Oral immunotherapy induces IgG antibodies that act through FcγRIIb to suppress IgE-mediated hypersensitivity

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Background: Food-induced anaphylaxis is triggered by specific IgE antibodies. Paradoxically, some subjects with significant IgE levels can ingest allergenic foods without incident. Similarly, subjects completing oral immunotherapy (OIT) tolerate food challenges despite persistent high-titer food-specific IgE.
Objective: We sought to test whether IgG antibodies induced by food immunotherapy prevent food-induced anaphylaxis and whether this occurs through the inhibitory receptor FcγRIIb.
Methods: Food allergy–susceptible *Il4raF709* mice were enterally sensitized to ovalbumin (OVA). Similarly sensitized IgE-deficient ($IgE^{-/-}$) *Il4raF709* mice, which can ingest OVA without anaphylaxis, were subjected to a high-dose enteral OVA desensitization protocol (OIT). Sera from both groups were tested for the ability to activate or inhibit bone marrow mast cells (BMMCs) exposed to allergen or to passively transfer allergy to naive hosts. In parallel experiments sera obtained from patients with peanut allergy before and after undergoing OIT were interrogated for their ability to enhance or suppress peanut-induced activation in an indirect assay by using basophils from nonallergic donors.
Results: *Il4raF709* mice exhibited strong OVA-specific IgE responses. Their sera efficiently sensitized BMMCs for activation by antigen challenge. Sera from *Il4raF709*/$IgE^{-/-}$ mice subjected to OVA OIT suppressed BMMC responses. This inhibition was IgG mediated and FcγRIIb dependent. Similarly, pre-OIT but not post-OIT sera from patients efficiently sensitized basophils for peanut-induced activation. IgG antibodies in post-OIT sera suppressed basophil activation by pre-OIT sera. This inhibition was blocked by antibodies against FcγRII.
Conclusion: Food-specific IgG antibodies, such as those induced during OIT, inhibit IgE-mediated reactions. Strategies that favor IgG responses might prove useful in the management of food allergy. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: *Food allergy, tolerance, anaphylaxis, IgE, desensitization, oral immunotherapy*

Food allergy is a growing health problem affecting children and adults worldwide.[1] Clinical evaluation for this disorder centers on assessment of food allergen–specific IgE antibodies, and anaphylaxis arises when these antibodies activate mast cells and basophils through FcεRI. However, the correlation between allergen-specific IgE levels and susceptibility to food-induced reactions is imperfect.[2,3] This inconsistency might be due to other immune parameters that are not usually evaluated, such as food-specific IgG antibodies or food-specific cellular immunity, that might protect against or suppress food-induced reactions, including anaphylaxis, the most significant manifestation of food allergy. A more complete understanding of these additional parameters affecting food sensitivity and tolerance is needed to enhance the evaluation and care of these patients.

A large body of evidence suggests that food-specific IgG antibodies might play a major role in protection against food-induced reactions. Injection immunotherapy to aeroallergens is known to induce allergen-specific IgG_4 antibodies, which have been postulated to function by blocking allergen interaction with FcεRI-bound IgE.[4] A similar mechanism might be operative in food allergy. Recovery from milk protein allergy has been associated with increased IgG levels,[5,6] and Caubet et al[7] reported that egg protein–specific IgG_4 antibodies are predictive of a successful baked egg challenge result. In patients successfully completing oral immunotherapy (OIT) or sublingual immunotherapy, desensitization is also associated with a strong food-specific IgG antibody response. Skripak et al[8] demonstrated that patients undergoing milk OIT exhibited no change in milk-specific IgE levels but had a marked induction of milk-specific IgG_4. Similarly, we found that OIT to milk, facilitated by a short course of omalizumab treatment, resulted in a 15-fold induction of

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Abbreviations used
BMMC: Bone marrow mast cell
LAMP-1: Lysosomal-associated membrane protein 1
OD: Oral desensitization
OIT: Oral immunotherapy
OVA: Ovalbumin
SCIT: Subcutaneous immunotherapy
TNP: Trinitrophenyl

milk-specific IgG_4.[9] Burks and colleagues reported similar increases in food-specific IgG_4 levels in patients receiving egg and peanut OIT and sublingual immunotherapy.[10-13] Although the association between IgG induction and food desensitization suggests a mechanistic effect, a suppressive function of the IgG antibodies induced in these clinical allergic responses has not been directly demonstrated.

In this study we evaluated the suppressive functions of IgG antibodies induced during food allergen desensitization using both experimental mouse models of food-induced anaphylaxis and analysis of sera from patients successfully completing peanut OIT.

METHODS

Sensitization of mice

Mice used in this study are described in the Methods section in this article's Online Repository at www.jacionline.org. For sensitization, *Il4raF709* and *Il4raF709*/IgE-deficient ($IgE^{-/-}$) mice were treated intragastrically with sterile PBS or 250 μg of ovalbumin (OVA; Sigma-Aldrich, St Louis, Mo) plus 20 μg of cholera toxin (List Biological Laboratories, Campbell, Calif) once a week for 3 weeks. Mice were challenged with 150 mg of OVA administered intragastrically to induce anaphylaxis. Some *Il4raF709*/$IgE^{-/-}$ mice were subsequently subjected to 3 weeks of daily desensitization therapy with 100 mg of OVA administered intragastrically.

Study population

To probe the induction of suppressive IgG antibodies in human subjects undergoing OIT, we used sera from our recently published trial of peanut OIT in high-risk patients with peanut allergy.[14] Thirteen patients with IgE-mediated peanut allergy and histories of significant symptoms, including urticaria, vomiting, and/or anaphylaxis, were enrolled in the study. The subjects included 8 boys and 5 girls, ranging in age from 8 to 16 years. The median peanut-specific IgE level was 229 kU/L. All children had a skin prick test wheal of at least 8.5 mm to peanut extract and failed an initial double-blind, placebo-controlled food challenge on week 0 at peanut protein doses of 50 mg or less (administered as peanut flour). After 12 weeks of pretreatment with omalizumab, patients were started on OIT, and 12 of 13 reached 2000 mg of peanut protein on week 32. Patients continued their daily ingestion of 2000 to 4000 mg of peanut protein daily. Sera used on this study were taken from patients on weeks 0 and 52. The Institutional Review Board at Boston Children's Hospital approved the clinical protocol, and written informed consent from all participants, their parents, or both was obtained before participation in the study. The trial was registered on ClinicalTrials.gov (NCT01290913).

Human basophil activation analysis

Basophil activation tests were performed with the Flow CAST Basophil Activation Test kit (Bühlmann Laboratories, Schönenbuch, Switzerland) per the manufacturer's instructions. Briefly, 50-μL aliquots of whole blood from patients without peanut allergy were sensitized with 20% pre- or post-OIT patient sera, IgG-depleted sera, eluted IgG fraction equal to original serum levels, and/or anti-CD32 antibody (2 μg/mL, FUN-2; BioLegend, San Diego, Calif) in 100 μL of basophil stimulation buffer at 37°C for 2 hours. Anti-FcεRI mAb was used as a positive control for basophil activation. Samples were incubated for 10 minutes at 37°C with 2 μg/mL complete peanut extract and Flow CAST staining reagent (including CCR3 and CD63). After erythrocyte lysis, cells were washed and subjected to flow cytometry. Basophils were identified as side scatter–low $CCR3^+$ cells, and activation was measured based on CD63 expression. In independent control experiments cells in this gate were confirmed to be uniformly $CD123^+CD3^-CD19^-Fc\varepsilon RI^+IgE^+c\text{-}Kit^-$. A minimum of 200 basophils was obtained for each sample. Peripheral blood from adult donors without peanut allergy was obtained with approval from the Institutional Review Board of Boston Children's Hospital.

