

Egg-specific IgE and basophil activation but not egg-specific T-cell counts correlate with phenotypes of clinical egg allergy



M. Cecilia Berin, PhD,^{a,*} Alexander Grishin, PhD,^{a,*} Madhan Masilamani, PhD,^{a,*} Donald Y. M. Leung, MD, PhD,^b Scott H. Sicherer, MD,^a Stacie M. Jones, MD,^c A. Wesley Burks, MD,^d Alice K. Henning, MS,^e Peter Dawson, PhD,^e Joanna Grabowska, MS,^a Charuta Agashe, MS,^a Wendy F. Davidson, PhD,^f Robert A. Wood, MD,^g and Hugh A. Sampson, MD^a *New York, NY, Denver, Colo, Little Rock, Ark, Chapel Hill, NC, and Rockville, Bethesda, and Baltimore, Md*

Background: Egg allergy is phenotypically heterogeneous. A subset of patients with egg allergy can tolerate egg in an extensively heated form. Inclusion of baked egg (BE) into the diet accelerates resolution of egg allergy. Conversely, BE reactivity is associated with persistent disease. The immune basis of this clinical heterogeneity is unknown.

From ^athe Department of Pediatrics, Icahn School of Medicine at Mount Sinai, New York; ^bthe Department of Pediatrics, National Jewish Health, Denver; ^cthe Department of Pediatrics, University of Arkansas for Medical Sciences and Arkansas Children's Hospital, Little Rock; ^dthe Department of Pediatrics, University of North Carolina, Chapel Hill; ^eEMMES Corporation, Rockville; ^fthe National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda; and ^gthe Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore.

*These authors contributed equally to this work.

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Corresponding author: M. Cecilia Berin, PhD, Pediatric Allergy and Immunology, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, Box 1198, New York, NY 10029. E-mail: cecilia.berin@mssm.edu.

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Objectives: We sought to study egg-specific antibody, basophil, and T-cell responses in children with reactivity or tolerance to BE.

Methods: All participants underwent double-blind, placebo-controlled challenges to BE, and those who tolerated BE were challenged with unheated egg white protein to confirm clinical egg reactivity. Laboratory studies included serum antibody measurements, basophil activation tests, and CD154-based detection of egg-responsive T cells by using flow cytometry.

Results: Of the 129 children studied, BE-reactive participants had significantly greater levels of egg-, ovalbumin-, and ovomucoid-specific IgE; lower ratios of egg-specific IgG₄/IgE; and increased basophil activation in response to egg. Among all participants, CD154-based profiling revealed egg-responsive T cells producing IL-4 and IL-13 but little IL-10 or IFN- γ , as well as the presence of egg-responsive Foxp3⁺CD25⁺CD127^{low} regulatory T cells. Egg-responsive T cells expressed CCR4, CCR6, and CXCR5, indicating capacity for homing to the skin, mucosa, and B-cell follicles. However, neither the frequency nor phenotype of egg-responsive T cells was different in those with tolerance or reactivity to BE.

Conclusions: Egg-specific antibody and basophil responses, but not T-cell responses, are greater in those with reactivity versus tolerance to BE. Egg-specific antibody and T-cell responses were highly heterogeneous in this cohort. The clinical implications of this immune heterogeneity will need to be studied longitudinally. (J Allergy Clin Immunol 2018;142:149-58.)

Key words: Egg allergy, food allergy, IgE, IgG₄, basophil, CD4⁺ T lymphocyte, regulatory T cell, T_H2

Over the past decade, it has become increasingly clear that a large subset of children with egg allergy are able to tolerate egg protein after it has been extensively heated (ie, heat denatured).¹⁻⁴ Specific predictors of those patients most likely to tolerate baked egg (BE) have been identified, including skin prick testing and egg-specific IgE and ovomucoid-specific IgE measurement.^{5,6} Children with tolerance to heated egg are more likely to outgrow their egg allergy by 2 years of life.⁷ Longitudinal studies have also demonstrated that exposure to heated egg enhances eventual tolerance to less heated forms of egg.^{8,9} In addition to these clinical outcomes, studies have also helped define the immunologic changes that occur with regular exposure to BE in patients with egg allergy.^{4,8}

Despite these advances, there is a lack of information about detailed immunologic characteristics that can differentiate patients with egg allergy who do or do not tolerate extensively

Abbreviations used

BE: Baked egg
OFC: Oral food challenge
Treg: Regulatory T

heated egg. In this study we had the opportunity to conduct detailed studies profiling basophil and T-cell responsiveness, as well as serologic measures, at baseline in a large cohort of children who underwent double-blind, placebo-controlled challenges (DBPCFCs) to baked or unheated egg.

METHODS**Participants**

Baseline blood samples were obtained from children aged 3 to 16 years at the time of enrollment (before egg exposure through diet or immunotherapy) in a multicenter Consortium of Food Allergy Research (CoFAR) intervention trial comparing the efficacy of BE diet or egg oral immunotherapy in the treatment of egg allergy (CoFAR7, NCT01846208). These participants included children with an egg-specific IgE level of greater than 5 kU_A/L who were avoiding all forms of egg in the diet.

Participants underwent an initial DBPCFC to BE in the form of a muffin. Those who reacted during the BE challenge were categorized as BE reactive. Those who tolerated the BE challenge then underwent a DBPCFC to unheated egg white protein to confirm reactivity to unheated egg. This group was categorized as BE tolerant. After enrollment of the BE-reactive group was complete (40 participants per the protocol), the baseline blood sample was drawn after BE challenge from the remaining BE-tolerant participants (to determine their qualification for the study before conducting immune profiling).

The National Institute of Allergy and Infectious Diseases Allergy and Asthma Data and Safety Monitoring Board and the Institutional Review Boards at Icahn School of Medicine at Mount Sinai, National Jewish Health, Johns Hopkins Medical School, University of Arkansas for Medical Sciences, and University of North Carolina, Chapel Hill, approved study procedures. Written informed consent was obtained from the parent or guardian.

Blood processing and PBMC isolation

Blood samples were obtained in sodium-heparin Vacutainer tubes at the 5 clinical sites. An aliquot was retained for basophil activation tests on site, and the remaining whole blood was shipped overnight in temperature-controlled Greenbox shipping containers (ThermoSafe, Arlington Heights, Ill) assembled according to standard operating procedures. Temperature loggers were included to ensure that temperatures were maintained between 20°C and 30°C. Samples from the Icahn School of Medicine at Mount Sinai clinical site were stored at room temperature and processed the next day to maintain consistency with the other sites. An aliquot of whole blood was used for phenotypic analysis by using flow cytometry with antibodies shown in Table E1 in this article's Online Repository at www.jacionline.org. Blood was centrifuged to obtain plasma before isolation of PBMCs by using Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden). Cells were washed, counted, and cultured in AimV (Thermo Fisher, Grand Island, NY) with 2.5% autologous plasma at a density of 4×10^6 cells in 1 mL in 24-well culture plates.

Basophil activation

Basophil activation assays were performed at clinical sites within 2 hours of blood draw. Whole blood was stimulated for 30 minutes with medium containing 2 ng/mL IL-3 plus egg white protein (1-0.001 µg/mL; Deb-El Food Products, Elizabeth, NJ), anti-IgE, or N-formylmethionine-leucyl-phenylalanine as positive controls or medium with or without IL-3 as negative controls.

After stopping degranulation with EDTA, samples were stained for the surface activation markers CD63 and CD203c (full antibody staining panel is shown in Table E2 in this article's Online Repository at www.jacionline.org).

PBMC stimulations

PBMCs (4×10^6) in 1 mL of AimV medium were stimulated with 300 µg/mL egg white protein (Deb-El Food Products) or 5 µL of anti-CD3/CD28 stimulation beads (Life Technologies AS, Oslo, Norway) for a bead/cell ratio of 1:20. Duplicate cultures were used for unstimulated and egg-stimulated conditions. Egg white had been cleaned of endotoxin by using Detoxi-Gel columns (Thermo Fisher Scientific, Waltham, Mass). Residual endotoxin levels were approximately 0.3 EU/mL of working solution of egg stimulant (99.85% removal and within the acceptable range for culture reagents). The antigen dose was based on pilot experiments conducted to optimize detection of egg-responsive T cells by using flow cytometry. Cells were cultured in standard tissue culture incubators. GolgiPlug (BD Biosciences, San Jose, Calif) was added 4 hours before harvesting.

