

ORIGINAL ARTICLE

Food Allergy and Gastrointestinal Disease

IgG1⁺ B-cell immunity predates IgE responses in epicutaneous sensitization to foods

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Abstract

Background: The generation of IgE-mediated food allergy in humans is silent and only diagnosed upon manifestation of clinical symptoms. While experimental models have been used to investigate some mechanisms of allergic sensitization, the generation of humoral immunity and memory remains to be elucidated. Here, we defined the evolution of allergen-specific B-cell responses during epicutaneous sensitization to foods.

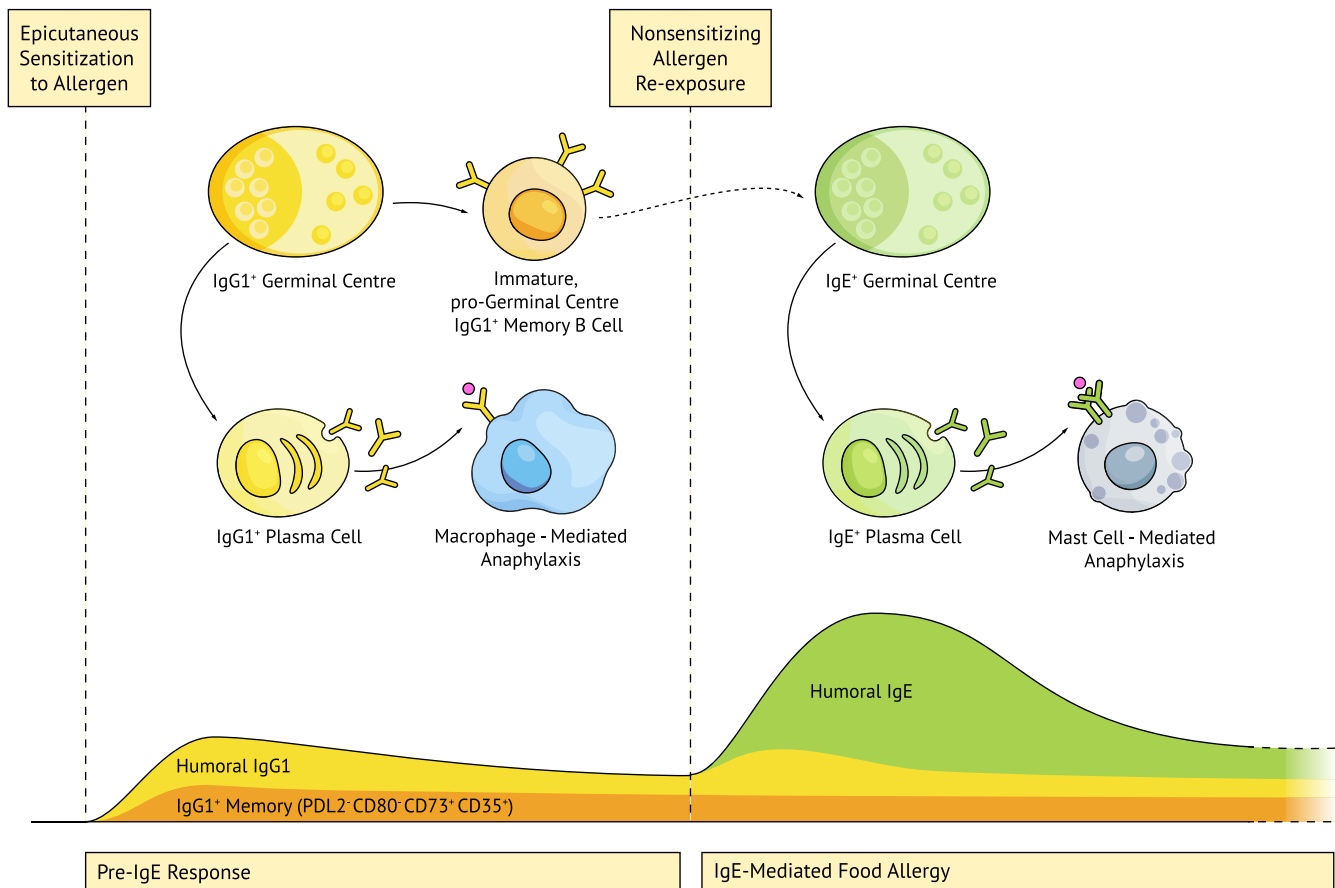
Methods: Wild-type and genetic knockout animals, and drug or antibody strategies for cell depletion and immunoglobulin signaling blockade were used to investigate epicutaneous sensitization and disease progression; we analyzed allergen-specific germinal centers and IgG1⁺ memory B cells by flow cytometry, evaluated humoral responses, and determined clinical reactivity (anaphylaxis).