Statistics

Statistical analyses were performed with Prism GraphPad Version 5.0f (GraphPad Software, San Diego, Calif). Unpaired *t* tests or ANOVA with Bonferroni posttests were used for comparisons between unlinked groups, including mouse bone marrow mast cell (BMMC) experiments and data from pooled patient sera. Repeated-measures ANOVA with Bonferroni posttests was used to analyze data from basophil activation tests of individual patients. Repeated-measures 2-way ANOVA was applied to anaphylaxis data. Because peanut-specific IgG values were spread across several orders of magnitude, the data were transformed by taking the logarithm before statistical analysis with paired *t* tests.

RESULTS

IgG antibodies generated during food allergen ingestion inhibit IgE-mediated mast cell activation and systemic anaphylaxis

We have previously used a genetic approach, targeted insertion of an activated form of the IL-4 receptor α-chain, to develop *Il4raF709* mice that are both susceptible to enteral allergen sensitization and capable of mounting robust systemic anaphylaxis on ingestion challenge.[15] Food-induced anaphylaxis in these animals is completely IgE dependent: neither $IgE^{-/-}$ nor $Fc\varepsilon RI\alpha^{-/-}$ animals carrying the *Il4raF709* allele exhibit allergic responses after ingestion challenge. We reasoned that IgE-deficient $IgE^{-/-}$/*Il4raF709* mice, which retain intact IgG antibody responses, would be useful for analysis of the biological effects of IgG antibodies generated in response to high-dose food allergen ingestion in an oral desensitization (OD) protocol.

$IgE^{-/-}$/*Il4raF709* and *Il4raF709* mice were enterally sensitized to OVA by means of low-dose gavage weekly over 3 weeks. Challenge was performed by using OVA gavage 1 week after the last sensitizing dose, and anaphylaxis was assessed by measuring core body temperature with implanted thermal transponders. As expected, *Il4raF709* but not $IgE^{-/-}$/*Il4raF709* mice enterally sensitized to OVA displayed robust anaphylactic responses, with intense and sustained temperature decreases and 2 deaths after oral OVA challenge (Fig 1, *A*). This anaphylactic response in *Il4raF709* animals was accompanied by a large increase in plasma levels of the mast cell–specific protease murine mast cell protease 1, which is indicative of intense mast cell activation (Fig 1, *B*).[16] No evidence of mast cell activation was detected in $IgE^{-/-}$/*Il4raF709* mice. Although OVA-sensitized $IgE^{-/-}$/*Il4raF709* mice exhibited evidence of immune sensitization, including OVA-specific T_H2 responses and mast cell expansion, they had no anti-OVA IgE (see Fig E1 in this article's Online Repository at www.jacionline.org and data not shown).

Because $IgE^{-/-}$/*Il4raF709* mice tolerated the oral OVA challenge, they were then treated with high enteral doses of OVA daily for an additional 3 weeks. We reasoned that OD in an IgE-free

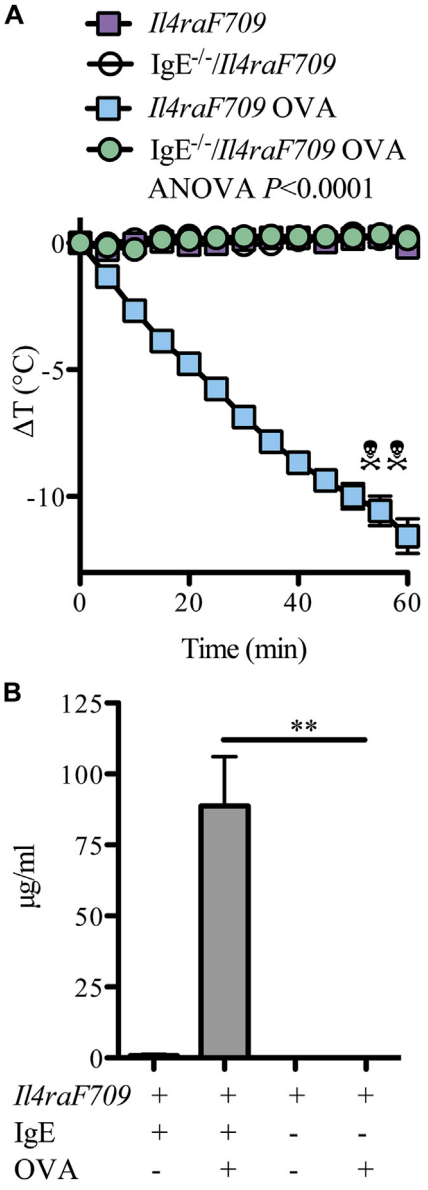

FIG 1. Food allergen–induced anaphylaxis and mast cell activation are IgE dependent. **A,** Systemic anaphylaxis (core body temperature) after enteral challenge of OVA-sensitized *Il4raF709* and IgE$^{-/-}$/*Il4raF709* mice. ☠Death. *ΔT*, Change in temperature. **B,** Plasma levels of mouse mast cell protease 1 (n = 5-8). Data are representative of 3 or more experiments. $^{**}P < .01$.

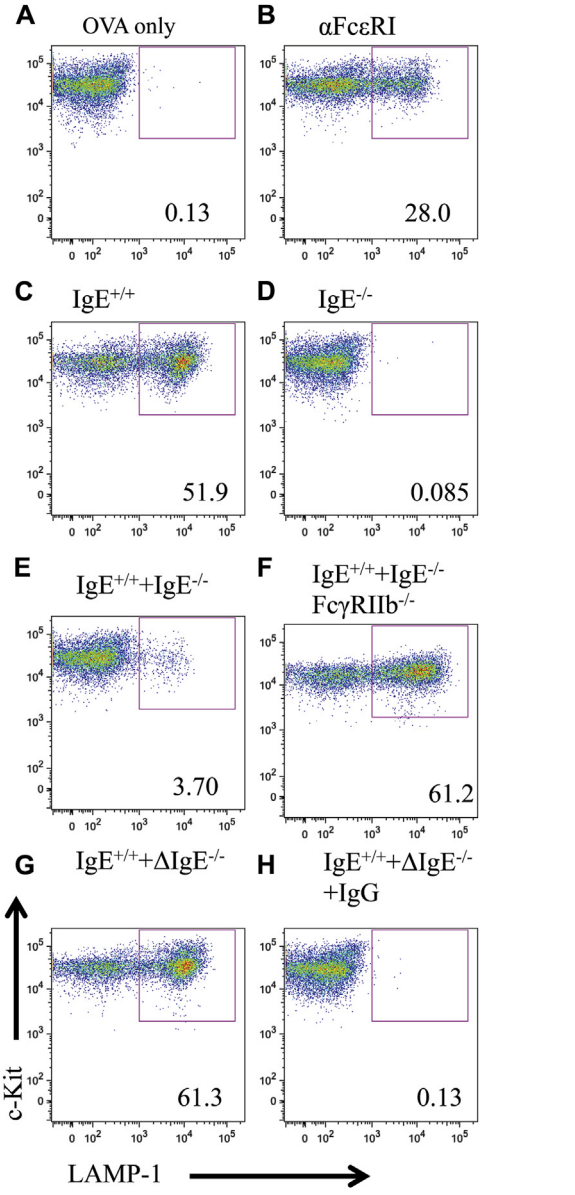

FIG 2. Antigen-induced activation of mast cells sensitized with sera of OVA-fed mice. OVA-induced LAMP-1 on serum-sensitized BMMCs is shown. **A,** No serum. **B,** Anti-FcεRI control. **C,** IgE$^{+/+}$/*Il4raF709* serum. **D,** High-dose OVA-fed IgE$^{-/-}$/*Il4raF709* serum. **E,** IgE$^{+/+}$ plus IgE$^{-/-}$ sera. **F,** FcγRIIb$^{-/-}$ BMMCs treated as in Fig 2, *E*. **G,** Wild-type BMMCs and IgE$^{+/+}$ plus IgG-depleted IgE$^{-/-}$ sera. **H,** As in Fig 2, *G*, but with IgG added back. Representative plots from one of 5 experiments are shown.