T-cell phenotypic analysis

Harvested PBMCs were stained for viability (Live/Dead Fixable stain; Invitrogen, Carlsbad, Calif), washed and stained for surface markers, and washed for fixation and permeabilization. Cells were fixed in 4% para-formaldehyde (Electron Microscopy Sciences, Hatfield, Pa) and treated with permeabilization buffer (eBioscience, San Diego, Calif) before staining with labeled antibodies to detect intracellular CD154 and cytokines. For FoxP3 staining, cells were processed with the FoxP3/Transcription Factor Staining Buffer Set (eBioscience) before staining with antibodies. Antibody panels used for surface and cytoplasmic staining are shown in Tables E3 and E4 in this article's Online Repository at www.jacionline.org. Stained cells were analyzed subsequently on an LSR II Cytometer (BD Biosciences, San Jose, Calif).

Skin prick tests

A skin prick test was performed while the participant was off antihistamines for an appropriate length of time (5 half-lives of the antihistamine being used). A skin test probe was pressed through a commercial extract of egg into the epidermis. Positive (histamine) and negative (saline-glycerin) controls were also used. The egg skin prick test score was calculated by subtracting the diameter of the saline-induced wheal from the diameter of the egg-induced wheal.

Immunoglobulin measurements

Frozen plasma samples were thawed, and IgE, IgG, and IgG₄ antibodies specific for egg, ovalbumin, and ovomucoid were measured by means of ImmunoCAP (Thermo Fisher Scientific). Ranges of detection for the assays were 0.01 to 100 kU_A/L for IgE, 2 to 200 mg_A/L for IgG, 0.07 to 30 mg_A/L for IgG₄, 5000 kU/L upper limit for total IgE. Values obtained outside of this range of detection were truncated for analysis as follows (lower and upper values): IgE, 0.05 and 101 kU_A/L; IgG, 1 and 201 mg_A/L; IgG₄, 0.035 and 31 mg_A/L; and total IgE, NA and 5001 kU/L.

Statistical analysis

The primary analysis sought to identify differences in immunologic markers between the BE-reactive and BE-tolerant groups. Continuous variables were summarized by medians and ranges and compared between groups by using a Wilcoxon rank sum test. Categorical variables were summarized by counts and percentages and compared between groups by using the Fisher exact test. Spearman correlations were used to assess associations between continuous variables. A secondary analysis looked at the same variables to determine whether there was a difference for participants with a sample drawn before or after BE oral food challenge (OFC). A *P* value of less than .01 was considered significant to control for multiple comparisons.

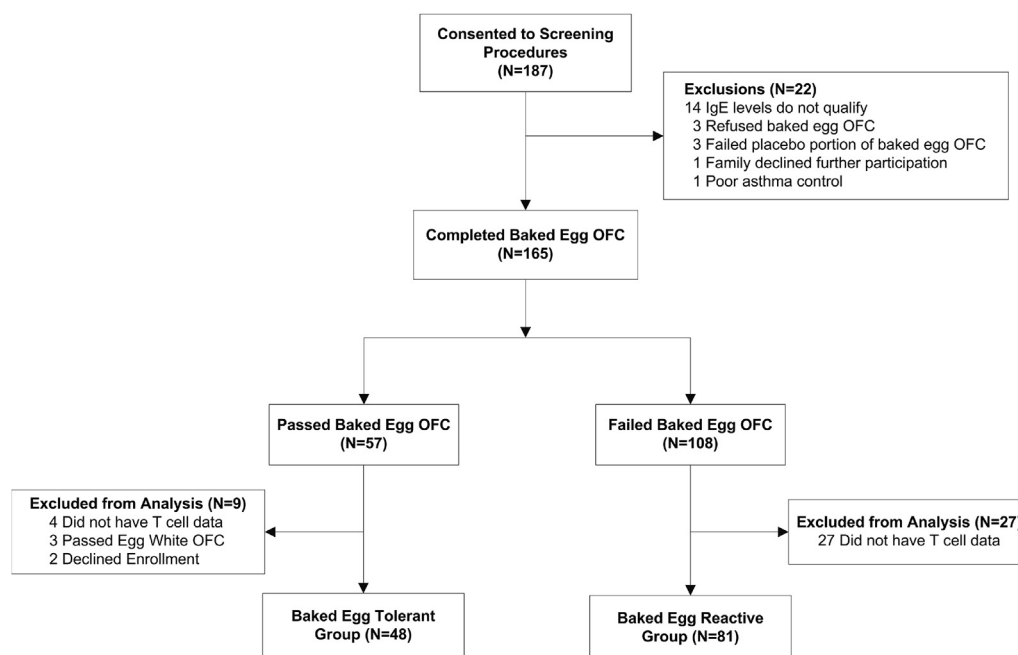


FIG 1. CONSORT diagram showing participant disposition.

RESULTS

Study population

The CONSORT diagram is shown in Fig 1, and participants' characteristics are shown in Table I. One hundred eighty-seven participants consented to screening procedures, and 165 underwent a BE OFC.

Fifty-seven participants successfully tolerated the BE OFC and were therefore categorized as BE tolerant. Forty-eight participants were included in analysis (BE-tolerant group). Nine participants were excluded from the analysis because of passing the egg white OFC (not egg allergic), declining to enroll, or not having T-cell data.

One hundred eight participants reacted to the BE OFC and were therefore categorized as BE reactive. Eighty-one participants were included in the analysis, and the remainder were excluded because of a lack of T-cell data, primarily due to completion of enrollment in the BE-reactive group and change in timing of specimen collection.

Enrollment occurred between July 2013 and August 2015 and did not reach the target enrollment (96) in the BE-tolerant group.

All participants were avoiding egg in the diet before enrollment. BE-reactive participants had a median successfully consumed dose of 300 mg of BE protein administered in the form of a muffin during the BE OFC. By definition, BE-tolerant participants tolerated the full challenge dose equivalent to 2000 mg of BE protein during the BE OFC. When rechallenged with unheated egg white powder to confirm egg allergy, the median successfully consumed dose was 144 mg of egg white protein.

Immune cell subsets

To determine whether there were any global differences in immune cell populations between BE-reactive and BE-tolerant groups, we compared the frequencies of immune cell subsets in

whole blood using flow cytometry. We identified CD14⁺ and CD16⁺ monocytes, plasmacytoid dendritic cells, classical dendritic cells, neutrophils, basophils, eosinophils, natural killer cells, and B and T cells (Table II). There was no significant difference in the representation of these populations between the BE-reactive and BE-tolerant groups.

Specific immunoglobulins

We compared skin prick tests and immunoglobulin levels in the 2 groups of participants with egg allergy, as shown in Fig 2 and Table I. Egg skin prick test scores were higher but did not reach the level of statistical significance in the BE-reactive versus BE-tolerant group (Fig 2, A). Egg-specific IgE levels were significantly ($P = .001$) increased in the BE-reactive group versus the BE-tolerant group (Fig 2, B). IgE binding to the major egg white allergens ovomucoid and ovalbumin was also significantly greater in the BE-reactive group versus the BE-tolerant group ($P = .001$ and $.0004$, respectively; Fig 2, C and D). Total IgE levels were similar between groups (data not shown). Egg-specific IgG (Fig 2, E) and egg-, ovalbumin-, and ovomucoid-specific IgG₄ levels (data not shown) were greater but did not reach the level of statistical significance in the BE-tolerant group compared with the BE-reactive group. Egg IgG₄/IgE ratios were significantly higher in BE-tolerant versus BE-reactive participants ($P = .0003$; Fig 2, F), whereas the percentage of egg IgE (ratio of egg-specific to total IgE) was not significantly different between groups (data not shown). Thus the dominant difference between groups when comparing egg-specific immunoglobulins was the magnitude of the egg- and egg component-specific IgE responses.