Results: Epicutaneous sensitization caused microscopic skin damage, inflammation, and recruitment of activated dendritic cells to the draining lymph nodes. This process generated allergen-specific IgG1⁺ germinal center B cells, serum IgG1, and anaphylaxis that was mediated by the alternative pathway. Whether we used peanut and/or ovalbumin from the egg white for sensitization, the allergen-specific IgG1⁺ memory compartment predominantly exhibited an immature, pro-germinal center phenotype (PDL-2⁺CD80⁺CD35⁺CD73⁺). Subsequent subclinical exposures to the allergen induced IgE⁺ germinal center B cells, serum IgE, and likely activated the classical pathway of anaphylaxis.

Conclusions: Our data demonstrate that IgG1⁺ B-cell immunity against food allergens in epicutaneous sensitization precedes the generation of IgE responses. Therefore, the assessment of allergen-specific cellular and humoral IgG1⁺ immunity may help to identify individuals at risk of developing IgE-mediated food allergy and hence provide a window for therapeutic interventions.

KEYWORDS

anaphylaxis, epicutaneous sensitization, IgE-mediated food allergy, IgG1⁺ memory B cells, Th2 immunity



GRAPHICAL ABSTRACT

IgG1⁺ B-cell immunity against food allergens in epicutaneous sensitization precedes the generation of IgE responses. Immature, pro-germinal centre, allergen-specific IgG1⁺ memory B cells are formed at the incipient stages of skin sensitization to foods. The assessment of allergen-specific cellular and humoral IgG1⁺ immunity may help to identify individuals at risk of developing IgE-mediated food allergy.

1 | INTRODUCTION

Food allergy is a worldwide health and economic concern. It currently affects ~15 million Americans, and its prevalence is increasing.¹⁻³ The clinical manifestations of food allergy are primarily mediated by immunoglobulin (Ig) E with symptoms ranging from hives, pruritus, wheezing, vomiting, and diarrhea to anaphylaxis: a rapid, systemic reaction that can lead to hypotension, respiratory failure, and death.⁴⁻⁶ Allergic sensitization—the series of events leading to the formation of allergen-specific IgE—is a clinically silent process; indeed, the majority of patients allergic to peanut, tree nuts, and egg react upon their first known exposure.^{7,8} Therefore, interventions to prevent or dismantle food allergy require understanding of the events (mechanisms) leading to the generation of IgE against foods.

Over the past 15 years, several clinical studies have proposed that the skin is a site for Th2 sensitization to foods.⁹⁻¹² Collectively, these studies postulate that defects in the integrity of the epidermis, whether due to gene mutations (eg, filaggrin) and/or damage (eg, eczema), are associated with a higher risk of developing food allergy. A number of animal models have been established to investigate the

immunological basis of food allergy induced via the epicutaneous route.¹³⁻¹⁹ These models have been shown to produce allergen-specific IgE against common food allergens such as peanut or ovalbumin (OVA)—the major allergen of the chicken egg white.^{20,21} However, the series of events that precede the generation of IgE-secreting cells plasma cells (PCs) remain unexplored. The primary objective of this study was to decipher the evolution of humoral immunity and immunological memory in a model of epicutaneous sensitization to foods.

We found that IgG1⁺ B-cell immunity prevailed in the initial stages of epicutaneous sensitization to food allergens in the germinal center (GC) as well as in the effector (ie, PC and clinical reactivity) and memory compartments. The memory compartment had predominantly an immature and pro-GC phenotype regardless of antigen specificity (OVA vs peanut). Accordingly, clinical reactivity at this stage was driven by the alternative pathway, that is, IgG, macrophages, and platelet-activating factor (PAF).^{22,23} Following subclinical, nonsensitizing allergen exposures, allergen-specific GCs formed, IgE⁺ GC B cells and PCs were generated, and allergen-specific IgE was detected in circulation.