system would be similar to human OIT performed under cover of omalizumab.[14,17] The ability of pooled sera from IgE$^{+/+}$/*Il4raF709* or high-dose OVA-treated IgE$^{-/-}$/*Il4raF709* mice to sensitize mast cells was assessed by using BMMCs from wild-type mice. Sensitized BMMCs were challenged *in vitro* with OVA, and activation was detected by measuring surface expression of lysosomal-associated membrane protein 1 (LAMP-1), a sensitive indicator of granule extrusion.[18,19] Data are presented both as flow cytometric plots for a single representative experiment (Fig 2) and as mean values for replicate activation assays (see Fig E2 in this article's Online Repository at www.jacionline.org). Exposure of BMMCs to OVA alone had no effect (Fig 2, *A*), whereas treatment with an anti-FcεRI antibody induced expression of LAMP-1 (Fig 2, *B*). Consistent with the anaphylaxis results *in vivo*, IgE$^{+/+}$ sera sensitized BMMCs for robust activation to OVA (51.2% ± 1.99% LAMP-1^{+}), whereas IgE$^{-/-}$ sera conferred no OVA sensitivity either before or after desensitization (Fig 2, *C* and *D*; see Fig E2; and data not shown). OVA challenge of BMMCs sensitized with a mixture of sera from IgE$^{-/-}$ and IgE$^{+/+}$ animals resulted in a strikingly weak response (4.12% ± 0.958%; Fig 2, *E*, and see Fig E2), a finding that indicated that sera from high-dose OVA–fed IgE$^{-/-}$ mice not only failed to sensitize mast cells for activation but also exerted a suppressive effect.

The hypothesis that IgG antibodies mediated the observed inhibition was tested by means of sensitization of BMMCs

with the same sera after IgG depletion. Removal of IgG from IgE$^{-/-}$ sera abrogated the suppressive activity (Fig 2, *G*, and see Fig E2). Replacement of the IgG fraction with equivalent amounts of purified IgG prepared from the same sera fully restored suppression (Fig 2, *H*, and see Fig E2). Taken together, these results establish that IgE antibodies drive mast cell activation and anaphylaxis in *Il4raF709* mice and that IgG antibodies produced after allergen ingestion exert a suppressive function.

IgG-mediated inhibition of food allergen–induced mast cell activation and anaphylaxis occurs through an FcγRIIb-dependent mechanism

Two non–mutually exclusive models have been proposed whereby IgG antibodies might inhibit IgE-mediated mast cell activation and hypersensitivity responses.[20-22] In the first putative mechanism IgG antibodies exert a "blocking" function, binding allergens in the extracellular milieu, masking their epitopes, and preventing their recognition by IgE antibodies. In an alternative model IgG antibodies bound to Fcγ receptors bind to the same allergenic proteins as FcεRI-bound IgE, resulting in delivery of an inhibitory signal. To discriminate between these mechanisms, we used FcγRIIb$^{-/-}$ mice, as well as BMMCs cultured from the same animals. Consistent with a role for FcγRIIb, sensitization of FcγRIIb$^{-/-}$ BMMCs with a mixture of sera from IgE$^{+/+}$ and IgE$^{-/-}$ mice resulted in strong activation (Figs 2, *G*, and 3, *A*). The suppressive effect of the IgE$^{-/-}$ serum was completely eliminated. We observed that the response of BMMCs sensitized with only IgE$^{+/+}$ serum was in fact enhanced in FcγRIIb$^{-/-}$ BMMCs, suggesting that elimination of inhibitory signals provided by IgG antibodies contained in this same serum triggered amplified degranulation.

A dose-response analysis of the ability of IgG to inhibit wild-type versus FcγRIIb$^{-/-}$ BMMC activation with trinitrophenyl (TNP)-specific mAbs revealed that, at high doses, inhibition could be achieved even in BMMCs lacking inhibitory IgG receptors, suggesting some contribution of a steric blocking effect (Fig 3, *B*). However, the IgG concentration required to mediate 50% inhibition of IgE-induced activation was greater than 10-fold higher for FcγRIIb$^{-/-}$ BMMCs, indicating that most of the inhibitory effect is mediated at the level of the Fc receptor.

To test the relevance of FcγRIIb-mediated inhibition of IgE-induced mast cell activation to anaphylactic responses *in vivo*, we passively sensitized wild-type or FcγRIIb$^{-/-}$ mice with sera from IgE$^{+/+}$ mice, IgE$^{-/-}$ mice, or both enterally sensitized to OVA. OVA challenge of both wild-type and FcγRIIb$^{-/-}$ recipients of IgE$^{+/+}$ serum resulted in robust anaphylactic responses (Fig 3, *C*). In contrast, wild-type, but not FcγRIIb$^{-/-}$, recipients of IgE$^{+/+}$ plus IgE$^{-/-}$ sera exhibited significant protection from hypothermia, a finding supportive of FcγRIIb-dependent IgG-mediated suppression of responses to OVA challenge in patients with systemic anaphylaxis.

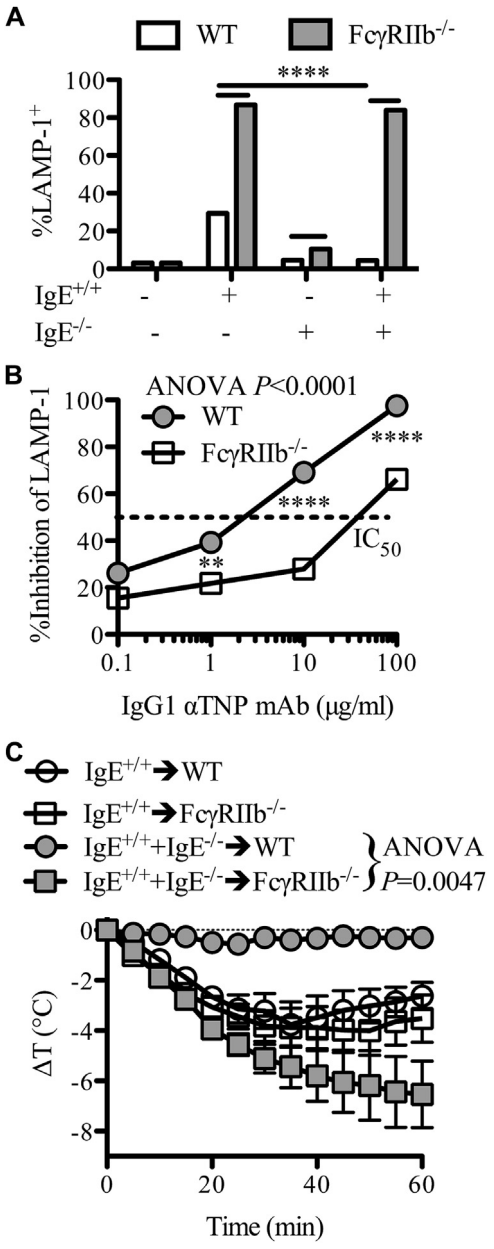

FIG 3. IgG inhibits mast cell activation through FcγRIIb. **A,** OVA-induced activation of wild-type *(WT)* or FcγRIIb$^{-/-}$ BMMCs sensitized with IgE$^{+/+}$ or high-dose OVA–fed IgE$^{-/-}$ sera. **B,** Monoclonal antibody–sensitized BMMCs activated with TNP. Values are presented as means ± SEMs of one of 3 or more experiments. **C,** Anaphylaxis (core temperature) in OVA-challenged WT or FcγRIIb$^{-/-}$ recipients of IgE$^{+/+}$ with or without high-dose OVA–fed IgE$^{-/-}$ sera. *ΔT,* Change in temperature. Values are presented as means ± SEMs (n = 3-5) of one of 2 experiments. ****$P < .0001$.

