



Letter

Cytokine responses to egg protein in previously allergic children who developed tolerance naturally

Egg allergy is the second most common food allergy in childhood, with 1% to 2% of Americans affected.^{1,2} Although approximately 70% of children outgrow this allergy,³ few studies have explored the mechanism by which children naturally outgrow the allergy (natural tolerance [NT]). Most studies have compared children with allergy with those who were never allergic, have not evaluated allergen-specific responses, or have only focused on treatment inducing desensitization (oral immunotherapy). Although not previously demonstrated in food allergy, tolerance to venom has been associated with elevation of interleukin (IL)-10.⁴ Our hypothesis was that peripheral blood mononuclear cells (PBMCs) stimulated with ovalbumin (OVA) in NT has increased allergen-specific IL-10 production. The objective was to determine the PBMC cytokine response to egg protein in previously allergic patients who developed NT and thus differentiate them from children with allergy.

Food allergy was defined by an allergist-immunologist as a history of IgE-mediated reaction to egg (anaphylaxis, urticaria, or significant vomiting) within 2 hours of food ingestion and positive laboratory testing (skin prick mean wheal diameter ≥ 4 mm larger than saline wheal and/or specific IgE [sIgE] level ≥ 2 kU/L). Non-allergic (control) was defined as no history of clinical reactivity to egg. NT was defined as a clinical and laboratory history of egg allergy but passing an open food challenge to 1 scrambled egg within 6 months of recruitment. The PBMCs were stimulated with or without OVA or with anti-CD3 and anti-CD28. After 48 hours, the supernatant was collected and cytokines were analyzed using multiplex assays (eMethods).

Patient characteristics, fold stimulation, and sIgE/sIgG4 ratio were compared using the Mann-Whitney or Kruskal-Wallis test. Cytokine data were logarithmically transformed, dose-dependent responses were analyzed with repeated-measures analysis of variance (ANOVA), and comparisons at specific doses were performed with ANOVA or *t* test. Data analysis was performed using SAS 9.3 (SAS Institute, Cary, North Carolina) and SPSS 14 (SPSS, Inc, Chicago, Illinois), with a 2-sided type I error rate of 5%.

Forty children (11 with NT, 20 with allergy, and 9 without allergy; median age 6 years, range 2–18 years) were recruited from a cross-sectional, case–control convenience sample (patient

characteristics are listed in eTable 1, available online). Patients with NT and those with allergy were more likely to have been diagnosed with other atopy. At diagnosis, there was no difference in skin prick size or original reactions between patients with NT and those with allergy. At recruitment, there was no difference in sIgG4 among groups (median 0.45 vs 1.2 vs 1.0 kU/L, respectively, $P = .38$).

With stimulation, IL-10 was more upregulated in patients with NT and control patients using a repeated-measures approach ($P < .01$) and was the only cytokine that differentiated patients with NT from those with allergy with fold stimulation at doses of at least 10 $\mu\text{g/mL}$ (Fig 1). At 100 $\mu\text{g/mL}$, the median increase was 68-fold compared with unstimulated samples.

The PBMCs from patients with NT produced significantly fewer T-helper cell type 2 cytokines (IL-4 and IL-5) than patients with allergy at baseline (Fig 1) and at every dose of OVA. Patients with NT produced similar amounts of IL-4 and slightly more IL-5 than controls. There was no dose response to OVA ($P = .41$ and $.42$ by repeated-measures ANOVA for IL-4 and IL-5, respectively). Patients with NT produced the most interferon- γ (IFN- γ) using repeated-measures ANOVA ($P = .02$), which was notable at doses of 1, 10, and 50 $\mu\text{g/mL}$, but was significant only in differentiating patients with NT from those with allergy with fold stimulation at 10 $\mu\text{g/mL}$ (Fig 1). Other cytokines that were not significant in differentiating NT from allergy also were IL-9, IL-13, IL-6, and IL-17 (eFig1).

Median IgE/IgG4 ratio to egg white was elevated in patients with allergy vs those with NT vs those without allergy (eFig 2). The 4 patients with allergy who tolerated baked egg had decreased sIgE/sIgG4 ratios compared with other patients with allergy but had increased levels compared with patients with NT (median 5.1 vs 35.7 vs 0.4, respectively, $P = .02$ and $.03$). The sIgE level was similar to the sIgE/sIgG4 ratio in predicting tolerance in a receiver operating characteristic curve (area under the curve 0.97 vs 0.96).