These data demonstrate that IgG1⁺ B-cell immunity against food allergens in epicutaneous sensitization precedes the generation of IgE responses. Therefore, the assessment of cellular and humoral allergen-specific IgG immunity may help to identify individuals at risk of developing IgE-mediated food allergy and hence provide a window for immunological interventions to prevent disease progression.

2 | METHODS

2.1 | MICE

Six- to eight-week-old females and vendor- and strain-matched controls were used in all the experiments. IgE-deficient mice (Igh-7^{tm1Ledy})²⁴ and PAF receptor KO (PAF-R^{-/-})²⁵ mice were kindly provided by Dr. H. Oettgen (Harvard Medical School, Boston, MA) and Dr. E. I. Tuomanen (St. Jude Children's Research Hospital, Memphis, TN), respectively, and bred in house. C57BL/6 and Balb/c mice were purchased from Charles River, and mast cell (MC)-deficient mice (B6.Cg-Kit^{W-sh}/HNIhrJaeBsmGlli) and CCR2^{-/-} mice (B6.129S4-Ccr2^{tm1lf}/J) were purchased from The Jackson Laboratory. IL-33 KO mice were generously provided by MedImmune (Gaithersburg, MD) from Taconic (Derwood, MD). All procedures were approved by the McMaster University Research Ethics Board.

2.2 | Food allergy model

Epicutaneous sensitization was conducted as previously published.²⁶ Serum was collected by retro-orbital bleeding and analyzed for peanut and/or OVA-specific Igs via sandwich ELISA.^{27,28} Mice were systemically challenged via intraperitoneal administration crude peanut extract (CPE) (5 mg). Core temperature was assessed telemetrically (see below), or rectally with a digital thermometer (23226-656, VWR), and hematocrit by centrifuging (HemataSTAT II, Fisher Scientific) anticoagulated blood 40 minutes after challenge.

2.3 | Telemetric blood pressure and body temperature measurements

Telemetric transmitters were used to measure mean arterial blood pressure (PA-C10, Data Science International) and core body temperature (TA-F10, Data Science International) in conscious unrestrained mice. The devices were implanted following the manufacturer's protocol as described.²⁹ Telemetric data were collected and analyzed with Dataquest A.R.T software (version 4.0; Data Science International).

2.4 | Peritoneal lavage

Peritoneal lavage was collected and processed as reported.³⁰ Cytospins of the cells were stained 24 hours later with the Hema 3 stain set (Fischer scientific). A minimum of 500 cells were counted to quantify percentages of MCs, mononuclear cells, neutrophils, and eosinophils.

2.5 | Histology

Skin segments were collected and fixed in 10% formalin for 24 hours and then washed with 70% ethanol and paraffin embedded. Sections were stained with hematoxylin and eosin (HE) for microscopic analysis (Nikon X-Cite 120 LED) using NIS-ELEMENTS software.

2.6 | Inguinal lymph nodes processing

Inguinal lymph nodes (iLNs) were triturated between frosted slides in Hank's balanced salt solution, washed, and filtered (40 µm).

2.7 | Flow cytometry

Antibodies were obtained from eBioscience, BD, or BioLegend. OVA-FITC was obtained from Molecular Probes (023020, Thermo Fisher), and biotinylated Ara h 1 and Ara h 2 from Indoor Biotechnologies Inc. In all assays, cells were incubated with anti-FcγRII/III before incubation with fluorochrome-conjugated antibodies. Dead cells were excluded by propidium iodide uptake (Sigma) or fixable viability dye eFluor780 (eBioscience) and gated on singlets. On average, a minimum of 10⁶ and 300 000 alive and singlet cells were analyzed for experiments evaluating GCs, PCs, and memory B cells, and dendritic cells (DCs), respectively. Experiments that involved detection of IgE⁺ B cells or PCs included unconjugated anti-IgE (clone RME-1) together with anti-FcγRII/III and exclusive detection of intracellular IgE via IgE-PE (clone RME-1) upon fixation/permeabilization of cells (BD Biosciences).³¹ Fluorescence minus one (FMO) and isotype controls were used for gating. Data were acquired on a LSR II or Fortessa (BD) and analyzed using FlowJo (Treestar).

