

CD4⁺CD25⁺ regulatory T cells are not functionally impaired in adult patients with IgE-mediated cow's milk allergy

To the Editor:

Peripheral tolerance is a mechanism by which the immune system prevents reactions against self and other harmless antigens (Ags), such as food allergens. A reduction in peripheral tolerance might lead to unwanted immune responses toward food allergens, the result being type I hypersensitivity reactions. Type I hypersensitivity is the most acute form of allergy; it is mediated by antigen-specific IgE and leads to clinical symptoms such as allergic rhinitis, asthma, and systemic anaphylaxis. Antigen-specific IgE production is induced by antigen-specific T_H2 cells that produce IL-4 and play a key role in allergy.¹ We have shown previously that T-cell clones from cow's milk-allergic infants displayed a T_H2 phenotype upon cow's milk antigen-specific stimulation.² Therefore, we hypothesized that in cow's milk-allergic patients the T_H2 response might be insufficiently controlled because of a reduction in peripheral tolerance. CD4⁺CD25⁺ regulatory T cells (Tregs) play an important role in maintaining peripheral tolerance.^{3,4} These cells have recently been described in human beings; they were shown to have a memory (CD45RO⁺, CD45RB^{low}) phenotype with increased expression of CTLA-4 (CD152).⁵ These CD4⁺CD25⁺ Tregs can be distinguished from activated CD4⁺CD25⁺ T cells because of their CD69⁻ and MHCII^{low} phenotype. CD4⁺CD25⁺ Tregs isolated from healthy donors suppressed ex vivo T-cell responses to anti-CD3 mAb, allo, and self Ags and, importantly, to cow's milk antigen (CMA) as well.⁶ To investigate whether cow's milk allergy is associated with abnormalities in CD4⁺CD25⁺ Tregs, we compared healthy and cow's milk-allergic donors with regard to percentage, phenotype, and function of the CD4⁺CD25⁺ T-cell subset.

Three adult cow's milk-allergic donors, each with a clinical history of systemic anaphylaxis upon ingestion of cow's milk, were selected. Their CMA-specific IgE levels were 9, 31.7, and 10.2 kU/L, as determined through use of the CAP system FEIA (Pharmacia Diagnostics, Uppsala, Sweden). All 3 allergic donors were monosensitized to casein; β -lactoglobulin and α -lactalbumin-specific IgE was not detectable (<0.35 kU/L). Five healthy adult donors were included as controls.

CD4⁺, CD4⁺CD25⁻, and CD4⁺CD25⁺ T cells were isolated from PBMCs and tested in proliferation assays through stimulation with anti-CD3 mAb (50,000 $\times$ dilution; CLB, Amsterdam, The Netherlands) for 3 days, CMA (50 μ g/mL)—consisting of casein, β -lactoglobulin, and α -lactalbumin (NIZO, Ede, the Netherlands)—or grass pollen (GP; 50 μ g/mL; HAL, Haarlem, The Netherlands) for 5 days.^{5,6} The suppressive capacity of CD4⁺CD25⁺ Tregs was investigated by coculture (ratio 1:1) with CD4⁺CD25⁻ T cells.^{5,6} Proliferative responses are expressed as the mean [³H]-TdR incorporation (in counts per minute) of triplicate wells $\pm$ SEM. Flow cytometry was performed with CD4, CD25, CD45RB, CD69, HLA-DR, CD45RO, CD45RA, and CD152 antibodies.^{5,6}

No differences were observed in the percentage of CD4⁺CD25⁺ Tregs present in peripheral blood between healthy donors and allergic donors (mean, 10.6% $\pm$ 2% [n = 5] versus 10% $\pm$ 3% [n = 3]).^{5,6} The phenotype of CD4⁺CD25⁺ Tregs from allergic donors was found to be CD69⁻, HLA DR^{low}, CD45RO⁺, and CD45RB^{low}; there was increased expression of CD152. This was similar to the phenotype of Tregs derived from healthy donors (data not shown).^{5,6}

Proliferative responses to anti-CD3 mAb and CMA were observed in the total CD4⁺ T-cell populations for both allergic patients and healthy controls (Fig 1). Upon depletion of the CD4⁺CD25⁺ Tregs, the proliferation of the remaining CD4⁺CD25⁻ T-cell population was increased in response to anti-CD3 mAb and CMA, providing indirect evidence that CD4⁺CD25⁺ Tregs suppressed the proliferative response. Proliferation of the CD4⁺CD25⁺ T cells alone in response to both stimuli was low, indicating that these cells, as in healthy controls, were anergic.