This study is the first to address the mechanisms of food challenge-proven NT using dose-dependent cytokine responses. When comparing patients with NT with those with allergy, IFN- γ and most significantly IL-10 were the only upregulated cytokines in those with NT. Importantly, upregulation of IL-10 was antigen-specific, because the increase was seen only with OVA and did not occur with anti-CD3 and anti-CD28 stimulation. This is in contrast to research on oral immunotherapy, which is not a model of NT, that has shown no increase or transient increases in IL-10. Lack of dose-dependent T-helper cell type 2 cytokine responses to certain cytokines (IL-4 and IL-5) in patients with allergy could be secondary to high background production, obscuring the contribution of antigen-specific responses to egg (in contrast to IL-10 and IFN- γ).⁵ Findings with IFN- γ have suggested that T-helper cell type 1 cytokines are involved in allergen-specific responses to food but are not clearly characteristic of NT.

This work was presented at the Pediatric Academic Societies meeting in April 2011, the American College of Allergy & Immunology meeting in October 2011, and the American Academy of Allergy, Asthma & Immunology meeting in March 2012. **Disclosure:** Authors have nothing to disclose.

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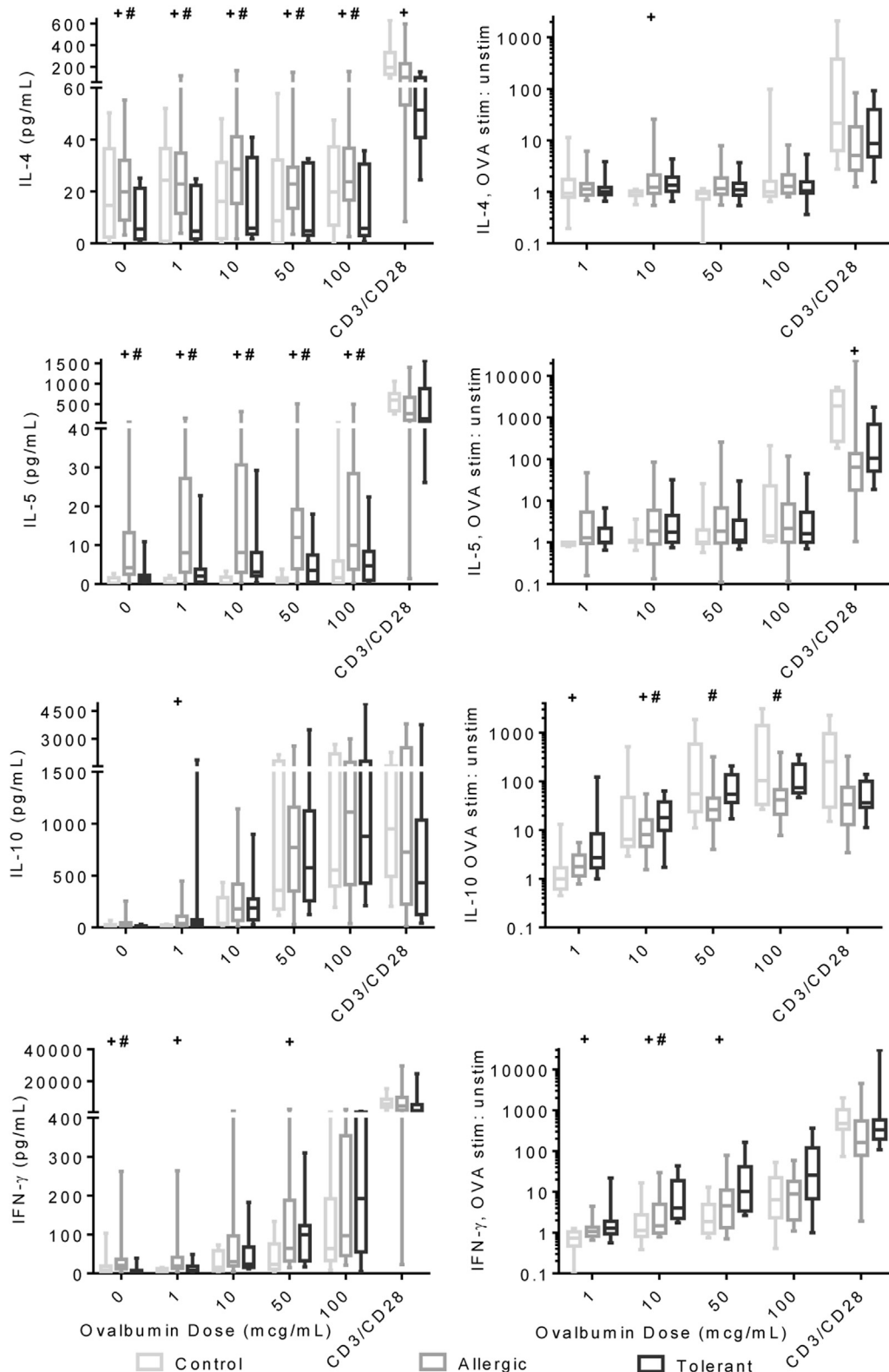