2.8 | Antibody and drug administration

Blockade of histamine receptors and IgG-mediated anaphylaxis was conducted as reported.^{32,33} Depletion of basophils, phagocytes, and neutrophils was conducted as previously shown.^{33,34}

2.9 | Spleen cell cultures and assessment of cytokine production

Spleens were processed and cultured as reported.³¹ After 4-5 days of culture, supernatants were stored at -80°C until further analysis. Cytokines in cell-free supernatants were quantified with the MAGPIX[®] system using MILLIPLEX[®] MAP magnetic bead-based multi-analyte panels (Millipore).

2.10 | Statistics

Data were analyzed and graphed with GraphPad Prism 6 software (GraphPad Software). Continuous data are expressed as means ± SEMs and were analyzed using 1-way ANOVA with Bonferroni post hoc tests and unpaired Student's *t* test. Ordinal data are

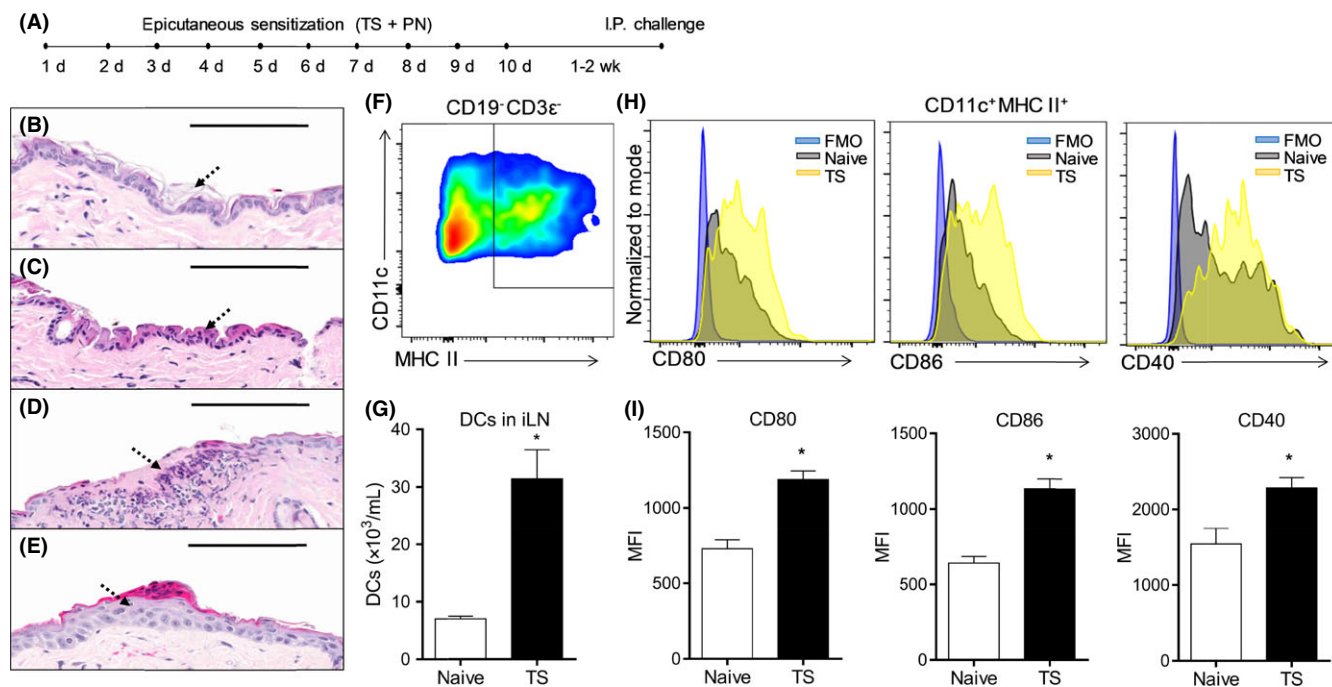


FIGURE 1 Epithelial damage causes an increase in activated DCs to iLNs. Separate groups of C57BL/6 and BALB/c mice were epicutaneously sensitized to peanut (PN) (A). Biopsies of the skin were taken (B-E) from naïve mice (B), or mice after 30 min (C), 6 h (D), and 24 h (E) of being tape-stripped (TS), and HE-stained. Following 3 d of sensitization, the draining lymph nodes (iLN) were collected to assess DC migration (F,G) and activation (H,I) by flow cytometry. Representative pictures from 3 mice were taken; scale bar is 100 μ m; arrows indicate significant features (B-E). Representative data from 2-3 independent experiments (F-I) represented as mean $\pm$ SEM, $n = 3-6$. * $P < .05$ vs naïve [Colour figure can be viewed at wileyonlinelibrary.com]

shown as individual mice with medians and were analyzed with Mann-Whitney U tests. Differences were considered statistically significant at a P value of $<.05$ or as indicated. Half-life was calculated using the following equation: $(\text{Elapsed time} \times \log_2)/(\log[\text{beginning amount/ending amount}])$.³¹

3 | RESULTS

3.1 | Epithelial damage causes DC activation and migration to lymph nodes