Basophil activation tests

We performed basophil activation tests in response to *in vitro* stimulation of whole blood with egg white protein. Expression of the degranulation marker CD63 was used to quantify the

TABLE I. Participant characteristics

	BE-reactive subjects	BE-tolerant subjects	P value
No.	81	48	
Sex (M/F)	42/39	31/17	.20
Age (y [range])	8.0 (3.7-16.0)	7.4 (3.7-16.8)	.49
Asthma, no. (%)	49 (60.5)	31 (64.6)	.71
Allergic rhinitis, no. (%)	66 (81.5)	40 (83.3)	1.00
Atopic dermatitis, no. (%)	51 (63.0)	30 (62.5)	1.00
Other food allergy, no. (%)	72 (88.9)	44 (91.7)	.77
Successfully consumed dose (mg protein), BE OFC (range)	300 (0-1500)	2000 (2000-2000)	
Successfully consumed dose (mg protein), egg white OFC (range)	ND	144 (1-444)	
Egg SPT (mm)	12 (7-16)	10 (6-13)	.05
Egg IgE (kU _A /L)	25.4 (12.4-70.0)	11.6 (6.7-26.7)	.001
Ovalbumin IgE (kU _A /L)	15.7 (5.9-31.4)	5.7 (3.3-16.6)	.0004
Ovomucoid IgE (kU _A /L)	15.1 (6.2-40.3)	7.0 (3.0-13.3)	.001
Egg IgG (mg _A /L)	5.2 (2.9-8.5)	7.4 (3.9-17.9)	.02
Egg IgG ₄ (mg _A /L)	0.6 (0.2-1.1)	0.7 (0.2-2.5)	.15
Ovalbumin IgG ₄ (mg _A /L)	0.34 (0.14-1.04)	0.48 (0.11-1.93)	.32
Ovomucoid IgG ₄ (mg _A /L)	0.11 (0.04-0.33)	0.15 (0.04-0.86)	.19
Egg IgG ₄ /IgE ratio	6.4 (3.4-14.9)	23.2 (6.8-80.8)	.0003

Medians with interquartile ranges are shown, unless otherwise stated.

F, Female; M, male; ND, not determined; SPT, skin prick test.

TABLE II. Leukocyte subset frequency (CD45 percentage)

	BE-reactive subjects			BE-tolerant subjects		
	Median	Maximum	Minimum	Median	Maximum	Minimum
Neutrophils	28.38	64.70	8.68	28.26	89.05	12.07
Eosinophils	7.25	20.43	1.07	6.90	64.70	1.66
Basophils	0.75	1.72	0.08	0.64	5.33	0.07
pDCs	0.20	0.74	0.01	0.17	0.88	0.01
mDCs	0.29	3.64	0.02	0.27	1.34	0.03
NK T cells	0.56	4.08	0.03	0.70	7.01	0.13
CD4 ⁺ T cells	18.41	33.33	0.59	18.99	73.10	3.74
CD8 ⁺ T cells	9.60	22.33	1.57	10.04	25.86	2.74
B cells	6.89	16.07	1.44	6.88	31.13	3.30
NK CD56 ^{Bright} cells	0.18	0.61	0.05	0.19	0.84	0.04
NK CD56 ^{dim} cells	2.60	9.18	0.71	2.96	11.08	0.34
CD14 ⁺ monocytes	3.66	7.85	0.11	3.62	21.54	0.02
CD16 ⁺ monocytes	0.29	2.91	0.06	0.41	2.24	0.04

mDCs, Myeloid dendritic cells; NK, natural killer; pDCs, plasmacytoid dendritic cells.

magnitude of activation. As shown in Fig 3, A, BE-reactive participants had higher basophil activation compared with BE-tolerant participants at intermediate stimulation levels of egg white (0.01 and 0.1 μ g), whereas the 2 groups reached similar levels of basophil activation at the lowest and highest levels of 0.001 and 1 μ g of egg white protein. When expressed as a stimulation index that normalized the egg-stimulated values to that of the positive control (anti-IgE stimulation), the differences in basophil activation between the BE-reactive and BE-tolerant participants at doses of 0.01 and 0.1 μ g were significantly different ($P = .002$ and $P = .004$, respectively; Fig 3, B). Again, maximal reactivity at 1 μ g of egg white protein was similar between the groups. These data suggest a lower threshold of basophil activation at intermediate antigen doses in the BE-reactive group.

Egg-responsive T cells

We stimulated fresh PBMCs with egg white protein and identified egg-responsive cells by means of upregulation of

CD154 after 6 or 18 hours of stimulation. Based on previous piloting of the kinetics of CD154 upregulation of different T-cell subsets, we examined CD154 expression on effector CD4⁺ T cells after 6 hours of stimulation and on Foxp3⁺CD25⁺CD127[−] regulatory T (Treg) cells after 18 hours of stimulation. Gating and representative flow cytometric plots for the 2 populations are shown in Figs E1 and E2 in this article's Online Repository at www.jacionline.org. As shown in Fig 4, A, there was an induction of CD154⁺ cells coexpressing IL-4 and IL-13 after 6 hours of stimulation with egg white antigen in both the BE-reactive and BE-tolerant groups. The frequency of CD154⁺IL-10⁺ and CD154⁺IFN- γ ⁺ coexpressing cells remained low after egg stimulation in both groups. Although the average frequencies of CD154⁺IL-4⁺ and CD154⁺IL-13⁺ cells in the BE-reactive and BE-tolerant groups were not statistically significantly different, there was a subset of subjects with high T_H2 responses in the BE-reactive group that was not observed in the BE-tolerant group. In both groups egg-responsive T_H2 cells (defined as CD154⁺IL-4⁺ or CD154⁺IL-13⁺ cells) correlated significantly with egg-specific IgE levels (data not shown).

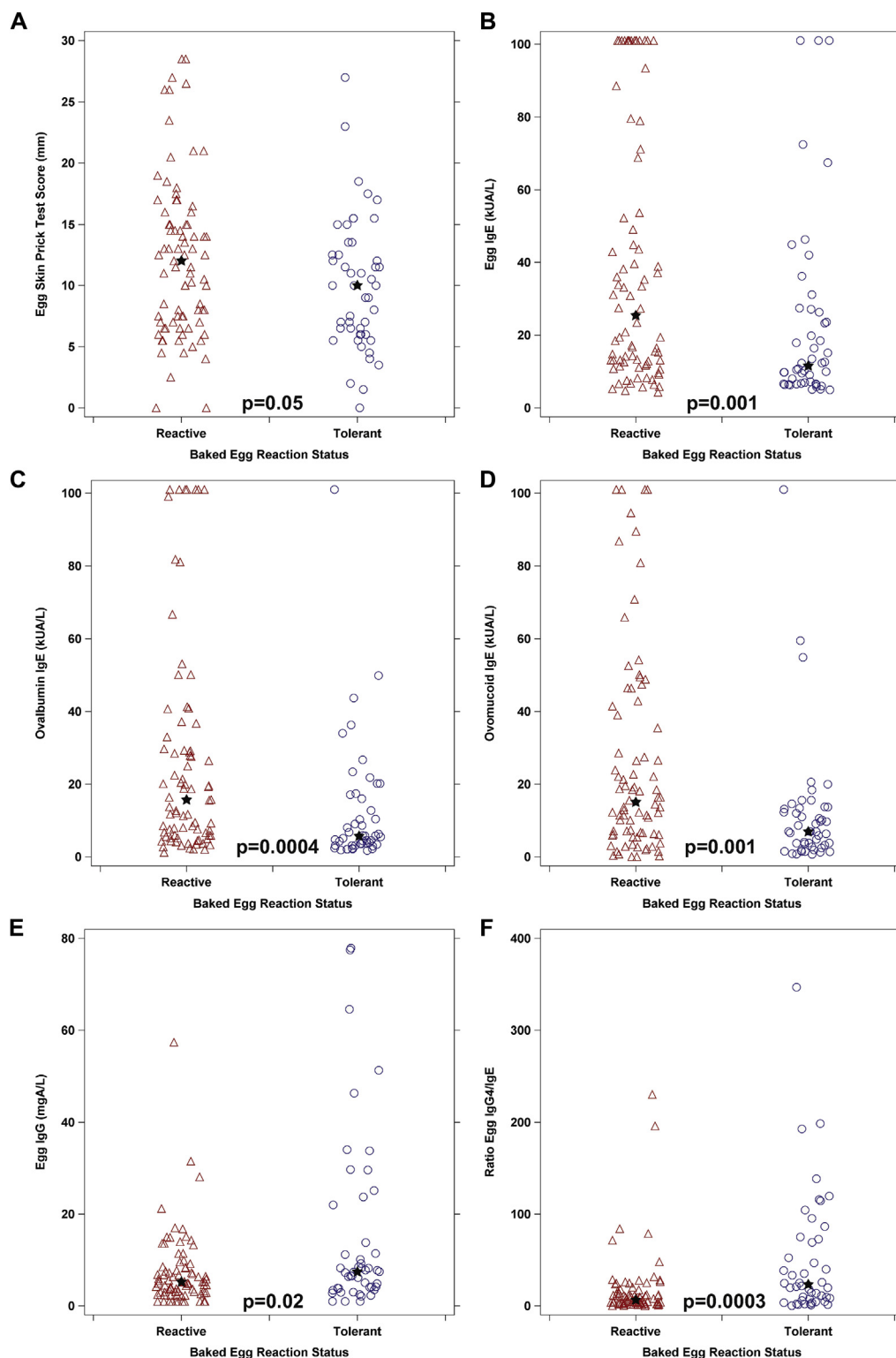


FIG 2. Egg-specific immunoglobulins. **A**, Egg skin prick test. **B**, Egg-specific IgE. **C**, Ovalbumin-specific IgE. **D**, Ovomucoid-specific IgE. **E**, Egg-specific IgG. **F**, Egg IgG₄/IgE ratio. The *star* indicates the group median, and *P* values from a Wilcoxon rank sum test comparing the groups are indicated on each graph.

