlingame, Calif). Cells were stained by using standard procedures in conjunction with 1 or more of the FITC-conjugated or PE-conjugated mAbs and then analyzed on a FACSCalibur (BD Biosciences).

Statistical analysis

Differences between the 2 groups were analyzed by the Student *t* test or the nonparametric Wilcoxon matched pairs test. A *P* value <.05 denoted the presence of a statistically significant difference.

RESULTS

Feeding OVA-TCR/IgE-Tg mice with OVA resulted in tolerance to OVA

Because it has been shown that high doses of oral antigen administration are required to achieve tolerance in TCR-Tg mice compared with non-Tg mice,¹² mice were fed 250 mg OVA. Ten percent of OVA-TCR/IgE-Tg

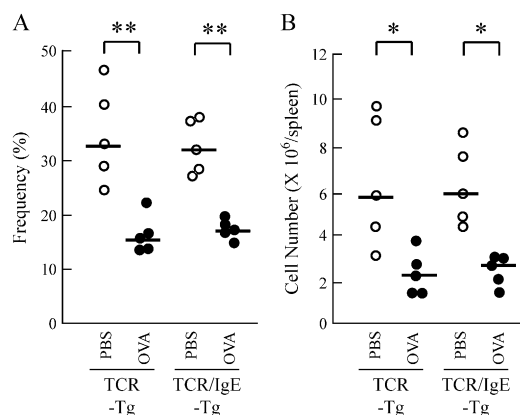


FIG 2. Effects of oral antigen administration on the number of OVA-specific TCR-bearing T cells in the spleen. The percentage (**A**) and the absolute number (**B**) of KJ1-26⁺ T cells in the spleen of OVA-fed or PBS-fed OVA-TCR/IgE-Tg mice and OVA-TCR-Tg littermates were determined. The bars indicate the median of 5 pooled independent experiments. **P* < .05; ***P* < .01.

mice and OVA-TCR-Tg mice exhibited diarrhea and lost weight after the second intragastric administration of OVA. Thereafter, repetitive OVA feeding allowed the mice to recover from diarrhea and to gain their weight again, indicating that both mouse strains developed clinical tolerance (data not shown).

To reveal whether oral antigen administration induced tolerance in T-cell levels, cells from the various lymphoid tissues were restimulated with OVA *in vitro*. As shown in Fig 1, when stimulated with suboptimal concentrations of OVA, splenocytes from PBS-fed OVA-TCR/IgE-Tg mice exhibited significantly higher proliferation than those of PBS-fed OVA-TCR-Tg mice, suggesting that the presence of antigen-specific IgE *in vivo* affects the antigen-specific responsiveness of spleen cells even though they had not encountered the antigen previously. After OVA administration, proliferative responses of the cells purified from the spleen, mesenteric lymph nodes, and Peyer's patches of either type of Tg mice were significantly decreased compared with those of the corresponding PBS-fed mice.

Oral OVA administration leads to clonal deletion of OVA-specific T cells

Next, we determined the numbers of OVA-TCR-bearing T cells in splenocytes by flow cytometry. The percentages and absolute numbers of splenic clonotype-specific KJ1-26⁺ cells of both OVA-TCR/IgE-Tg and OVA-TCR-Tg mice fed with OVA were lower than those of the corresponding PBS-fed mice (Fig 2, *A and B*). There was no significant difference in the percentages and absolute numbers of splenic KJ1-26⁺ T cells between OVA-TCR/IgE-Tg and OVA-TCR-Tg mice, suggesting that irrespective of the presence of OVA-specific IgE *in vivo*, OVA-TCR-Tg mice developed oral tolerance, which seems to be partially dependent on the clonal deletion of OVA-specific T cells.