Detection of inhibitory antibodies in the sera of subjects undergoing peanut OIT

We have recently reported the results of peanut OIT performed under cover of omalizumab in a group of 13 high-risk subjects.[14] Patients had a median peanut-specific IgE level of 229 kU$_A$/L and reacted to peanut flour challenge doses of 100 mg or less (Table I). Twelve patients eventually reached a dose of 4000 mg of peanut flour over a median time of 8 weeks. We reasoned that these highly peanut-sensitive subjects, all of whom successfully ingested significantly more peanut after OIT, would provide an ideal group in which to assess the hypothesis that inhibitory IgG antibodies are induced in the course of OIT.

Pre-OIT sera (W0) and post-OIT sera (W52) were used for this analysis. At the 52-week time point, subjects had been off omalizumab for 8 months (>10 elimination half-lives for omalizumab). Two of 13 patients withdrew from the study and were not included in our evaluation. Because the ability to detect

TABLE I. Clinical characteristics of enrolled subjects

Subject	Age (y)	Sex	W0 peanut-specific IgE (kU_A/L)	W52 peanut-specific IgE (kU_A/L)	W0 skin test wheal (mm)	W52 skin test wheal (mm)
1	8	M	436	85	12.5	11.5
2*	8	M	58	38.2	20.5	8
3	9	F	617	578	15	5
5	14	M	150	62.1	16.5	7
6	14	M	229	84.1	10.5	3.5
8	14	F	290	>100	24	8.5
9*	7	M	65	17	9.5	5.5
10	10	F	327	61.7	9.5	6
11*	11	F	21	16.5	8.5	4
12	12	F	307	42.9	24.5	6
13	8	M	172	92.5	18	Not done

Of the 13 subjects initially enrolled in the study, subjects 4 and 7 withdrew before completion of the 52-week protocol. Their clinical information is not presented here.
F, Female; *M*, male.
*Subjects not included in basophil activation analysis secondary to less than 30% basophil activation by W0 patient serum.

suppressive activity in post-OIT sera was dependent on a minimum 30% basophil activation by pretreatment sera, we additionally excluded 3 patients (Table I) for whom basophil activation decreased to less than this threshold.

Peanut OIT induces allergen-specific suppressive IgG antibodies that act through FcγRIIb

We evaluated peanut-specific IgG by subclass and observed significant increases in all of them (Fig 4). The more than 2-log increases in peanut-specific IgG_2 and IgG_4 were most striking, and in the face of decreasing IgE levels, the peanut IgG_4/IgE ratio increased by more than 3 logs. Significant increases were evident for IgG_1 and IgG_3 levels as well. Peanut-specific IgA levels also increased (see Fig E3 in this article's Online Repository at www.jacionline.org).

Basophil assays were used to analyze the effects of these peanut-specific antibodies on IgE-mediated activation. Previous investigations have demonstrated decreased basophil sensitivity to antigen challenge after peanut OIT.[23] To determine whether such hyporesponsiveness might be the result of alterations in the balance of activating versus inhibitory antibodies interacting with Fc receptors, we developed an indirect assay analogous to the BMMC system used in the murine model.

Basophils from healthy donors without peanut allergy (hence uniform in their intrinsic susceptibility to activation) were incubated with study sera and then exposed to peanut antigen. Expression of CD63, a granule membrane protein, which is rapidly induced by FcεRI signaling and closely linked to anaphylactic degranulation,[24] was used as an indicator of activation. In preliminary and control experiments, this assay consistently provided a unimodal increase in CD63 expression in all but 3 of the study patients, with negligible expression on resting cells or on cells exposed to peanut allergen but not sensitized by allergic serum. The ability of the sera to activate basophils was concentration dependent (see Fig E4 in this article's Online Repository at www.jacionline.org).

Basophils were incubated with the following combinations of sera before peanut stimulation and activation analysis: (1) pre-OIT (W0); (2) post-OIT (W52); (3) a mixture of pre-OIT and post-OIT (W0 + W52); and (4) W0 plus W52 plus anti-FcγRII antibody (W0 + W52 + αCD32). Data are presented for a representative patient (3, Fig 5), as well as for all subjects (Fig 6, *A*). Additional informative manipulations and controls were performed with pooled sera from all subjects tested (Fig 6, *B*) and individual patient sera (see Fig E5 in this article's Online Repository at www.jacionline.org). Exposure of donor basophils to peanut alone resulted in no appreciable activation (Fig 5, *A*). FcεRI cross-linking with a polyclonal serum induced 62% to 92.9% CD63 expression (Fig 5, *A*, and data not shown). Pre-OIT sera sensitized basophils for 63.33% ± 5.64% activation (Fig 6, *A*). W52 sera conferred markedly less activation (32.53% ± 6.06%), a finding that is striking in light of the fact that the W52 study subjects still had markedly increased peanut-specific IgE levels, with a median peanut-specific IgE level of 62.1 kU_A/L.

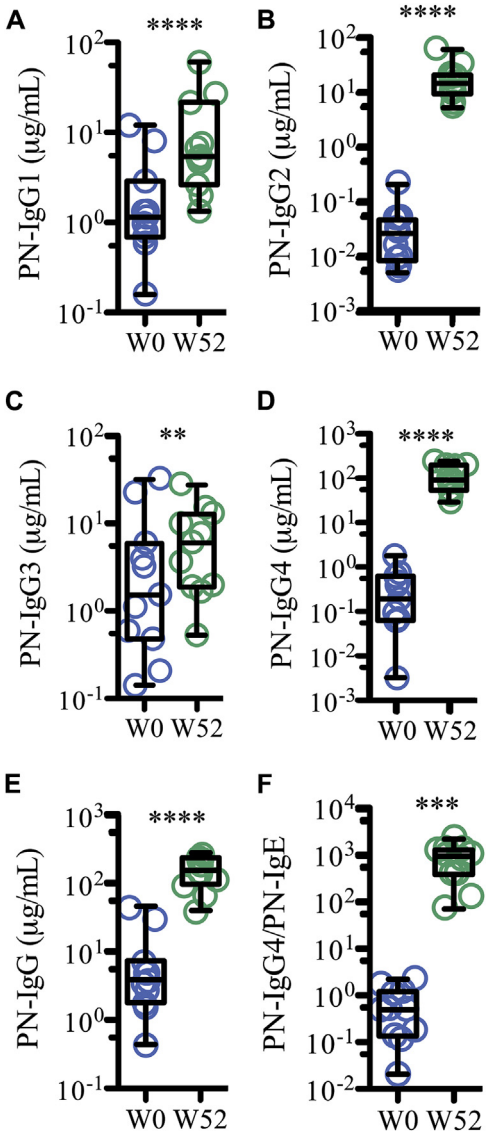

FIG 4. Peanut *(PN)*–specific IgG levels increase after OIT. ELISA analysis of peanut-specific IgG levels in serum samples taken before and after OIT: **A,** IgG_1; **B,** IgG_2; **C,** IgG_3; **D,** IgG_4; **E,** IgG (all isotypes); and **F,** IgG_4/IgE ratio. *Circles* represent individual patient values, with box plots overlaid. *W0,* Before OIT at week 0; *W52,* after OIT at 52 weeks. $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$.