Because some of the BE-tolerant group had T-cell assays performed after the BE OFC, the frequency of egg-responsive T_H2 cells was compared in those analyzed before BE OFC (*n* = 31) versus those analyzed after BE OFC (*n* = 17). There was no

significant difference in the frequency of CD154⁺ T_H2 cells when assayed before or after BE OFC in the BE-tolerant group.

We examined the expression of the chemokine receptors CCR4, CCR6, and CXCR5 on CD154⁺ cells from the

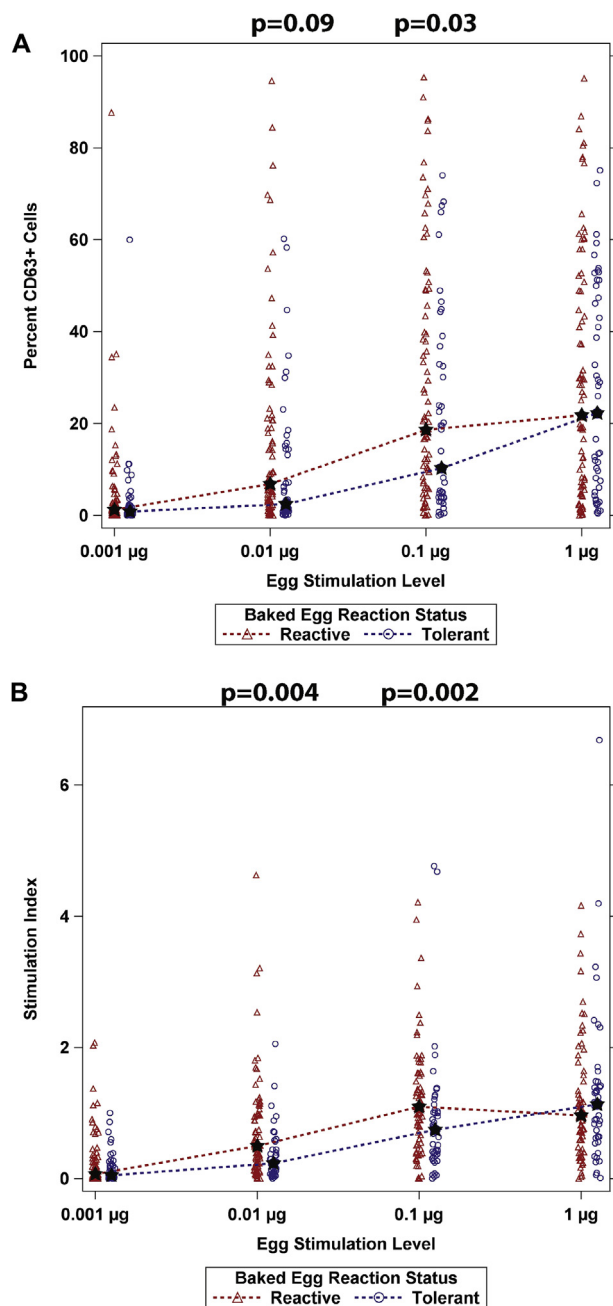


FIG 3. Basophil activation test. **A**, Percentage of CD63⁺ cells by egg stimulation level. **B**, Stimulation index was calculated as the percentage of cells positive for CD63 after egg stimulation divided by the percentage of cells positive for CD63 after positive control anti-IgE stimulation. The *star* indicates the group median, and *P* values from a Wilcoxon rank sum test at each stimulation level comparing the groups are shown.

BE-tolerant and BE-reactive participants after stimulation with egg or anti-CD3/CD28 antibody stimulation for 6 hours. All 3 chemokine receptors were enriched on egg-activated T cells compared with polyclonally activated T cells, and no significant differences were observed between the BE-tolerant and BE-reactive participants (Fig 4, B). These data indicate that egg-specific CD4⁺ T-cell populations include cells with the capacity to home to the skin (CCR4⁺), mucosa (CCR6⁺), and B-cell follicles (CXCR5⁺). We also quantified Treg cells (Foxp3⁺CD25⁺CD127^{low}) upregulating CD154 after 18 hours

of stimulation with egg antigen. We observed a large induction of CD154⁺ on cells expressing Treg cell markers. This activation of Treg cells was not significantly different when comparing BE-reactive and BE-tolerant participants.

Age and egg-specific immune response

We examined the association between age and specific IgE levels to egg, ovalbumin, and ovomucoid (see Fig E3 in this article's Online Repository at www.jacionline.org) and between age and egg-responsive CD154⁺IL-4⁺ or CD154⁺IL-13⁺ cells (see Fig E4 in this article's Online Repository at www.jacionline.org) in the BE-reactive and BE-tolerant groups. We found no significant association between age and any of these parameters.

DISCUSSION

We investigated the immune basis underlying reactivity or tolerance to BE among 129 pediatric participants with egg allergy who underwent oral egg challenge during baseline evaluations for a clinical trial comparing the efficacy of BE diet with egg oral immunotherapy. A large subset of children with egg allergy has been reported to tolerate extensively heated or baked forms of egg.²⁻⁴ The proportion of BE-reactive and BE-tolerant subjects screened and enrolled in this study does not reflect those findings. Most subjects screened were BE reactive, and we were not able to reach target enrollment in the BE-tolerant group, most likely because of the fact that many BE-tolerant children are now identified in their local clinical centers and were no longer eligible for this study.

We found significant differences between the BE-reactive and BE-tolerant groups at the level of egg-specific immunoglobulins and egg-induced basophil activation but not in egg-specific T-cell responses. Heating alters the conformational structure and gastrointestinal absorption of heat-sensitive allergens in egg.¹⁰ Of the 2 major egg allergens, ovomucoid is more resistant to heating and digestion than ovalbumin, and therefore a difference in specificity of the IgE response could explain reactivity versus tolerance to BE. We found that BE reactivity was associated with significantly higher levels of IgE specific for ovomucoid, as well as ovalbumin and total egg white. These results are consistent with previous single-center studies^{4,11} and indicate that the magnitude rather than the specificity of the IgE response to whole or component proteins is the most relevant difference between BE-reactive and BE-tolerant participants. Previous studies comparing immunoglobulin responses in patients with reactivity or tolerance to heated milk identified the affinity of IgE binding as an important factor discriminating the 2 groups.¹² Furthermore, there might be important differences at the level of binding to clinically relevant epitopes (ie, those that survive heating and digestion and are absorbed) that are obscured by measurement of IgE binding to the entire allergen. In patients with milk allergy, IgE from sera of baked milk-reactive subjects binds to a greater number of epitopes compared with baked milk-tolerant subjects.¹² Egg-specific IgG and IgG₄, which could function to suppress reactivity to egg, were not significantly different between BE-reactive or BE-tolerant participants, likely reflecting the lack of exposure to egg in both groups. Basophil activation as a functional assay reflected this lower level of egg-specific IgE and higher threshold of reactivity in BE-tolerant participants.