We employed a murine model to investigate the humoral arm of food sensitization via the epicutaneous route.²⁶ This 10-day model involves application of allergen onto previously shaved and tape-stripped skin on the lower backs of the mice (Figure 1A). This is shorter than other models in which the length of allergen exposure ranges from 14 to 49 days.¹³⁻¹⁹ While tape-stripping did not cause macroscopic damage (Figure S1A), histologic analysis revealed the signs of microscopic damage and inflammation of the skin epithelium (Figure 1B-E). Shortly after tape-stripping (30 minutes), the *stratum corneum* (the outer layer of the epidermis) was absent, the extracellular space stained intensely with eosin—which is suggestive of the release of cellular contents and damage—and the normal morphology of the cells in the epidermis (ie, shape and nuclear staining) was altered (Figure 1C); 6 hours following tape-stripping, the epidermis remained altered, and immune cell recruitment into the dermis was

observed (Figure 1D); 24 hours after tape-stripping, the inflammation of the dermis was no longer apparent, while the epidermis showed hyperplasia and partial regeneration of the *stratum corneum* (Figure 1E).

We next assessed the impact of this local inflammatory response on the initiation of adaptive immunity by evaluating DC migration and activation in the draining inguinal lymph nodes (iLNs). After 3 days of tape-stripping and allergen exposure, the absolute number of DCs was significantly higher in mice undergoing epicutaneous sensitization compared to naïve mice (Figure 1F-G); DCs in mice undergoing sensitization were activated, borne out by upregulation of the costimulatory markers CD80, CD86, and CD40^{35,36} (Figure 1H-I). These data suggest that microscopic skin damage and local inflammation lead to the migration of activated DCs to the iLNs, thus indicating an ongoing immune response.

3.2 | Allergen-specific GC activity and humoral IgG1 immunity in epicutaneous sensitization

We then assessed whether the immune response initiated during epicutaneous sensitization entailed GC activity, as this process is central to the evolution of humoral responses and memory.³⁷ To accomplish this, we delineated the kinetics of allergen-specific GC B cells in the spleen and iLNs using a validated flow cytometric method³¹ (Figure 2A,B). Allergen-specific GC B cells were not detected in the spleen at any time point. Conversely, the number of