Figure 1. Cytokine responses to ovalbumin (OVA) in patients without allergy (control), with allergy, and with natural tolerance. Boxplots depict cytokine responses to OVA and anti-CD3 and anti-CD28 (0.2 mg/mL) after 48-hour culture of peripheral blood mononuclear cells for interleukin (IL)-4, IL-5, IL-10, and interferon- γ (IFN- γ) (left) and upregulation after stimulation compared with unstimulated samples (right). Boxplots display median values (horizontal lines within bars), lower and upper quartiles (areas within bars below and above lines, respectively), and minimum and maximum values (whiskers). Within-dose comparisons: $^+P < .05$ among all 3 groups, $^{\#}P < .05$ between allergic and tolerant groups.

It is unlikely IL-10 is the only immunomodulatory cytokine involved in NT. IL-6 showed OVA responsiveness but did not differ between patients with NT and those with allergy but was important

in the response of those without allergy. This suggests a role of IL-6 in food allergen nonresponsiveness but not necessarily in the acquisition of NT. These data and those from other mechanistic

studies^{6,7} support that PBMC-derived IL-10 is a significant contributor to allergic suppression and an important biomarker to explore.⁸ In addition, IL-10 was elevated in control patients, supporting the finding that IL-10 differentiates between allergy and tolerance.

Although a recent study with egg oral immunotherapy reported an increase in IgG4 level to egg in patients with successful desensitization, this relation did not hold 2 months after cessation of oral immunotherapy.⁹ We did not find a difference in sIgG4, and this may imply that sIgG4 is important early in desensitization or unresponsiveness but not after sustained unresponsiveness.

However, our patients with NT were selected for challenge based on an sIgE level lower than 2 kU/L, which might explain why we found that sIgE is a better biomarker than sIgG4. In this study, sIgE explains the relevance of the sIgE/sIgG4 ratio, suggesting this test is no better than sIgE alone in predicting tolerance. Although this was a cross-sectional study and sIgE was lower at the time of diagnosis of NT, this does not diminish the main finding that children with NT have unique immunologic mechanisms allowing them to outgrow their allergy.

Future work on the source and role of IL-10 in NT will help identify mechanisms by which children outgrow their disease naturally and should have significant implications for the evaluation of treatments of food allergy and in the development of intervention strategies for this life-threatening disease.

Supplementary Data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.anai.2014.09.002>.