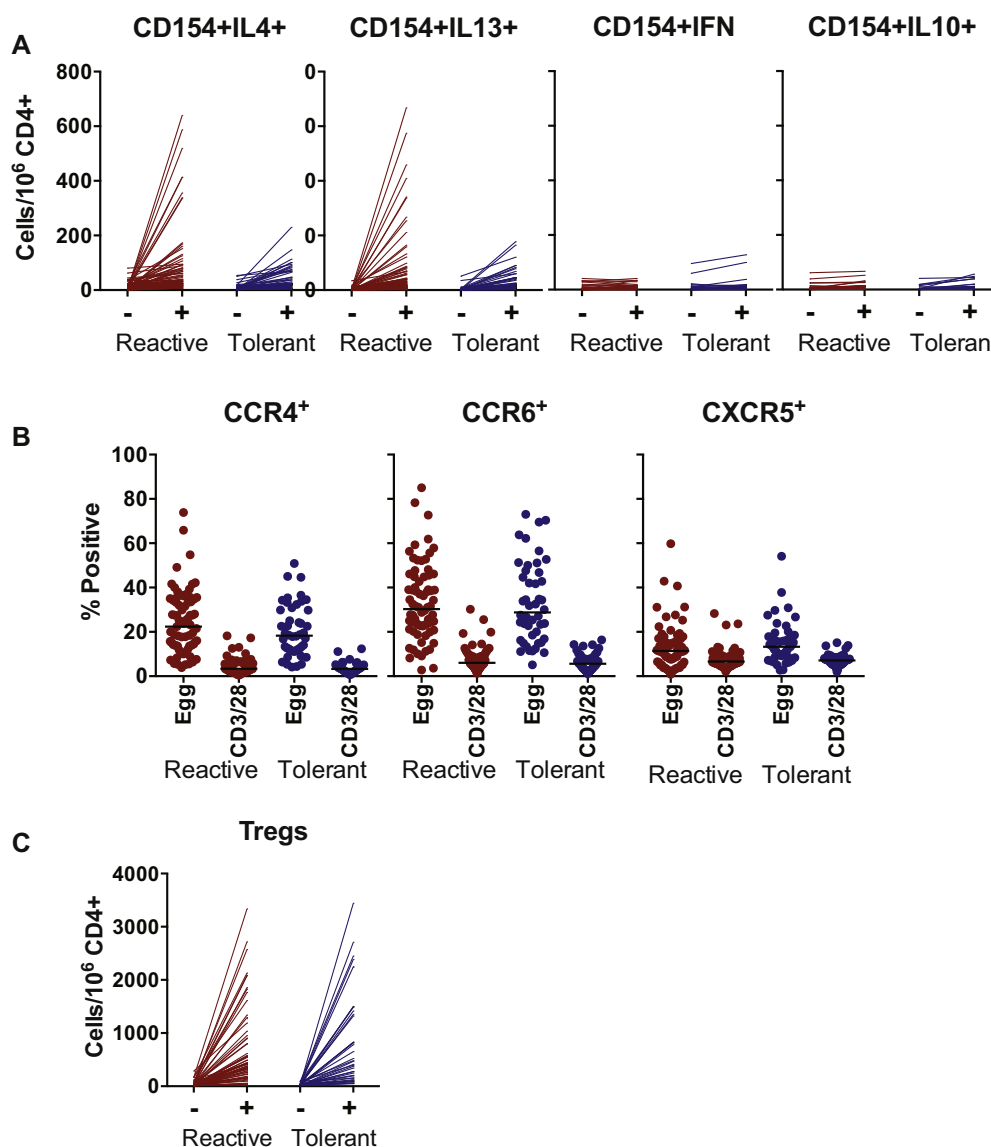


FIG 4. Egg-responsive T cells. **A**, Frequency of cells coexpressing CD154 and the cytokines IL-4, IL-13, IFN- γ , or IL-10 after 6 hours of stimulation with egg antigen (+) or medium alone. Reactive and tolerant refers to responsiveness to BE. **B**, Percentage of CD154⁺ cells after stimulation with egg or anti-CD3/CD28 that coexpress CCR4, CCR6, or CXCR5. Bars indicate medians. **C**, Frequency of Foxp3⁺ CD25⁺ CD127⁻ cells that express CD154 after 18 hours of stimulation with egg allergen.

It should be noted that despite the significant difference between groups, there was a great deal of overlap in IgE binding and basophil activation, indicating that other factors must contribute to the difference in clinical reactivity of these participants to BE. In addition to immune parameters, these differences might also include factors upstream of immune activation, such as differences in digestion or absorption of BE.

We examined the immunoglobulin, basophil, and T-cell responses to unheated egg protein or components. This was chosen over using BE for several reasons. First, BE-reactive and BE-tolerant subjects have a different prognosis in their resolution of allergy to unheated egg, with BE-tolerant subjects more likely to outgrow their allergy to unheated egg. The primary outcome of the clinical trial in which these participants were enrolled is testing the effect of intervention (immunotherapy vs BE diet) on

the development of sustained unresponsiveness to unheated egg. Furthermore, tolerance to BE is a phenotype associated with an increased rate of resolution of allergy to unheated egg. Thus there is evidence for a clinical difference between the 2 groups in development of tolerance to unheated egg, and our goal was to examine whether there was an immune basis for that difference.

There is also a question of the immune basis underlying the difference in clinical reactivity to BE. This is a question that we have not addressed directly here through assessment of immune reactivity to BE *in vitro*. We and others have previously examined how heating of egg influences clinical reactivity by using *in vitro* model systems and *in vivo* mouse models.^{1,10,13-15} These previous findings are summarized in Fig 5, A. Heating destroys conformational epitopes, makes antigens more susceptible to degradation by digestive enzymes, and suppresses uptake across intestinal