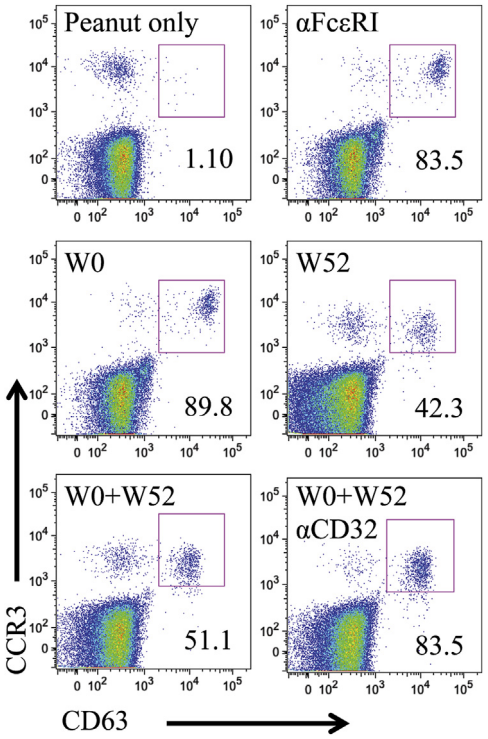

FIG 5. Peanut OIT induces IgG that inhibits basophil activation. Basophil activation was measured by means of flow cytometry in peanut-challenged whole-blood samples from a nonallergic donor. Basophils were sensitized with sera taken before or after OIT and stained for CD63. *αCD32*, αCD32 antibody; *W0*, before OIT at week 0; *W52*, after OIT at 52 weeks.

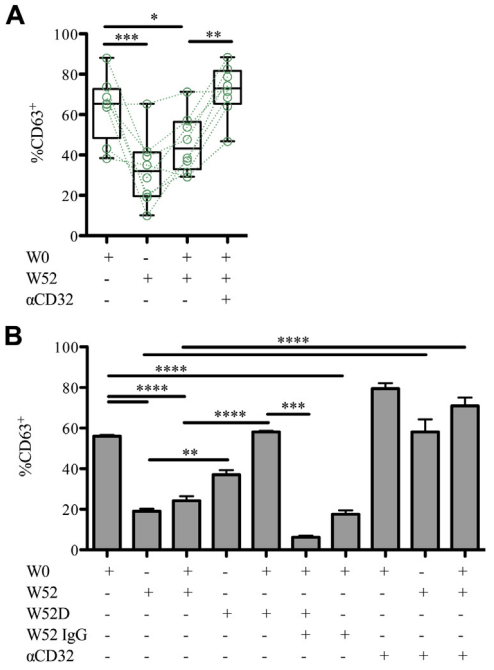

FIG 6. IgG inhibits basophil activation by allergic sera through CD32. **A,** Peanut-induced activation of basophils sensitized with serum from individual patients (n = 11), with box plots overlaid. **B,** CD63 upregulation in basophils sensitized with serum pooled from all patients. Values are presented as means ± SEMs of independent replicates (n = 4). *αCD32*, αCD32 antibody; *W0*, before OIT at week 0; *W52*, after OIT at 52 weeks; *W52D*, W52 depleted of IgG; *W52 IgG*, IgG purified from post-OIT serum. *$P < .05$, **$P < .01$, ***$P < .001$, and ****$P < .0001$.

When basophils were exposed to both the same amount of W0 serum used under the initial activating conditions and an additional equal amount of W52 serum, a marked reduction in responsiveness was observed (45.81% ± 5.10% vs 63.33% ± 5.64%, Figs 5 and 6), which is consistent with a suppressive activity within the W52 sera. This suppressive activity was lessened when W52 sera were passed over protein G Sepharose to remove IgG antibodies (Fig 6, *B*, and see Fig E5) and was restored ($P < .001$) by replacement of the IgG fractions. The suppression exerted by post-OIT sera was similarly ablated by addition of anti-CD32 antibodies. These findings provide strong evidence that peanut OIT induces suppressive IgG antibody responses that directly inhibit FcεRI-mediated basophil activation through an FcγRIIb-dependent mechanism.

DISCUSSION

Previous investigations have established that both natural loss of food allergen sensitivity and successful completion of OIT protocols are associated with induction of food allergen–specific IgG responses.[5-9,11-13] However, the functional effects of food-specific IgG antibodies induced during OIT on allergen/IgE-mediated hypersensitivity reactions have not been evaluated. This study provides evidence that food allergen administration, as occurs in OIT, drives IgG production and that the IgG antibodies formed during such responses can suppress IgE-mediated responses, including anaphylaxis.

We used both a mouse model system and sera from a human OIT trial to test the hypothesis that food-specific IgG antibodies act through FcγRIIb to inhibit FcεRI-mediated activation. Application of the murine model system allowed for highly consistent conditions of food allergen exposure in a genetically homogeneous population and for analysis of the effects of sera on primary mast cells. The use of FcγRIIb$^{-/-}$ mice and their mast cells permitted a direct genetic test of the contribution of this receptor to the alterations in mast cell responsiveness to pre-OD versus post-OD sera. A key novel observation in this study was that IgE$^{-/-}$*/Il4raF709* mice not only did not express anaphylaxis but that their sera, taken after repeated high-dose OVA gavage, also suppressed IgE-mediated anaphylaxis. This provides strong evidence that IgG antibodies generated in the course of allergen ingestion and acting through FcγRIIb can suppress anaphylactic reactions. However, it is important to note that IgE-deficient mice are not equivalent to omalizumab-treated patients. Unlike patients, the mice have lacked IgE throughout their lives, with possible effects on the development, homeostasis, and function of FcεRI^{+} cells, including mast cells and dendritic cells. Furthermore, the IgE$^{-/-}$ animals have absolutely no FcεRI-bound IgE, whereas omalizumab does not completely remove IgE from cellular receptors in patients.

Our studies on the high-risk patients with peanut allergy undergoing OIT indicate that the same mechanisms are operative in patients with food allergy and are therefore clinically relevant. These subjects exhibited marked increases in peanut-specific IgG antibody levels and were uniformly able to tolerate 160 to 400 times the dose tolerated before desensitization. This was despite the presence of peanut-specific IgE levels normally associated with a high risk of peanut reaction after desensitization at week 52

of the trial. On the basis of our CD32 blocking assays, this was likely due to the FcγRIIb-mediated suppression of IgE signals.