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References

- [1] Sicherer SH, Sampson HA. Food allergy. *J Allergy Clin Immunol*. 2010;125:S116–S125.
- [2] Eggesbo M, Botten G, Halvorsen R, Magnus P. The prevalence of allergy to egg: a population-based study in young children. *Allergy*. 2001;56:403–411.
- [3] Savage JH, Matsui EC, Skripak JM, Wood RA. The natural history of egg allergy. *J Allergy Clin Immunol*. 2007;120:1413–1417.
- [4] Meiler F, Zumkehr J, Klunker S, Ruckert B, Akdis CA, Akdis M. In vivo switch to IL-10-secreting T regulatory cells in high dose allergen exposure. *J Exp Med*. 2008;205:2887–2898.
- [5] Sicherer SH, Wood RA, Stablein D, et al. Immunologic features of infants with milk or egg allergy enrolled in an observational study (Consortium of Food Allergy Research) of food allergy. *J Allergy Clin Immunol*. 2010;125:1077–1083.e8.
- [6] Frossard CP, Eigenmann PA. The role of IL-10 in preventing food-induced anaphylaxis. *Expert Opin Biol Ther*. 2008;8:1309–1317.
- [7] Krogulska A, Borowiec M, Polakowska E, Dynowski J, Mlynarski W, Wasowska-Krolikowska K. FOXP3, IL-10, and TGF-beta genes expression in children with IgE-dependent food allergy. *J Clin Immunol*. 2011;31:205–215.
- [8] Campos Alberto EJ, Shimojo N, Suzuki Y, et al. IL-10 gene polymorphism, but not TGF-beta1 gene polymorphisms, is associated with food allergy in a Japanese population. *Pediatr Allergy Immunol*. 2008;19:716–721.
- [9] Burks AW, Jones SM, Wood RA, et al. Oral immunotherapy for treatment of egg allergy in children. *N Engl J Med*. 2012;367:233–243.

eMethods

Patient Recruitment

Children 1 to 18 years of age with egg allergy, NT, or without egg allergy were invited to participate, and written informed consent or assent was obtained. Institutional review board approval was obtained from the Ann & Robert H. Lurie Children's Hospital (Chicago, Illinois; formerly Children's Memorial Hospital) for patient recruitment, study protocols, and informed consent.

Food allergy was defined by an allergist-immunologist as a history of typical signs and symptoms of IgE-mediated allergy to egg (anaphylaxis, urticaria, or significant vomiting) within 2 hours of food ingestion in addition to positive laboratory test reactions (skin prick test or sIgE to egg white, with mean wheal diameter ≥ 4 mm larger than saline control wheal and/or sIgE level ≥ 2 kU/L). No allergy (control) was defined as no history of clinical reactivity to egg. NT was defined as a clinical and laboratory history of egg allergy in patients who passed an open food challenge to 1 scrambled egg without any clinical reaction (food challenge scheduled at patient's convenience).

Exclusion criteria included current or recent use of oral steroids, use of other systemic immunomodulators (biologics, immunoglobulins, or plasmapheresis), history of non-IgE-mediated food allergies, non-immediate type reactions to egg (eg, atopic dermatitis), or severe illness (requiring admission to the hospital within previous month).

Specific IgE and sIgG4 levels were detected from plasma at the time of recruitment, not during food challenge, with the ImmunoCAP system (Phadia, Uppsala, Sweden). Skin prick testing was performed at the time of diagnosis, as part of our standard clinic procedure, using a bifurcated needle with egg white extract, phenol-saline solution, and 1 mg/mL of histamine solution (Greer Laboratories, Lenoir, North Carolina).

PBMC Isolation and OVA Stimulation

Five milliliters to 10 mL of peripheral whole blood was collected from each patient in a heparinized tube. Using validated methods,^{1,2} PBMCs were isolated using Ficoll gradient, washed with phosphate buffered saline (sterile Dulbecco phosphate buffered saline), and separated into equal aliquots. Stimulations of 2×10^6 PBMCs per 15-mL conical tube occurred with or without the egg white protein OVA or with nonspecific T-cell stimulation (anti-CD3 and anti-CD28). Doses of 1, 10, 50, or 100 mg/mL of sterile, filtered, endotoxin-free, commercially obtained grade V lyophilized OVA

(Sigma, Poole, United Kingdom) was used.³ In addition, PBMCs were stimulated with nonspecific T-cell stimulation and 0.2 mg/mL of anti-CD3 and anti-CD28 (BD Pharmingen, San Diego, California). Samples were incubated for 48 hours at 37°C in AIM-V medium with human serum albumin (Gibco, Grand Island, New York).

Cytokine Analysis

After 48 hours of stimulation (maximal secretion of cytokines, data not shown), samples were centrifuged, and the supernatant was collected and frozen at -80°C . Samples were thawed and subjected to cytokine analysis using commercially available multiplex assays (Luminex xMAP, Millipore, Austin, Texas) with the Luminex 200 IS system. T-helper cell type 1 (IFN- γ), T-helper cell type 2 (IL-4, IL-5, IL-9, and IL-13), and regulatory and inflammatory (IL-10, IL-6, and IL-17) cytokines were measured.