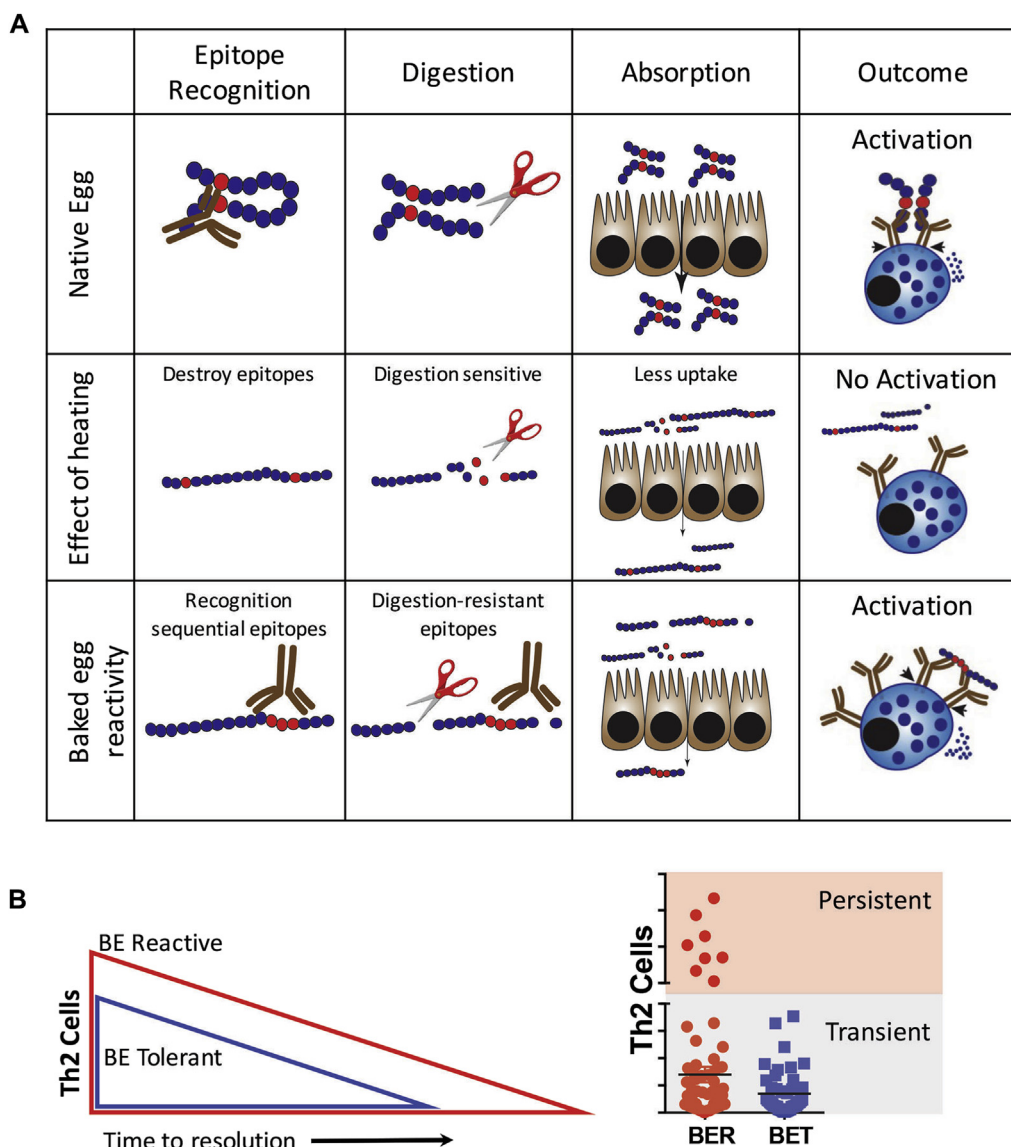


FIG 5. **A**, Mechanisms contributing to clinical reactions to BE. Native egg antigens that contain IgE-binding epitopes, survive digestion, and are absorbed across the epithelial barrier can trigger effector cells in patients with egg allergy. Heating destroys conformational epitopes, increases susceptibility to digestion, and reduces uptake across the epithelium, resulting in no effector cell activation in BE-tolerant subjects. BE-reactive subjects recognize sequential epitopes in digestion-resistant regions of egg antigens. Furthermore, increased levels of egg-specific IgE decrease the reaction threshold, resulting in effector cell activation. **B**, Hypothesized relationship between egg-specific T_H2 cell frequency and resolution of egg allergy. *Left*, If resolution is dependent on a loss of egg-specific T_H2 cells and the rate of T_H2 cell loss is similar between BE-reactive and BE-tolerant subjects, a higher starting value in the BE-reactive group would be associated with delayed resolution. *Right*, A high T_H2 frequency can be associated with persistence of T_H2 cells and egg allergy. The finding of high T_H2 frequency only in BE-reactive subjects would confer an increased persistence of egg allergy in this group. *BER*, BE-reactive subjects; *BET*, BE-tolerant subjects.

epithelium, leading to suppression of immune activation. Mechanisms that contribute to maintained reactivity to heated egg in BE-reactive subjects include IgE recognition of linear epitopes that are maintained after heat denaturation and recognition of epitopes that are not destroyed by means of digestion. Furthermore, as we show here, BE-reactive subjects had increased egg- and component-specific IgE levels and a lower threshold of reactivity of basophils to egg, indicating that they are more likely to respond despite the lower amount of antigen that can survive digestion and be absorbed intact across the epithelial barrier (Fig 5, A).

We observed no significant differences in proportions of peripheral blood immune subsets between those who were reactive or tolerant to BE. In a previous study using a subset of this cohort, we identified differences between groups in the transcriptional response to egg stimulation.¹⁶ We found that genes associated with virally infected dendritic cells, type I interferons, and IFN- γ were expressed differentially between BE-tolerant and BE-reactive participants. Here we did not find any numeric difference in numbers of dendritic cells or other innate cells, but there might be functional differences

within the innate populations that differ between egg allergy phenotypes.

This is the first phenotypic analysis of the T-cell response to egg using CD154-based flow cytometric profiling, an approach that minimizes time in culture while identifying functional parameters, such as cytokine production and homing receptor expression. Egg-responsive T cells were T_H2 skewed, with an absence of detectable T_H1 cells and only low frequencies of IL-10⁺ cells. This is consistent with T_H2 skewing identified by using proliferation-based approaches.¹⁷ Although we do not have egg-tolerant control subjects in this study, we have previously shown that T_H2 -related and proinflammatory cytokine secretion (IL-5, IL-9, and TNF- α) is significantly increased in patients with egg allergy compared with atopic control subjects who were egg tolerant.¹⁶ In addition to their T_H2 profile, egg-responsive T cells were enriched for homing receptors, which would support trafficking to the skin or intestinal mucosa or to the B-cell follicle. The phenotype of egg-responsive T cells was similar to that described for peanut by using a similar profiling approach,¹⁸ with the exception of CD25, which was more highly expressed on egg-responsive T cells. We also identified a population of Foxp3⁺CD25⁺CD127^{low} Treg cells expressing CD154 after 18 hours of egg allergen exposure. We hypothesized that there would be differences in the egg-responsive T_H2 and Treg cell profiles when comparing BE-reactive and BE-tolerant participants because BE tolerance has been associated with an increased frequency of egg allergy resolution.⁷ Tolerance to extensively heated forms of milk, which is associated also with an increased rate of allergy resolution, was associated with an increased frequency of milk-responsive Treg cells.¹⁹ However, T_H2 and Treg cell responses were not statistically different when comparing those with reactivity or tolerance to BE. The similarity in the distribution of egg-reactive Treg cells in the BE-reactive and BE-tolerant participants suggests that a deficit in Treg cell numbers is unlikely to explain the increased IgE response in the BE-reactive participants, although the possibility of a difference in Treg cell function cannot be excluded. Interestingly, there was a subset of subjects with a high frequency of egg-responsive T_H2 cells that was observed only in the BE-reactive group. We speculate that these high T_H2 responders might be more resistant to resolution of their egg allergy. In Fig 5, B, we speculate on the relationship of T_H2 responses to the rate of egg allergy resolution in the BE-reactive and BE-tolerant groups. In follow-up studies of this cohort, it will be of interest to determine how the “ T_H2 -high” participants respond to egg immunotherapy.

In summary, an examination of antibody and T-cell responses to egg demonstrated that BE-reactive participants have significantly higher egg-specific IgE levels and basophil responses, but T_H2 and Treg cell responses to egg were not different when comparing these 2 clinical phenotypes. A limitation of the study is the reductionist approach of *in vitro* activation that does not incorporate important factors, such as gastrointestinal digestion or absorption of antigens known to be influenced by heating of egg. We examine the immune basis for differences in reactivity to unheated egg, which is the basis of the clinical trial, but not the immune mechanisms responsible for differences in reactivity to BE. Furthermore, greater resolution at the level of epitope-specific immune responses might be needed to discriminate between these phenotypes of egg allergy. The data emphasizes the considerable heterogeneity of egg-specific antibody and T-cell

responses in this cohort; it will be of significant interest to determine whether the immune heterogeneity observed at baseline has clinical implications in follow-up studies.

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Key messages

- BE reactivity is associated with increased egg- and egg component-specific IgE antibodies, as well as basophil activation compared with BE tolerance.
- Egg-responsive T cells in the peripheral blood of patients with egg allergy are dominated by IL-4 and IL-13 expression and express the skin and mucosal homing receptors CCR4 and CCR6.
- Frequency of egg-responsive T_H2 and Treg cells did not differ between BE-reactive and BE-tolerant subjects, but a subset of subjects with increased frequency of egg-responsive T_H2 cells was observed within the BE-reactive group.

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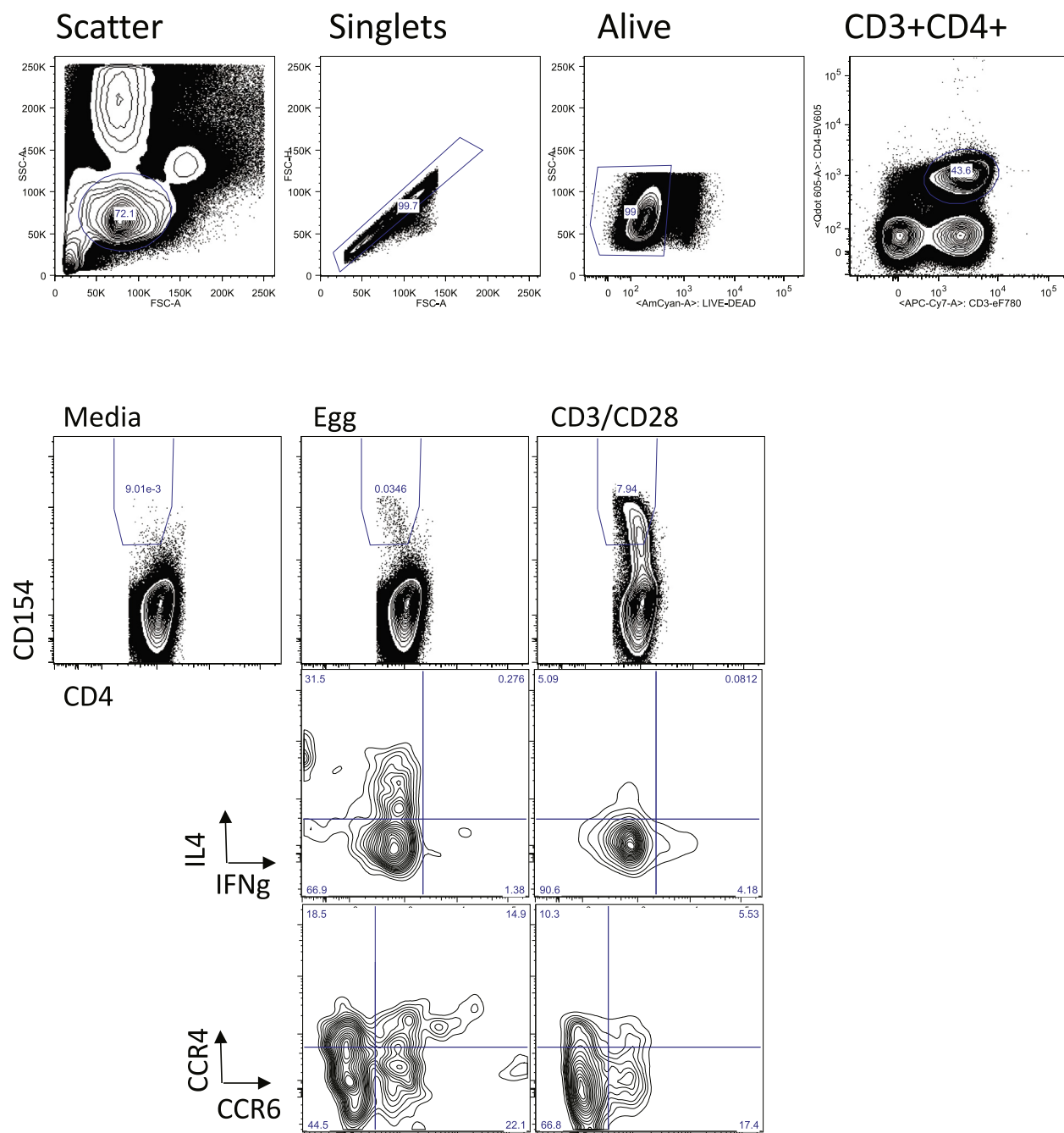