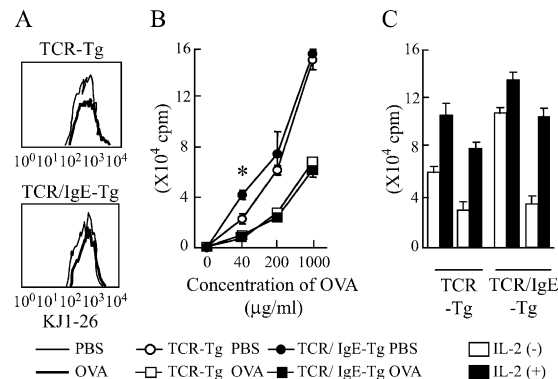


FIG 3. Oral OVA administration induces T-cell anergy. TCR expression levels of splenic T cells were determined by gating KJ1-26⁺ T cells (**A**). Splenic CD4⁺ T cells were restimulated with OVA and irradiated splenocytes of nontreated wild-type mice (**B**). Splenocytes were restimulated with OVA in the presence of recombinant IL-2 (**C**). Shown are the means ± SDs of 7 experiments.

T-cell anergy was involved in the hyporesponsiveness of orally tolerated splenocytes

Because there was no change in the fluorescence intensity of clonotype-specific TCR, KJ1-26 expressed on the T cells between OVA-TCR/IgE-Tg or OVA-TCR-Tg mice after OVA feeding (Fig 3, *A*), the hyporesponsiveness of the orally tolerated splenocytes was not explained by the downregulation of TCR in our experimental system as reported by Benson et al.¹³ Next, CD4⁺ T cells were purified from orally tolerated splenocytes and then stimulated with OVA and irradiated splenocytes purified from nontreated Balb/c mice. As shown in Fig 3, *B*, the proliferation of OVA-specific T cells of both OVA-TCR/IgE-Tg and OVA-TCR-Tg mice decreased after OVA feeding without any significant difference between these OVA-fed mice. The decreased proliferative response of the splenocytes from OVA-fed OVA TCR/IgE-Tg mice and OVA-TCR-Tg mice was rescued significantly by exogenous IL-2 (Fig 3, *C*). These results collectively suggest that clonal anergy is also involved in the hyporesponsiveness of orally tolerated splenocytes irrespective of antigen-specific IgE *in vivo*.

Cytokine producing profiles of splenocytes from OVA TCR/IgE-Tg or OVA-TCR-Tg mice

We measured cytokine production of splenocytes restimulated with 1000 µg/mL OVA *in vitro*. Of note, there was a difference in the cytokine profiles between PBS-fed OVA-TCR/IgE-Tg mice and OVA-TCR-Tg mice; the splenocytes from OVA-TCR/IgE-Tg mice produced higher levels of IL-4 and IL-10 and lower levels of IFN-γ than those from OVA-TCR-Tg mice (Fig 4). After feeding of OVA, both IL-4 and IFN-γ production levels were decreased in splenocytes from either OVA-TCR/IgE-Tg mice or OVA-TCR-Tg mice. IL-10 production by splenocytes from OVA-TCR/IgE-Tg mice, but not from

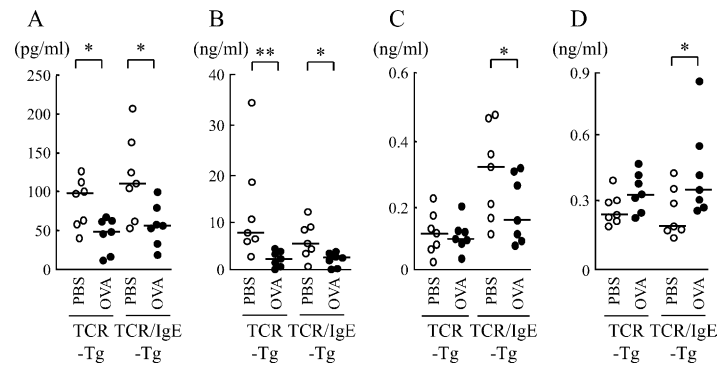


FIG 4. Oral OVA administration modulated cytokine-producing profiles of splenocytes. The splenocytes of OVA-fed or PBS-fed OVA-TCR/IgE-Tg mice and OVA-TCR-Tg littermates were restimulated with 1000 μ g/mL OVA. Supernatants were tested by ELISA to determine the production of IL-4 (A), IFN- γ (B), IL-10 (C), and TGF- β 1 (D). The bars indicate the median of 7 pooled independent experiments. * P < .05; ** P < .01.