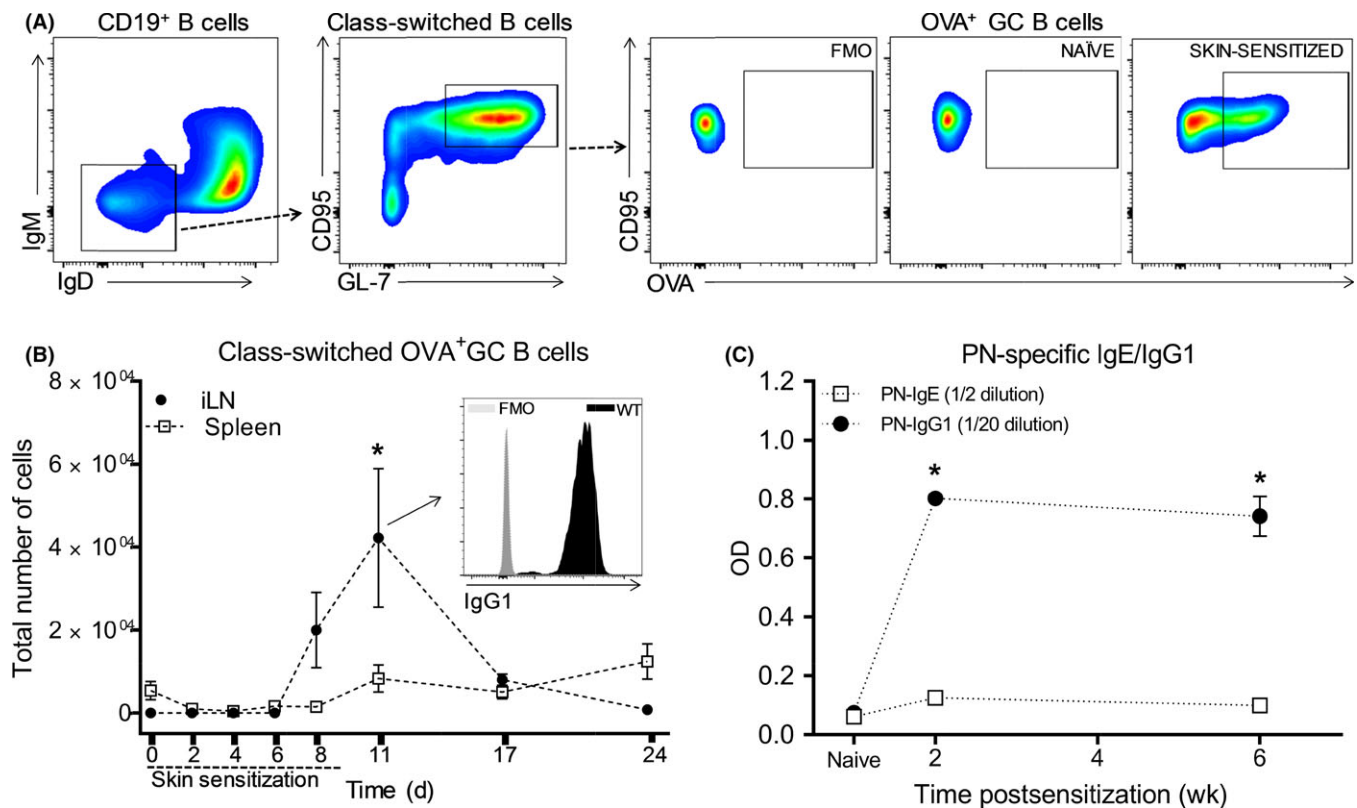


FIGURE 2 Epicutaneous sensitization entails IgG1⁺ GC B-cell activity and humoral IgG1. Gating strategy to identify OVA-specific, class-switched, GC B cells by flow cytometry (A). Separate groups of C57BL/6 wild-type (WT) mice were epicutaneously sensitized to peanut (PN) or OVA, and the kinetics of allergen-specific GC activity were assessed in the draining iLNs and the spleen (B). Serum levels of PN-specific IgE and IgG1 were measured at different times postsensitization by ELISA (C). Representative (A,C) or pooled (B) data from 3 independent experiments represented as mean $\pm$ SEM, $n = 3$ –6. * $P < .01$ vs naïve (ie, day 0) [Colour figure can be viewed at wileyonlinelibrary.com]

allergen-specific GC B cells increased in the iLNs after 7 days. They peaked after 10 days and returned to baseline 1 week later (day 17). The calculated half-life of allergen-specific GC reactions in this system was 2.48 days (95% CI, 0.98–4.63 days). The majority ($\geq 95\%$) of class-switched allergen-specific GC B cells generated at 11 days expressed cell-surface IgG1 (Figure 2B), while IgE⁺ GC B cells were undetectable (data not shown). We also assessed serum Igs at 2 and 6 weeks after sensitization by ELISA (Figure 2C). Mirroring the GC responses, allergen-specific IgE levels were not significantly increased compared to untreated mice and allergen-specific IgG1 levels were elevated for at least 6 weeks (Figure 2C). Altogether, these data show that epicutaneous exposure to food allergens induces allergen-specific GC activity, which is dominated by IgG1-expressing B cells, and a serum allergen-specific IgG1 humoral response.