FIG E1. Gating strategy (*top row*) and representative flow cytometric results showing CD154 expression of stimulation with egg, medium (negative control), or anti-CD3/CD28 (positive control). *Bottom panels* show expression of IL-4 and IFN- γ or CCR4 and CCR6 in CD154⁺ cells after stimulation with egg or anti-CD3/CD28. FSC, Forward scatter; SSC, side scatter.

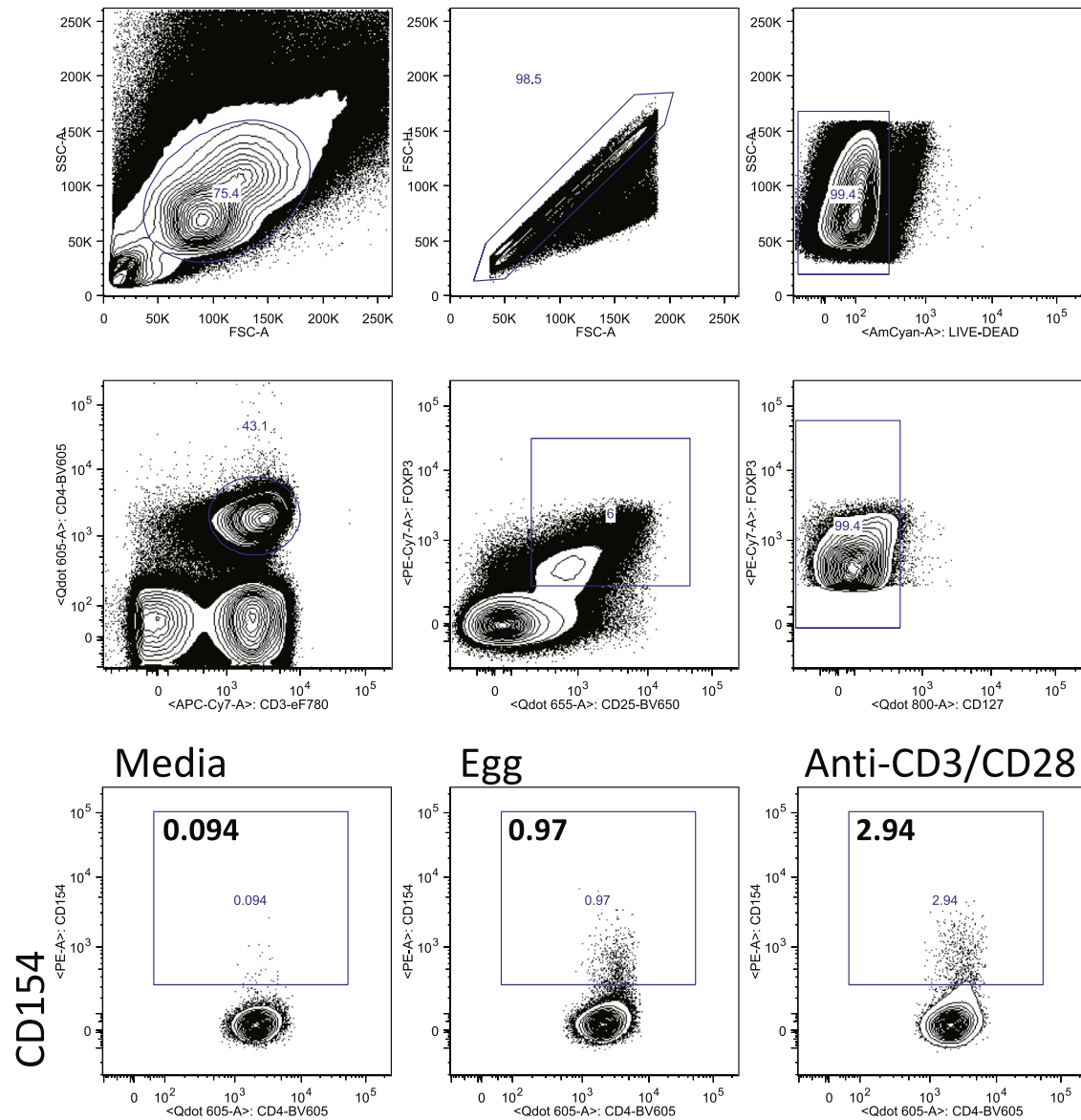


FIG E2. Gating strategy for identification of Treg cells ($CD3^{+}CD4^{+}Foxp3^{+}CD25^{+}CD127^{low}$) and representative flow cytometric results showing CD154 expression in gated Treg cells after stimulation with egg, medium (negative control), or anti-CD3/CD28 (positive control). *FSC*, Forward scatter; *SSC*, side scatter.