Statistical Analysis

Patient characteristics were compared between patients with allergy and those with NT using the Mann-Whitney test or a 3-way comparison was performed using the Kruskal-Wallis test for continuous variables. Logarithmic transformation of cytokine data was performed, and dose-dependent responses were analyzed using repeated-measures ANOVA. Comparison of cytokine data at specific doses was made using ANOVA among groups and unpaired 2-tailed *t* test was used to make comparisons between 2 groups. For fold stimulation, data were compared at individual doses using the Mann-Whitney test. Specific IgE/sIgG4 data were compared between groups using the Mann-Whitney test or a 3-way comparison was performed using the Kruskal-Wallis test. Simple linear regression was performed to compare sIgG4 levels of patients with NT with other biomarkers. Data analysis was performed using SAS 9.3 and SPSS 14. All analyses were run at a 2-sided type I error rate of 5%.

eReferences

- [1] Turcanu V, Maleki SJ, Lack G. Characterization of lymphocyte responses to peanuts in normal children, peanut-allergic children, and allergic children who acquired tolerance to peanuts. *J Clin Invest*. 2003;111:1065–1072.
- [2] Thottingal TB, Stefura BP, Simons FE, Bannion GA, Burks W, HayGlass KT. Human subjects without peanut allergy demonstrate T cell-dependent, TH2-biased, peanut-specific cytokine and chemokine responses independent of TH1 expression. *J Allergy Clin Immunol*. 2006;118:905–914.
- [3] Tay SS, Clark AT, Deighton J, King Y, Ewan PW. T cell proliferation and cytokine responses to ovalbumin and ovomucoid detected in children with and without egg allergy. *Clin Exp Allergy*. 2007;37:1519–1527.