OVA-TCR-Tg mice, was consistently suppressed. In contrast, TGF- β 1 production in OVA-TCR/IgE-Tg mice was clearly increased (Fig 4, D).

Role of TGF- β 1 in the hyporesponsiveness of orally tolerated splenocytes of OVA-TCR/IgE-Tg mice

The reduced proliferation of splenocytes from either OVA-TCR/IgE-Tg mice or OVA-TCR-Tg mice was partially reverted by the addition of anti-TGF- β 1 neutralizing antibody but not by anti-IL-10 antibody (Fig 5). The combination of anti-TGF- β 1 antibody and anti-IL-10 antibody did not show any further increase of cell proliferation in OVA-fed mice. On the other hand, cell proliferation of splenocytes from PBS-fed OVA-TCR/IgE-Tg mice increased by the addition of anti-IL-10 antibody and was synergistically enhanced by a combination with anti-TGF- β 1 antibody. These data indicated that endogenous IL-10 inhibited splenocyte proliferation of control PBS-fed OVA-TCR/IgE-Tg mice, but endogenous TGF- β 1 rather than IL-10 may be involved in the hyporesponsiveness of OVA-fed mice (either OVA-TCR/IgE-Tg mice or OVA-TCR-Tg mice).

CD25⁺CD4⁺ T cells in the splenocytes of OVA TCR/IgE-Tg mice

Because regulatory CD25⁺CD4⁺ T cells have been shown to play an important role in controlling oral tolerance of TCR-Tg mice, we measured the percentage of CD25⁺CD4⁺ T cells in splenocytes.^{14,15} As shown in Fig 6, OVA feeding increased the percentage of splenic CD25⁺CD4⁺ T cells from OVA-TCR/IgE-Tg mice as well as that from OVA-TCR-Tg mice.

DISCUSSION

In this study, we demonstrated that oral tolerance could be induced after repetitive oral administration of high doses of OVA, even in the presence of high levels of OVA-specific IgE, and that clonal deletion/anergy and

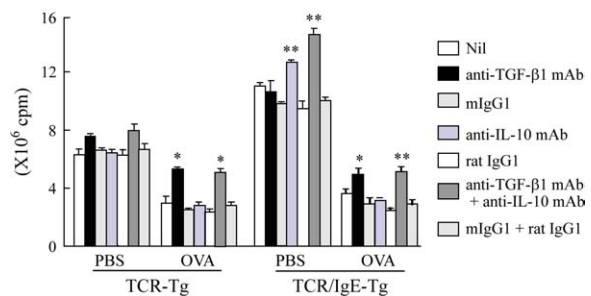


FIG 5. Roles of TGF- β 1 and IL-10 in the hyporesponsiveness of orally tolerated splenocytes. Splenocytes of the fed mice were restimulated with 200 μ g/mL OVA in the presence or absence of antibodies as shown. The data indicate the means $\pm$ SDs of triplicate cultures. Similar results were observed in 3 independent experiments. * P < .05; ** P < .01.

active suppression by TGF- β 1 may play important roles in the development of tolerance. The dose of fed antigen has long been suggested to influence the mechanism of oral tolerance induction, with repeated and/or low doses inducing active suppression, whereas high doses result in clonal deletion and/or anergy.⁷ Our study shows that these mechanisms are not mutually exclusive and may occur simultaneously.