The function of IgG antibodies in modulating immune responses to allergens has been most extensively studied in the setting of subcutaneous immunotherapy (SCIT).[4,22] Almost 80 years ago, using Prausnitz-Küstner passive cutaneous skin testing analyses, Cooke et al[25] demonstrated that serum from patients completing immunotherapy contained a suppressive activity that inhibited passive sensitization by the "reagin" contained in pretreatment sera. This inhibitory factor was eventually identified as IgG, and the ability of immunotherapy preparations to induce IgG versus IgE responses has been used to guide the development of optimal extracts for SCIT.[26]

Lichtenstein et al[27] demonstrated a correlation between induction of blocking IgG and the reduction in allergen-specific IgE levels in patients undergoing SCIT. SCIT induces predominantly IgG_1 and IgG_4 responses, with IgG_4 responses becoming a focus of particular interest because of their very consistent induction, correlation with clinical improvement, and unique biochemical characteristics.[28] IgG_4 antibodies undergo immunoglobulin chain reassortment, generating chimeric structures with dual specificity, a property that prevents immune complex formation.[29] However, it is notable that IgG_4 antibodies have a relatively low binding affinity for FcγRIIb,[30] suggesting that the OIT-induced suppressive IgG activity observed in our study might be mediated by another isotype or isotypes. We detected significant increases in all 4 IgG subclasses, and additional studies will be required to assess whether they exert differential contributions to inhibit IgE responses. We also observed significant increases in peanut-specific IgA levels after OIT, and it is possible that IgA contributes to the inhibitory effect of post-OIT serum through allergen neutralization.

The ability of IgG antibodies to suppress IgE-mediated anaphylaxis *in vivo* has previously been demonstrated by Strait and colleagues.[31,32] Our findings in both the mouse model and in the human OIT serum analyses provide important evidence for the relevance of these inhibitory antibodies under physiologic conditions. In contrast to the findings of Strait and colleagues, who found IgG suppression of allergic diarrhea to be FcγRIIb independent, we observed that IgG-mediated inhibition of food allergy in both the mouse model and the basophil reporter system was mediated by CD32 (FcγRII), with the murine experiments implicating FcγRIIb. The reason for this discrepancy might reside in differing mass amounts of IgG present in active versus reconstituted systems. High-titer monospecific IgG might provide a stoichiometric excess of IgG sufficient to make direct steric blocking of the antigen/IgE/FcεRI interaction the prevailing mechanism, removing a requirement for functional FcγRIIb.

Negative regulation of FcεRI-mediated mast cell and basophil activation has previously been studied in a number of experimental systems.[33] Inhibition of IgE signaling by IgG was first demonstrated by Daeron et al[20] in RBL-2H3 cells stably transfected with FcγRIIb cDNA. The same group subsequently reported that IgE-mediated activation of BMMCs is similarly subject to suppression by IgG antibodies of the same specificity.[34] Tam et al[35] demonstrated that cross-linking of FcεRI and FcγRIIb on human basophils by using a bispecific antibody against human IgE and FcγRII inhibits basophil histamine release, and as in BMMCs, the inhibitory effect of IgG antibodies seems to dominate over the activating response to IgG in human basophils.

The relevance of inhibitory IgG receptor signaling to allergic pathogenesis has been suggested by animal models showing augmented anaphylaxis and allergic rhinitis.[36-38] One previous study evaluating the effects of "blocking" antibodies induced during Bet v 1 immunotherapy in patients with birch allergy showed inhibition of basophil activation by allergen/IgG immune complexes but no effect of anti-CD32, implying a "blocking" IgG effect rather than receptor-mediated inhibition.[39] In contrast to our study, this group used basophils from the allergic subjects themselves. We believe this suggests that the allergic state might alter responsiveness to negative signaling pathways, a fact that might account for the lack of complete correlation between the presence of allergen-specific IgG and protection from allergic reactions.

Until recently, the treatment of patients with established IgE-mediated food allergy was limited to counseling strict allergen avoidance. Recent trials of OIT have provided evidence that safe, graded food allergen administration is possible and can have a very significant effect on the patient's ability to safely ingest allergenic foods. We believe that delineation of the mechanisms operative in successful OIT will provide important insights into new opportunities for the treatment of food allergy. The FcγRIIb-mediated effects of OIT-induced IgG antibodies described in our study, for instance, lead us to speculate that passive immunization[40] with polyclonal food-specific IgG, the application of small-molecule inhibitors of FcγRIIb signaling regulators, or the generation of recombinant heterobivalent constructs that cross-link FcεRI and FcγRIIb in an allergen-specific manner[41] might all be strategies to pursue in the future. In the meantime, further characterization of the full spectrum of immunoregulatory mechanisms operative in controlling food allergy should remain a high priority.

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Clinical implications: OIT induces IgG antibodies that can act through the inhibitory IgG receptor FcγRIIb to block IgE-mediated immediate hypersensitivity reactions.

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METHODS

Animals

All mice were bred and housed in a specific pathogen-free environment and were 6 to 12 weeks old when used. Wild-type BALB/c mice were originally purchased from Taconic Farms (Germantown, NY). C57BL/6 and FcγRIIb$^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, Me). *C.129.Il4ra^{F709} (Il4raF709)* mice were bred onto BALB/c and BALB/c/*Igh-7*$^{-/-}$ (IgE$^{-/-}$) backgrounds, as previously described.[E1] All experiments were carried out in accordance with the Institutional Animal Care and Use Committee policies and procedures of Children's Hospital.

Measurement of anaphylaxis

Anaphylaxis was assessed in challenged mice by measuring changes in body temperature with transponders placed subcutaneously 2 days before challenge (Implantable Programmable Temperature Transponder; Biomedic Data Systems, Seaford, Del), as we have previously described.[E1]

BMMC culture

BMMCs were cultured from bone marrow precursors of C57BL/6 and FcγRIIb$^{-/-}$ mice by using IL-3 and stem cell factor, as previously described.[E2] Mast cells were sensitized overnight with 2% mouse serum in 100 μL of RPMI containing 10 ng/mL IL-3 and 20 ng/mL stem cell factor (both from Shenandoah Biotechnology, Warwick, Pa) and challenged for 10 minutes at 37°C with 100 ng/mL OVA. Serum from PBS-treated *Il4raF709* mice was used as a negative control, and anti-FcεRI (10 μg/mL, MAR-1; Affymetrix eBioscience, San Diego, Calif) was used as a positive control for activation. In some experiments BMMCs were sensitized with IgE anti-TNP (10 ng/mL) and increasing amounts of IgG_1 anti-TNP before challenge with TNP-OVA (20 ng/mL). BMMCs were stained during the challenge period with anti–LAMP-1 (CD107a)–peridinin-chlorophyll-protein complex–eFluor710 (1D4B, Affymetrix eBioscience) anti–c-Kit–phycoerythrin (ACK2; BioLegend), and Fixable Viability Dye eFluor780 (Affymetrix eBioscience) at 1:1000 dilutions. After challenge, BMMCs were promptly washed twice with calcium-free ice-cold 0.5% BSA in PBS and analyzed on an LSR Fortessa (BD Biosciences, Franklin Lakes, NJ). Doublets and nonviable cells were excluded from analyses.

Passive anaphylaxis studies

BALB/c mice were sensitized intraperitoneally with 2 μg of IgE anti-TNP (C38-2, BD Biosciences) and challenged 16 hours later by means of intraperitoneal injection with 2 μg of TNP-OVA (Biosearch Technologies, Petaluma, Calif). IgG_1 anti-TNP (200 μg, 1B7.11; ATCC, Manassas, Va) was injected at the same time as IgE. C57BL/6 and FcγRIIb$^{-/-}$ mice were sensitized intraperitoneally with 100 μL of sera pooled from OVA-sensitized *Il4raF709* mice. Some mice additionally received 100 μL of serum from *Il4raF709*/IgE$^{-/-}$ mice that had been sensitized and then subjected to OVA OIT. In these experiments mice were challenged 24 hours later with 100 μg of OVA intraperitoneally. BALB/c mice were sensitized by means of intraperitoneal injection with 2 μg of IgE anti-TNP and challenged 24 hours later with 2 μg of TNP-OVA intraperitoneally. IgG_1 anti-TNP (200 μg) was injected along with IgE, where indicated.