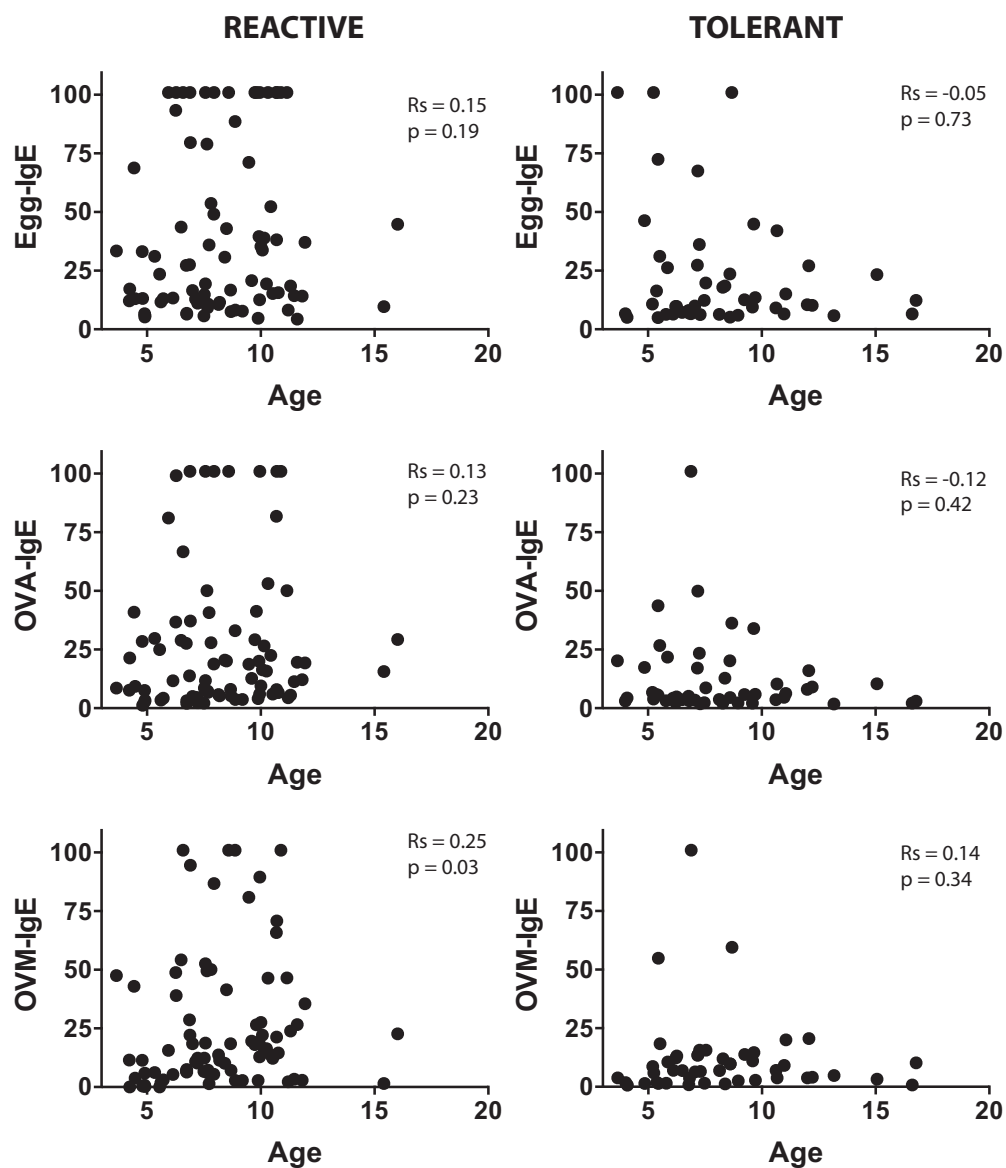


FIG E3. Egg-specific IgE, ovalbumin (OVA)-specific IgE, and ovomucoid (OVM)-specific IgE versus age of participant in the BE-reactive and BE-tolerant groups. Spearman correlation coefficient (r) and P value are provided on each graph.

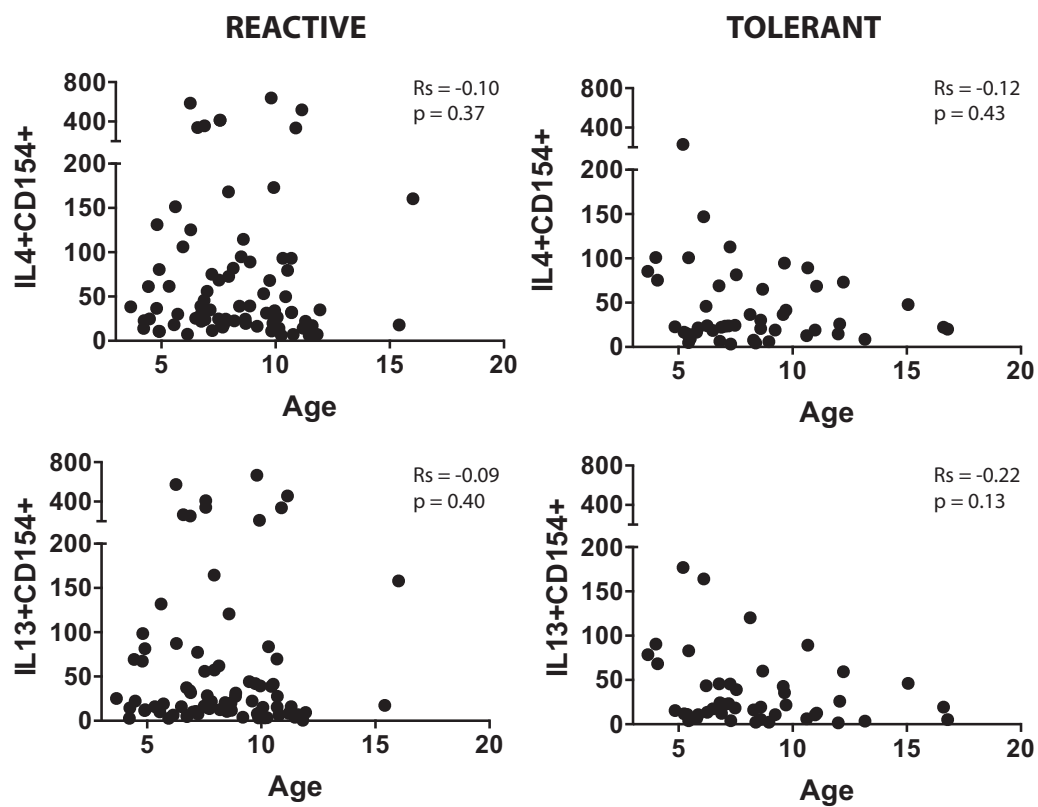


FIG E4. Frequency of CD154⁺IL-4⁺ and CD154⁺IL-13⁺ cells after egg stimulation versus age of participants in the BE-reactive and BE-tolerant groups. Spearman correlation coefficient (r) and P value are provided on each graph.

TABLE E1. Whole-blood phenotyping panel

Target	Clone	Fluorophore	Company
CD45	H130	Pacific Orange	Invitrogen, Carlsbad, Calif
CD3	UCHT1	AF700	BD Biosciences, San Jose, Calif
CD4	rpa-t4	PE-CF594	BD Biosciences
CD8	SK1	APC	BioLegend, San Diego, Calif
CD19	HIB19	PE	BioLegend
CD56	HCD56	BV605	BioLegend
CD14	HCD14	PE-Cy7	BioLegend
CD16	3G8	APC-H7	BD Biosciences
HLADR	LN3	AF488	BioLegend
CD123	6H6	BV650	BioLegend
CD11c	3.9	Pacific Blue	BioLegend

AF, Alexa Fluor; APC, allophycocyanin; BV, Brilliant Violet; PE, phycoerythrin.

TABLE E2. Basophil activation staining panel

Marker	Clone	Fluorophore	Company
CD3	UCHT1	APC	BD Biosciences, San Jose, Calif
CD14	M5E2	APC	BD Biosciences
CD19	HIB19	APC	BD Biosciences
CD41a	HIP8	APC	BD Biosciences
HLA-DR	L243	PE-Cy7	BD Biosciences
CD123	9F5	PE-Cy5	BD Biosciences
CD63	557288	FITC	BD Biosciences
CD203c	97A6	PE	Beckman Coulter Life Sciences, Fullerton, Calif

APC, Allophycocyanin; *FITC*, fluorescein isothiocyanate; *PE*, phycoerythrin.

TABLE E3. Effector T-cell staining panel (6 hours)

Marker	Clone	Fluorophore	Company
Live/Dead Aqua	NA	AmCyan	Thermo Fisher Scientific, Waltham, Mass
CD3	SK7	APC-Cy7	eBioscience, San Diego, Calif
CD4	OKT4	Brilliant Violet 605	BioLegend, San Diego, Calif
CD25	BC96	Brilliant Violet 650	BioLegend
CD127	A019D5	Brilliant Violet 785	BioLegend
CXCR5	RF8B2	Alexa Fluor 488	BD Biosciences
CCR4	1G1	PerCP-Cy5.5	BD Biosciences
CCR6	11A9	PE-Cy7	BD Biosciences
CD154	24-31	PE	eBioscience
IFN- γ	B27	Alexa Fluor 700	BD Biosciences
IL-4	MP4-25D2	Alexa Fluor 647	BioLegend
IL-10	JES3-19F1	PE-CF594	BD Biosciences
IL-13	JES10-5A2	BD Horizon V450	BD Biosciences

APC, Allophycocyanin; NA, not applicable; PE, phycoerythrin.

TABLE E4. Treg cell staining panel (18 hours)

Marker	Clone	Fluorophore	Company
Live/Dead Aqua	NA	AmCyan	Thermo Fisher Scientific, Waltham, Mass
CD3	SK7	APC-Cy7	eBioscience, San Diego Calif
CD4	OKT4	Brilliant Violet 605	BioLegend, San Diego, Calif
CD25	BC96	Brilliant Violet 650	BioLegend
CD127	A019D5	Brilliant Violet 785	BioLegend
CCR4	1G1	PerCP-Cy5.5	BD Biosciences, San Jose, Calif
CCR6	11A9	PE-Cy7	BD Biosciences
CCR9	112509	Alexa Fluor 488	BD Biosciences
CD154	24-31	PE	eBioscience
IFN- γ	B27	Alexa Fluor 700	BD Biosciences
IL-10	JES3-19F1	PE-CF594	BD Biosciences
FoxP3	206D	Alexa Fluor 647	BioLegend

APC, Allophycocyanin; NA, not applicable; PE, phycoerythrin; PerCP, peridinin-chlorophyll protein complex.