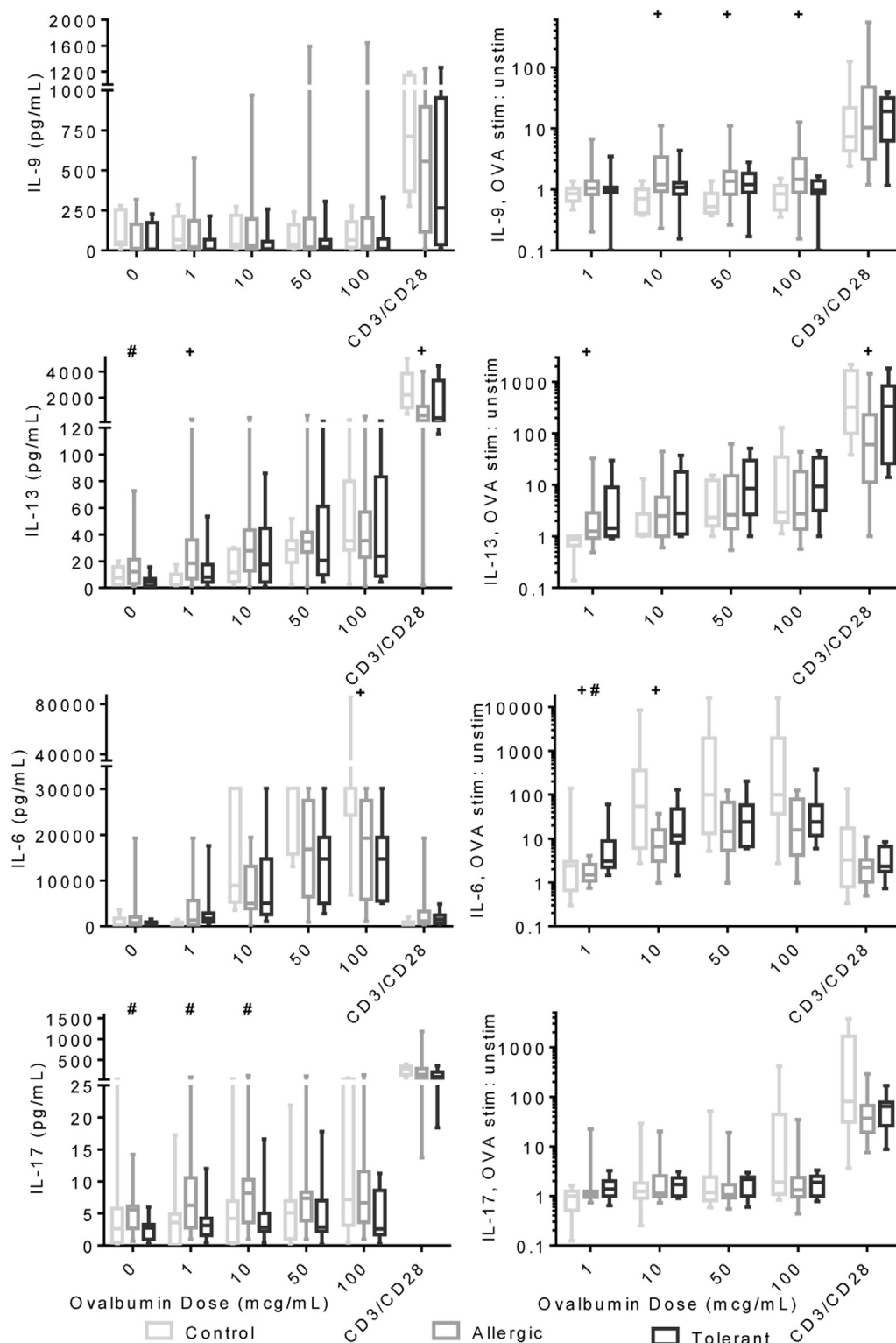


Figure 1. Cytokine responses to ovalbumin (OVA) in patients without allergy (control), those with allergy, and those with natural tolerance. Boxplots depict cytokine responses to OVA and anti-CD3 and anti-CD28 (0.2 mg/mL) after 48-hour culture of peripheral blood mononuclear cells for interleukin (IL)-9, IL-13, IL-6, and IL-17 (left) and upregulation after stimulation compared with unstimulated samples (right). IL-9 was significantly produced only in patients with allergy ($P < .01$), with IL-13 trending toward upregulation in these patients ($P = .06$). IL-6 showed a robust dose-dependent response in control patients ($P < .01$ by repeated-measures ANOVA) and was the only cytokine out-produced by OVA stimulation compared with anti-CD3 and anti-CD28. Overall, IL-17 production was not different among groups when using a repeated-measures approach ($P = .5$). Boxplots display median values (horizontal lines within bars), lower and upper quartiles (areas within bars below and above lines, respectively), and minimum and maximum values (whiskers). Within-dose comparisons: + $P < .05$ among all 3 groups, # $P < .05$ between allergic and tolerant groups.