3.3 | Epicutaneously sensitized mice undergo anaphylaxis by the alternative pathway

To evaluate the functionality of the effector B-cell response generated on epicutaneous sensitization, we assessed clinical reactivity on allergen challenge 2 weeks after the end sensitization. Compared to naïve mice, epicutaneously sensitized mice underwent anaphylaxis,

as evidenced by the development of hypothermia (Figure 3A), hypotension (Figure 3B), hemoconcentration (Figure 3C), and clinical signs (Figure 3D). In addition, inflammation (Figure S1C) and eosinophilia (Figure S1D) were detected in the peritoneal cavity (the site of allergen challenge) of epicutaneously sensitized mice 72 hours postchallenge indicating a late phase, T cell-mediated allergic response.

As the Ig response was mainly comprised of IgG1, we tested the importance of the classical and alternative pathways of anaphylaxis^{32,33} in epicutaneously sensitized mice. Therefore, we assessed clinical reactivity in IgE-deficient mice and mice treated with anti-Fc γ RII/III prior to challenge (Figure 3E). IgE-deficient mice underwent anaphylaxis comparable to wild-type animals. However, blockade of IgG signaling significantly reduced the allergic reaction, which was fully abrogated in IgE-deficient mice treated with anti-Fc γ RII/III. Next, we evaluated the contribution of immune cells potentially relevant to clinical reactivity. The depletion of MCs, basophils, or neutrophils did not significantly ameliorate anaphylaxis (Figure 3F). In contrast, depletion of phagocytes using clodronate-containing liposomes completely abrogated clinical reactivity (Figure 3F). However, CCR2^{-/-} mice, which are monocytopenic due to defective bone marrow egress of these cells,³⁸ developed anaphylaxis similar to wild-type animals (Figure S2). Because CCR2^{-/-} mice have an intact