To investigate the direct suppressive capacity of CD4⁺CD25⁺ Tregs from healthy and allergic donors, we cocultured CD4⁺CD25⁻ T cells with CD4⁺CD25⁺ T cells and stimulated them with anti-CD3 mAb and CMA. Of note, when CD4⁺CD25⁺ Tregs were added to the coculture in both healthy donors and allergic donors suppression was observed in response to anti-CD3 and CMA (Fig 2, A and B). The mean percentage inhibition values ($\pm$ SEM) of the T-cell response to anti-CD3 and CMA in the healthy controls were 74% $\pm$ 8% and 53% $\pm$ 22%, respectively (n = 4); in allergic patients, the values were 71% $\pm$ 4% and 79% $\pm$ 12% (n = 3), respectively.

Purified CMA consists of 3 different proteins: casein, α -lactalbumin and β -lactoglobulin. The patients in this study were

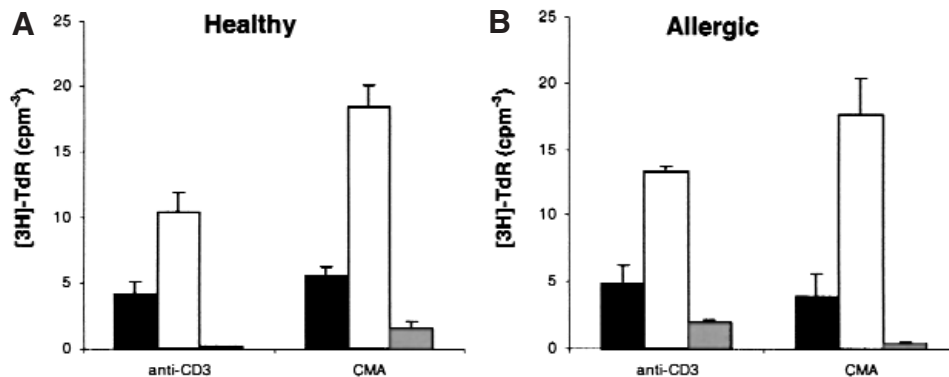


FIG 1. CD4⁺CD25⁺ T cells are anergic and suppressive on Ag-specific stimulation. CD4⁺ (black bars), CD4⁺CD25⁻ (open bars), and CD4⁺CD25⁺ T cells (gray bars) were isolated from PBMCs from healthy donors and cow's milk-allergic donors. The T cells were stimulated with irradiated autologous PBMCs as APCs and the indicated antigens. The graph is a representative example of 3 independent experiments.