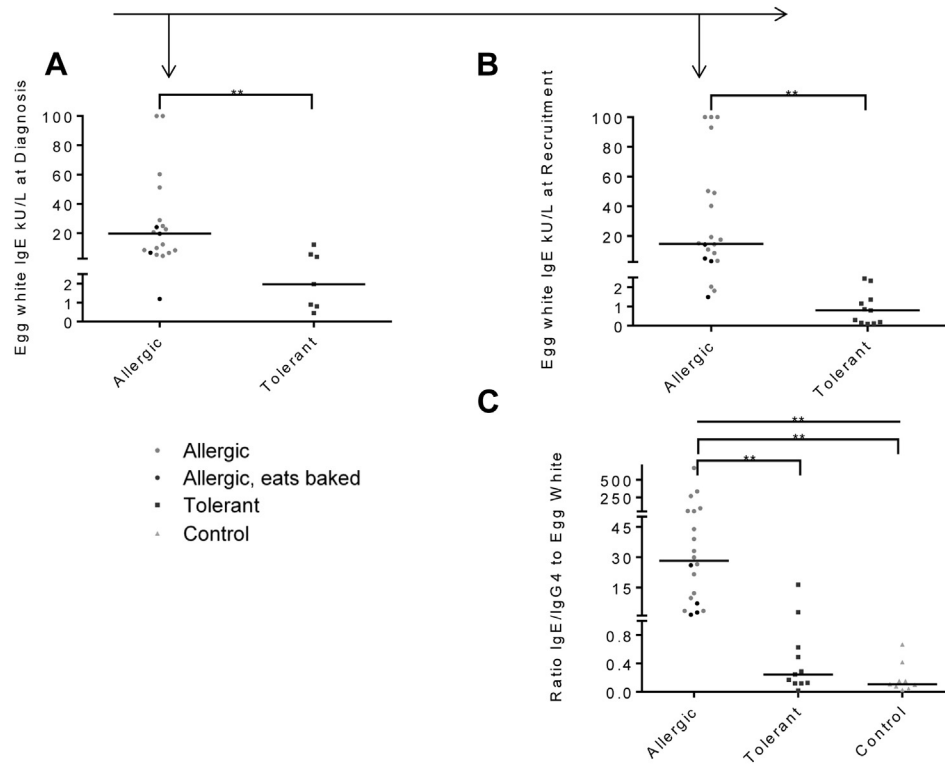


Figure 2. Egg white IgE and egg white IgE/IgG4 ratio. Scatterplots show a comparison of patients with allergy, those with natural tolerance, and those without allergy (control). (A) Egg white IgE at diagnosis. (B) Egg white IgE at recruitment. (C) IgE/IgG4 ratio at recruitment. Median values are displayed (horizontal lines). ** $P < .01$ among all groups by Kruskal-Wallis test and between allergic and tolerant groups by Mann-Whitney test.

eTable 1

Patient characteristics at recruitment

Group	Age (y)	Other food allergies	Egg White Specific IgE (kU/L)	Egg White Specific IgG4 (kU/L)	Months after recruitment patient challenged with scrambled egg
Allergic	4.1	Y	93.0	9.6	NA
Allergic	7.0	Y	>100	3.4	NA
Allergic	3.0	Y	1.8	<0.2	2 ^a
Allergic	3.0	Y	19.3	0.4	NA
Allergic	9.3	Y	10.9	0.3	NA
Allergic	6.05	Y	49.0	0.9	NA
Allergic	6.3	Y	2.0	0.6	NA
Allergic	18.3	Y	40.3	<0.2	NA
Allergic	6.3	Y	3.2	<0.2	NA
Allergic	8.3	Y	14.4	<0.2	NA
Allergic	5.8	Y	>100	29.4	NA
Allergic	8.8	Y	>100	<0.2	NA
Allergic	16.6	Y	17.5	0.5	NA
Allergic	7.7	Y	15.1	0.6	NA
Allergic	7.4	Y	8.5	<0.2	NA
Allergic	17.8	Y	50.3	<0.2	NA
Median	7.2		18.4	0.4	
Allergic, eats baked	5.8	Y	3.0	0.4	NA
Allergic, eats baked	4.1	Y	1.5	1.0	1 ^a
Allergic, eats baked	5.3	Y	5.0	1.9	NA
Allergic, eats baked	12.9	Y	14.3	0.6	NA
Median	5.6		4	0.8	
Tolerant	2.5	Y	0.1	<0.2	9
Tolerant	2.2	Y	0.2	1.6	3
Tolerant	2.3	Y	0.9	5.1	9
Tolerant	2.0	Y	1.2	0.4	3
Tolerant	6.9	Y	0.8	1.6	12
Tolerant	5.1	N	2.3	19.7	2
Tolerant	9.9	Y	0.1	0.5	1
Tolerant	17.1	Y	2.5	<0.2	14
Tolerant	2.1	N	0.2	0.2	2
Tolerant	9.9	Y	1.4	4.7	1
Tolerant	3.9	N	0.3	2.4	14
Median	3.9		0.8	1.6	3
Control	10.1	N	<0.1	1.3	eating egg ad libitum
Control	14.9	N	<0.1	0.2	eating egg ad libitum
Control	4.0	N	<0.1	2.3	eating egg ad libitum
Control	6.9	N	<0.1	0.9	eating egg ad libitum
Control	16.4	N	<0.1	3.8	eating egg ad libitum
Control	3.6	N	<0.1	0.7	eating egg ad libitum
Control	17.4	N	<0.1	<0.2	eating egg ad libitum
Control	9.3	N	0.26	1.7	eating egg ad libitum
Control	10.5	Y	<0.1	1.0	eating egg ad libitum
Median	10.1		<0.1	1	

Abbreviations: N, no; NA, not available; Y, yes.

^aFailed oral food challenge.