Accumulating evidence supports the notion that mucosal dendritic cells play a central role in the induction of tolerance.¹⁶ In human beings, the presence of high levels of IgE has been shown to enhance Fc ϵ RI expression on dendritic cells as well as mast cells.^{17,18} To date, whether mice dendritic cells express Fc ϵ RI is still unknown. Because the mast cells in OVA-IgE-Tg mice expressed high levels of Fc ϵ RI, it is presumable that high levels of OVA-specific IgE upregulated Fc ϵ RI on dendritic cells, too, facilitating antigen uptake and antigen presentation.⁵ Cross-linking of Fc ϵ RI on monocytes alters differentiation toward a macrophage species away from a dendritic cell phenotype.¹⁹ Although the role of B-cell presentation of antigen in oral tolerance induction remains controversial,^{20,21} the upregulated expression of IgE-loaded CD23

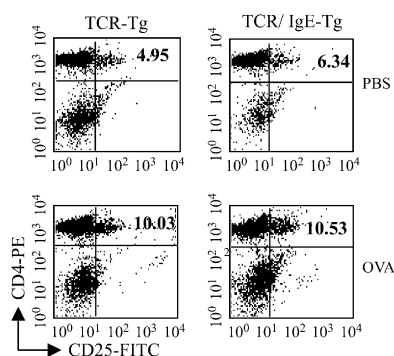


FIG 6. Effects of oral OVA administration on the frequency of CD25⁺CD4⁺ T cells in the spleen. OVA-TCR/IgE-Tg mice and OVA-TCR-Tg littermates were fed OVA or control PBS. The percentage of CD25⁺CD4⁺ T cells in the splenocytes was determined by flow cytometry. Values represent the percentages of CD25⁺CD4⁺ cells. This experiment represents 3 independent experiments with similar results.

on B cells in OVA-IgE-Tg mice might enhance antigen uptake. Moreover, CD23 has been identified on the apical membrane of enterocytes with increased expression in individuals with food allergies.²² IgE antibodies bound to CD23 on epithelial cells contribute to the specific and rapid intestinal transepithelial antigen transport.²² Given that the antigen dose is a crucial factor to determine the mechanisms of oral tolerance, high levels of IgE seem to modulate the underlying mechanisms of oral tolerance indirectly via modulation of antigen uptake and antigen-presenting cell function. It is interesting to examine whether OVA-TCR/IgE-Tg mice fed low doses of OVA develop a different type of oral tolerance from that in OVA-TCR-Tg mice.

Oral antigen administration has been shown to induce functional anergy of antigen-specific T cells. Several forms of anergy exist, including clonal anergy and division arrest.²³ Asai et al²⁴ reported that long-term feeding with high-dose antigen induced a unique type of T-cell hyporesponsiveness that was not rescued by IL-2 addition. In the current study, however, the hyporesponsiveness was reversed by exogenous IL-2, suggesting that clonal anergy could be induced by a distinct feeding procedure.

Zhang et al¹⁴ demonstrated that oral administration of OVA to OVA-TCR-Tg mice activated CD25⁺CD4⁺ T cells in an antigen-specific fashion and that CD25⁺CD4⁺ T cells from OVA-fed mice exhibited more potent regulatory activity, secreting more TGF- β 1 than that from unfed mice. In this study, OVA feeding increased the percentage of CD25⁺CD4⁺ T cells, and endogenous TGF- β 1 seems to be involved in the hyporesponsiveness of splenocytes from OVA-TCR/IgE-Tg mice. It has been reported that CD25 was transiently expressed on antigen-exposed CD4 T cells, whereas late expression of CD25 was seen only after tolerogenic antigen exposure.¹⁵ This circumstantial evidence suggested that CD25⁺CD4⁺ T cells detected in OVA-TCR/IgE-Tg mice may exert suppressive function as regulatory T cells.

In conclusion, although high levels of antigen-specific IgE are presumed to modulate the development of oral tolerance, dietary antigens that cause food allergies could induce oral tolerance even in the presence of antigen-specific IgE. The process of outgrowing food allergies varies a great deal for different foods and among individual patients. Some children may lose their food allergy in a matter of months.²⁵ It is conceivable that tolerance induction differs in the maturing immune system. Further study using childhood OVA-TCR/IgE-Tg mice instead of adult ones could have higher clinical relevance.

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