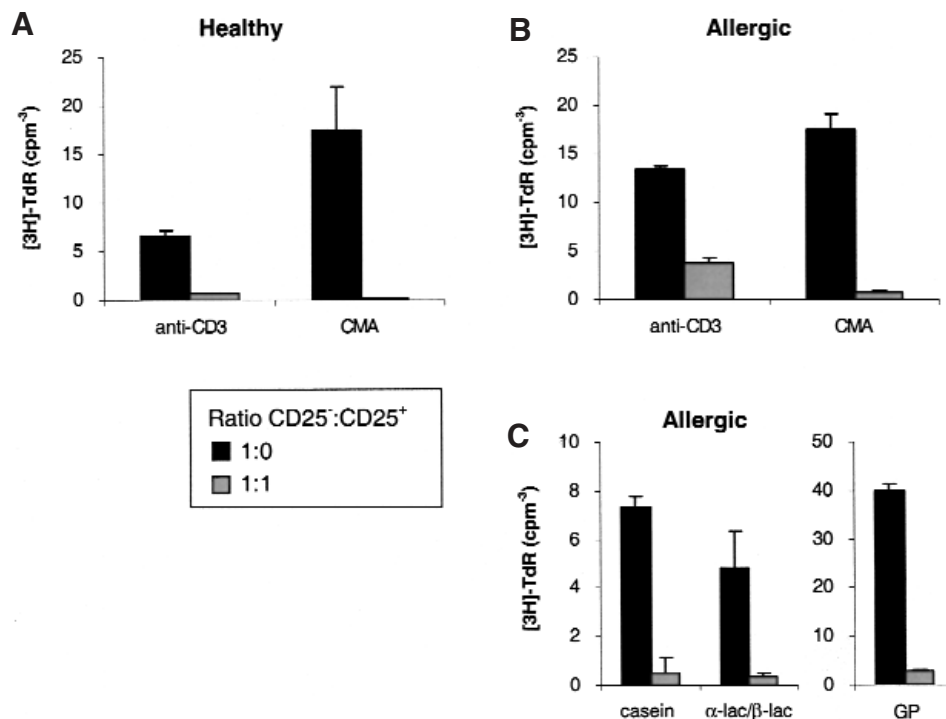


FIG 2. CD4⁺CD25⁺ Tregs derived from allergic donors are not functionally impaired. CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells were isolated from PBMCs from healthy (A) and cow's milk-allergic donors (B and C). CD4⁺CD25⁻ T cells were stimulated with irradiated autologous PBMCs as APCs and the indicated antigens. CD4⁺CD25⁻ T cells were cultured either alone (1:0 ratio; black bars) or in the presence of CD4⁺CD25⁺ T cells (1:1 ratio; gray bars). The graph is a representative example of 3 independent experiments.

monosensitized to casein, and thus it was possible that the observed suppressive response toward CMA was directed toward α -lactalbumin and β -lactoglobulin instead of casein. We therefore compared suppression toward purified casein and a mix of α -lactalbumin and β -lactoglobulin. We observed no difference in suppression by CD4⁺CD25⁺ Tregs to these Ags (Fig 2, C). As an additional control, we studied the response to GP outside the GP season and found that CD4⁺CD25⁺ Tregs from allergic patients suppressed the T-cell response to GP (Fig 2, C), for which these patients also had specific IgE. The findings indicate that in these patients the suppressive response to food allergens and inhalant allergens was intact. Together, these data demonstrate that CD4⁺CD25⁺ Tregs from cow's milk-allergic patients are not impaired with regard to percentage, phenotype, and, most importantly, suppressive function.

In cow's milk-allergic patients, accidental ingestion of cow's milk leads to (a) immediate clinical reactions provoked by allergen-specific IgE and (b) activation of allergen-specific T cells. Therefore, elimination of the allergen from the diet is required. We here demonstrate, in an in vitro system, that the CD4⁺CD25⁺ Tregs appear to function normally during the allergen-elimination phase of an IgE-mediated allergic disease. It is still possible, however, that their percentage or function was impaired during the onset of the allergy, thus allowing the activation of T_H2 cells and the induction of allergen-specific IgE. Indeed, studies in mice have demonstrated that CD4⁺CD25⁺ Tregs can suppress the activation and expansion of inflammatory T cells through a mechanism involving IL-10, thereby preventing inflammatory bowel disease.⁷ A functional impairment of Tregs at the time of allergen exposure might thus lead to a disturbed

balance between immunity and tolerance. Even though we demonstrated that the function of CD4⁺CD25⁺ Tregs is normal in allergic patients, this suppressive effect is clearly not sufficient to prevent anaphylactic reactions after ingestion of cow's milk.

Recent studies in mice have shown that oral antigen administration led to an increase in the percentage and suppressive function of CD4⁺CD25⁺ Tregs.^{8,9} Interestingly, in human beings it was found that bee venom-specific immunotherapy led to an increase in the number of IL-10-producing CD4⁺CD25⁺ T cells in the treated patients that was comparable to what is seen in nonallergic bee venom-hyperimmune beekeepers.¹⁰ These findings suggest that administration of the allergen in a tolerizing manner results in an enhancement of the percentage and suppressive capacity of CD4⁺CD25⁺ Tregs, partly through the production of IL-10.