Wild-type and FcγRIIb$^{-/-}$ mice were injected intraperitoneally with 100 μL of pooled serum from *Il4raF709* mice subjected to enteral low-dose OVA sensitization. Some groups additionally received 100 μL of pooled serum from IgE$^{-/-}$/*Il4raF709* mice that had undergone high-does enteral OVA OIT. After 24 hours, mice were challenged by means of intraperitoneal injection of 200 μg of OVA, and core body temperatures were recorded as a measure of anaphylaxis severity.

IgG depletion protocols

IgG depletion of 100 to 200 μL of post-OIT patient sera (week 52) was performed with the Nab Protein G Spin Kit, according to the manufacturer's instructions (Thermo Scientific, Rockford, Ill). Flow-through fractions were confirmed to be free of IgG by using ELISA. IgG-containing eluates were applied to Amicon Ultra-0.5 Centrifugal Filter Devices to concentrate and desalt the antibody (EMD Millipore, Billerica, Mass). The same procedures were applied to murine sera samples.

ELISAs

Murine mast cell protease 1 release into serum was measured by using a standard sandwich ELISA, according to the manufacturer's instructions (Affymetrix eBioscience). Murine antibody levels were evaluated through capture of serum onto OVA-coated plates, followed by detection with biotinylated isotype-specific antibodies (Southern Biotech, Birmingham, Ala). Values were calculated in arbitrary units by comparison with a reference serum obtained from mice intraperitoneally immunized with OVA and alum or to the monoclonal IgE anti-OVA TOε, as previously described.[E1] Peanut-specific IgG subclasses and IgA concentrations were measured in pre- and post-OIT patient sera by using an indirect ELISA. A post-OIT serum sample was used to construct a standard curve, and absolute concentrations of peanut-specific isotypes within the standard were calculated in comparison with known concentrations of each isotype (obtained from Sigma-Aldrich) coated directly onto the ELISA plate and developed under the same conditions. Antibodies used include biotinylated IgG_1, IgG_2, IgG_4, $IgA_{1/2}$ (BD PharMingen, San Jose, Calif), and IgG_3 (Invitrogen Life Technologies, Grand Island, NY). Total peanut-specific IgG was calculated as the sum of mass units of all IgG isotypes. Total IgG and IgG_4 levels were quantified by using Ready-Set-Go! kits (Affymetrix eBioscience).

Flow cytometry

Mesenteric lymph nodes were analyzed for T_H2 cells by using intracellular cytokine staining. Cells were stimulated directly *ex vivo* with 500 ng/mL phorbol 12,13-dibutyrate, 500 ng/mL ionomycin, and 1000 ng/mL Brefeldin A (Sigma-Aldrich) for 4 hours, followed by staining with Fixable Viability Dye e780 (Affymetrix/eBioscience), CD4–peridinin-chlorophyll-protein complex–Cy5.5 (RM4-5) and IL-4–Alexa Fluor 488 (11B11; both from BioLegend) by using the Cytofix/Cytoperm kit from BD Biosciences.

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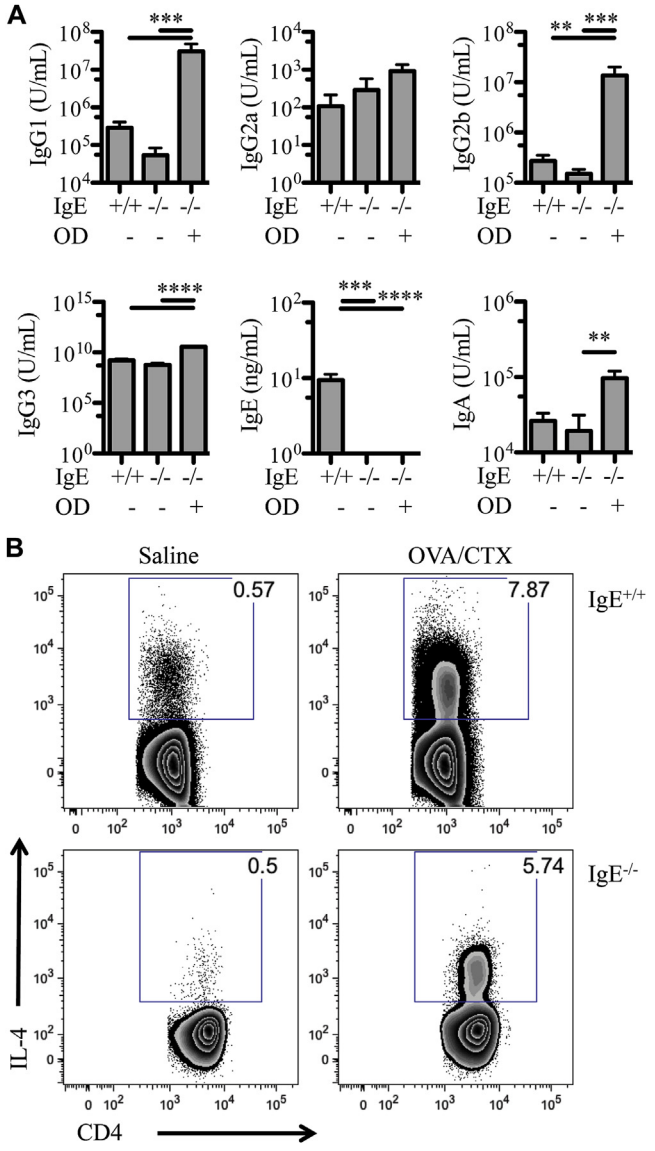

FIG E1. Effect of IgE deficiency on sensitization of *Il4raF709* mice with allergen and adjuvant. **A,** OVA-specific immunoglobulin levels in sensitized $IgE^{+/+}$/*Il4raF709* mice, sensitized $IgE^{-/-}$/*Il4raF709* mice, and $IgE^{-/-}$/*Il4raF709* mice after receiving enteral high-dose OVA (n = 7-9 per group). **B,** Representative flow cytometric plots showing IL-4 staining in $CD4^+$ T cells from the mesenteric lymph nodes. $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$ by using the Bonferroni posttest on ANOVA. *CTX*, Cholera toxin.