In conclusion, the CD4⁺CD25⁺ Tregs in allergic patients appear to function normally with regard to suppression of allergen-specific T-cell proliferation in vitro. This indicates that specific stimulation of these T cells through oral tolerance induction and/or immunotherapy in vivo could reestablish clinical tolerance to food allergens.

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Anaphylaxis after ingestion of gummy bears

To the Editor:

Despite the vast number of agents that are added to foods, there are relatively few documented hypersensitivity reactions to food additives. The primary food additives that have been implicated in classic IgE-mediated anaphylactic reactions include sulfites and parabens.¹ Other additives that are known to cause adverse reactions include dyes, nitrates, nitrites, antioxidants, and flavorings; however, the mechanisms underlying these reactions remain unclear.¹ Gelatin, a stabilizer in food products and vaccines, such as measles, mumps, and rubella (MMR) vaccine, has been implicated in anaphylaxis.^{2,3} We report a case of anaphylaxis immediately after ingestion of gummy bears that led to an evaluation, including a negative food challenge result to gelatin, thus implicating "natural and artificial flavors" as the offending antigen.

A 3½-year-old white girl was referred for evaluation after 2 episodes of anaphylaxis after ingestion of gummy products. The first episode occurred within 5 minutes of consuming 2 packages of Farley's Fruit Snacks with development of generalized urticaria and erythema, which resolved after receiving oral antihistamines. Approximately 6 months later, a second episode occurred after ingesting gummy products from a local restaurant. Ten minutes after ingestion, she had a generalized rash, angioedema, and pruritus with subsequent lethargy, wheezing, and hypotension. She received 2 doses of subcutaneous epinephrine, nebulized albuterol, and intravenous diphenhydramine and ranitidine. In the interval between the 2 incidents, parents report that she consumed gummy products, with development of facial erythema only. This patient had an unrestricted diet and no prior history of reactions to foods, dyes, or medications.

Approximately 8 weeks after the second episode of anaphylaxis, skin prick test responses with a red gummy bear soaked in water were negative for both the gel and solution, whereas positive reactions were seen to MMR vaccine, pork extract, porcine gelatin, and Knox gelatin. Subsequent CAP-RAST test results to bovine and porcine gelatin and to common foods (milk, egg, soy, wheat, codfish, shrimp, and peanut) were negative. An open food challenge to Farley's Fruit Snacks resulted in no symptoms, whereas open challenge to Haribo Gold Bear Candy resulted in urticaria after ingestion of 16 pieces. Open challenge to Nabisco Gummi Savers and Trolli Brite-crawlers also produced pruritus and urticaria. Double-blind, placebo-controlled food challenge results to porcine (Nabisco) and Knox gelatin were negative. Because gelatin is an ingredient found in most gummy products, vaccinations were postponed. However, after a negative double-blind, placebo-controlled food challenge and CAP-RAST result to gelatin, MMR and varicella vaccines were given by means of incremental dosing without adverse reaction. The common component among the products to which this child had a positive reaction was "natural and artificial" flavors. Attempts to contact company representatives to obtain natural flavoring for objective testing were unsuccessful. Presently, the patient continues to avoid all gummy products and carries injectable epinephrine.

To our knowledge, this is the first reported pediatric case of food additive anaphylaxis after ingestion of gummy candies. In 1989, Wahl and Kleinhaus⁴ reported a case of urticaria and oral allergy in a 32-year-old woman after ingestion of fruit gum candies. RAST testing revealed IgE to gelatin, and RAST inhibition showed cross-reactivity to gelatin and gelatin-containing products.⁴ Although adverse reactions to gelatin have been described in association with vaccinations and some food products, objective testing did not reveal gelatin as the cause of anaphylaxis in our index case. Rather, the common ingredient "natural and artificial flavors" was suspected as the culprit in our patient. Currently, vendors are reluctant to reveal the variable components of these flavorings. In light of the potentially life-threatening complications that can ensue from consumption of these products, cooperation with industry and improved labeling of all ingredients is essential. Collaboration between allergy organizations and the Food and Drug Administration could serve to accomplish these efforts.

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