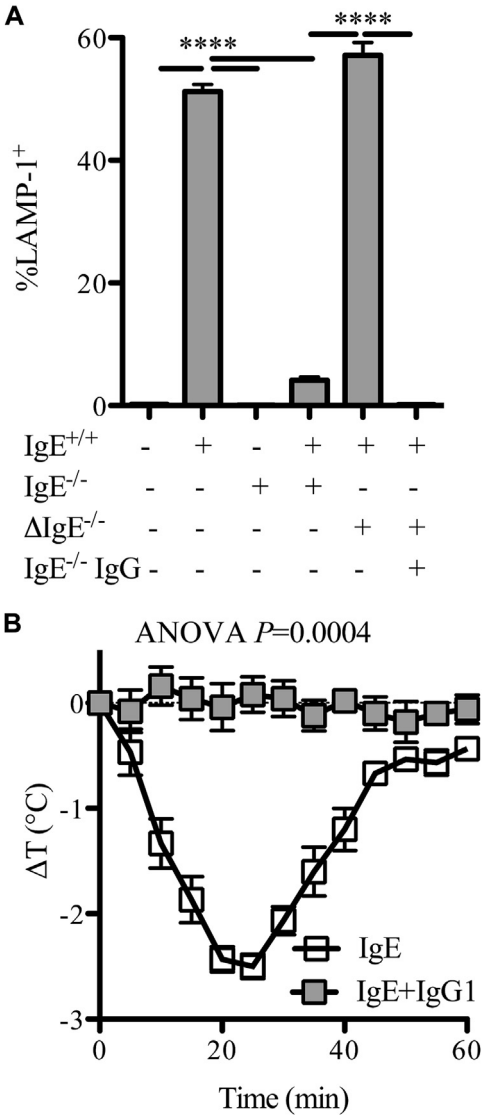

FIG E2. Effect of IgG on mast cell degranulation and anaphylaxis. **A,** Effect of IgG depletion or replacement on LAMP-1 expression by wild-type BMMCs after OVA challenge. BMMCs were sensitized with OVA-sensitized *Il4raF709*, high-dose OVA–fed *IgE*$^{-/-}$/*Il4raF709*, IgG-depleted *IgE*$^{-/-}$/*Il4raF709*, and/or IgG-reconstituted sera. ****$P < .0001$, Bonferroni posttest on 2-way ANOVA. Values are presented as means ± SEMs of *in vitro* replicates and are representative of at least 3 independent experiments. **B,** Effects of monoclonal IgG_1 on systemic anaphylaxis induced by monoclonal IgE. BALB/c mice were sensitized by means of intraperitoneal injection with 2 μg of IgE anti-TNP and challenged 24 hours later with 2 μg of TNP-OVA administered intraperitoneally. IgG_1 anti-TNP (200 μg) was injected along with IgE, where indicated. The overall *P* value derives from repeated-measures 2-way ANOVA. Values are presented as means ± SEMs from 3 to 5 mice per group and are representative of 2 independent experiments. *ΔT*, Change in temperature.

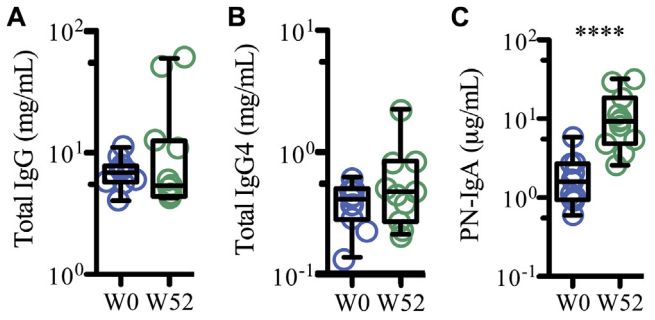

FIG E3. Peanut-specific and total immunoglobulin levels in patients before *(W0)* and after (W52) OIT with omalizumab adjunct therapy: **A,** total IgG; **B,** total IgG_4; and **C,** peanut *(PN)*–specific IgA. ****$P < .0001$, paired *t* test on log-transformed values. Data shown represent individual patient values *(circles)*, with box plots in overlay.

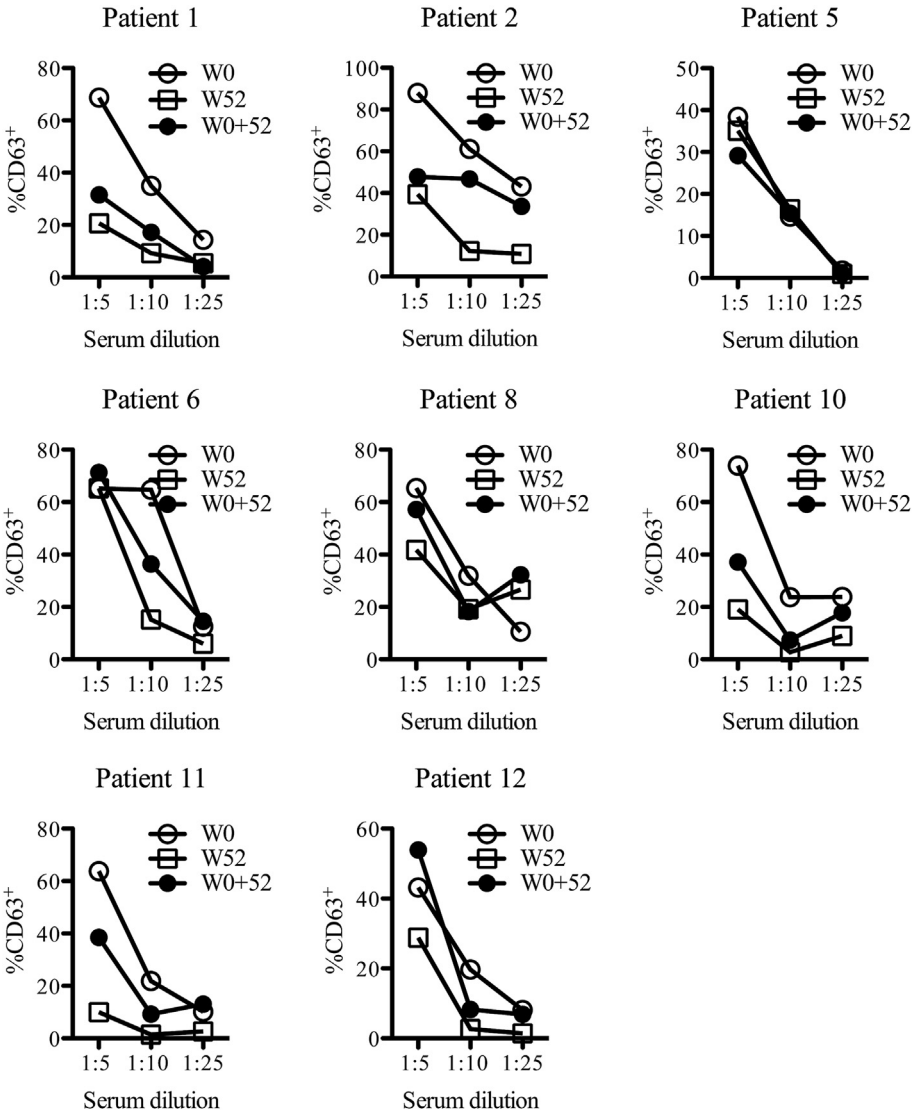

FIG E4. Basophil-sensitizing properties of patient sera as a function of concentration. Basophil activation tests were performed with patient sera to sensitize basophils at 1:5, 1:10, and 1:25 dilutions, followed by peanut challenge. *W0*, Before OIT at week 0; *W52*, after OIT at 52 weeks.

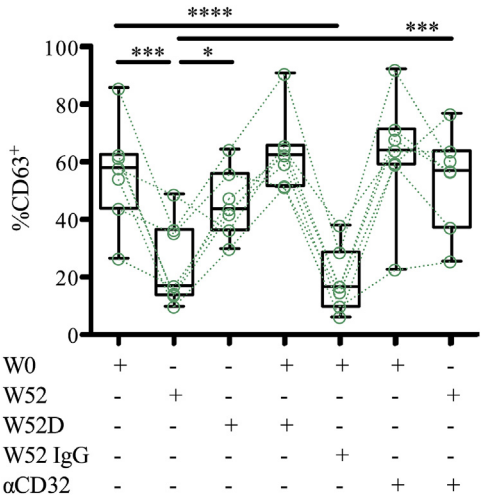

FIG E5. Effects of IgG and CD32 on basophil activation by patient sera before and after OIT. Sera from individual patients were used to sensitize donor basophils for activation by peanut. Data are shown as box plots, with individual data points overlaid as *circles* and tracing lines between conditions. *αCD32*, Anti-CD32 antibody; *W0*, pre-OIT serum; *W52*, post-OIT serum; *W52D*, post-OIT serum depleted of IgG; *W52 IgG*, IgG purified from post-OIT serum. **P* < .05, ****P* < .001, and *****P* < .0001, Bonferroni posttest on repeated-measures ANOVA.