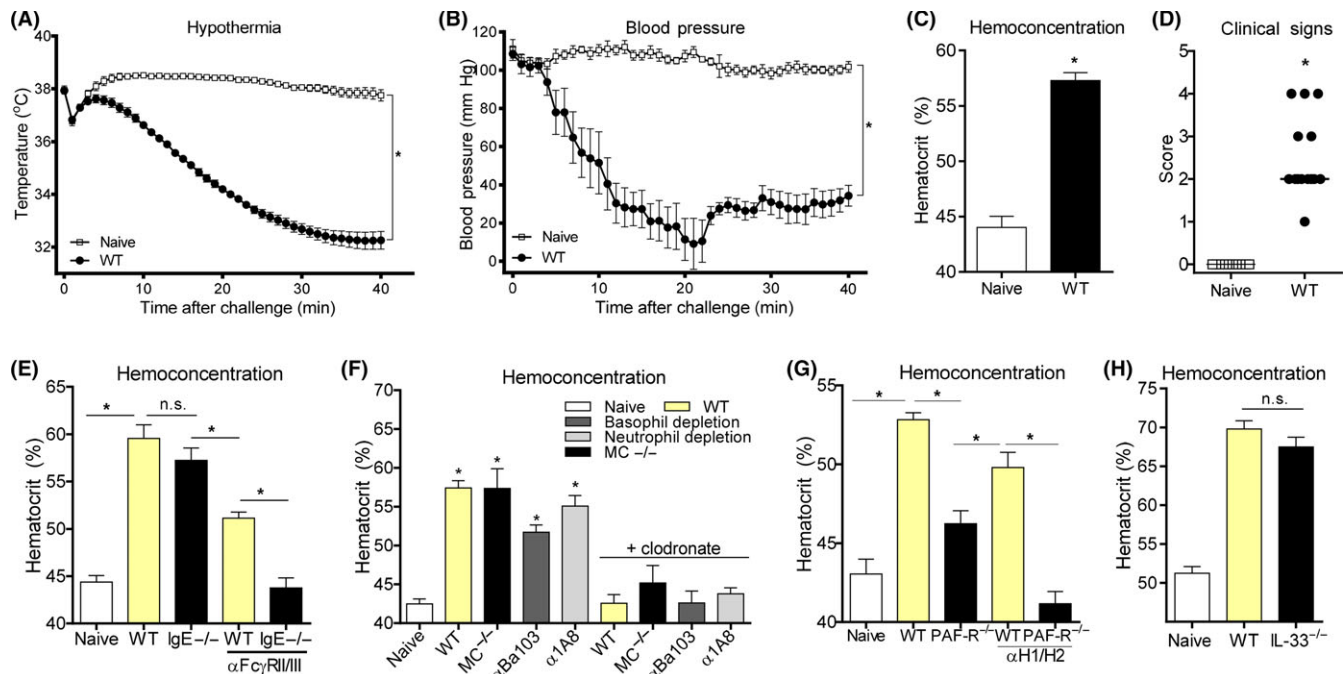


FIGURE 3 Epicutaneously sensitized mice undergo anaphylaxis mediated by IgG-macrophage-PAF and independent of IL-33. Separate groups of C57BL/6 and BALB/c wild-type (WT) mice, and mice deficient in IgE (E, BALB/c), MCs (F, C57BL/6), PAF-R (G, C57BL/6), and IL-33 (BALB/c) were epicutaneously sensitized to peanut. Clinical reactivity was assessed by measuring hypothermia (A), blood pressure (B), hemoconcentration (C,E-H), and clinical signs (D). Some groups of sensitized mice were treated prior to allergen challenge with drugs or antibodies to inhibit IgG signaling (E, anti-FcγRII/III), to deplete basophils (F, anti-Ba103), neutrophils (F, anti-1A8), and phagocytes (F, clodronate-containing liposomes) or to block histamine receptors 1 (mepyramine) and 2 (cimetidine) (G). Pooled data from 2 to 3 experiments represented as mean ± SEM (A-C,E-H), or individual mice with medians (D), $n = 6-15$. * $P < .05$ vs naïve or as indicated [Colour figure can be viewed at wileyonlinelibrary.com]

macrophage compartment,³⁹ this indicates that macrophages were necessary to cause anaphylaxis.

We then proceeded to investigate the contribution of a number of anaphylactic mediators potentially relevant to epicutaneous sensitization.^{16,32,40} Clinical reactivity was significantly reduced in PAF-R^{-/-} mice (Figure 3G) despite having the same serum levels of peanut-specific IgG1 than wild-type mice (data not shown). The co-administration of H1 and H2 receptor antagonists had a modest, nonsignificant effect on clinical reactivity. However, the combined blockade of H1 and H2 receptors in PAF-R^{-/-} mice fully prevented clinical reactivity (Figure 3G), which is consistent with our previous work in a model of oral sensitization.³² Galand et al¹⁶ recently showed that IL-33 promotes IgE- and MC-mediated anaphylaxis. Expectedly, we found that IL-33 KO mice underwent anaphylaxis similar to wild-type mice on allergen challenge (Figure 3H). Collectively, these data show that the IgG-macrophage-PAF axis is central in mediating clinical reactivity at the early stages of epicutaneous sensitization to foods.

3.4 | Phenotypic characterization of allergen-specific IgG1⁺ memory B cells

To better understand the B-cell compartment driving IgG1-dominant epicutaneous sensitization to foods, we analyzed allergen-specific memory B cells generated against major peanut (Ara h 1 and 2)⁴¹ and egg (OVA)⁴² allergens. To identify features in the memory

compartment that are inherent to the allergen, mice were sensitized to peanut extract or OVA, either alone or concomitantly. Mice were rested after sensitization for 3-4 weeks—well beyond the duration of allergen-specific GC activity (2.48 days (95% CI, 0.98-4.63 days); Figure 2B)—before the memory compartment in the iLNs was analyzed (Figure 4). Following exclusion of lymphocytes expressing IgD, IgM, CD138, and CD4, a clear population of IgG1⁺ memory B cells was found while class-switched B cells expressing IgE were undetectable (Figure 4A). Then, we assessed allergen specificity via binding to either Ara h 1 and 2, or OVA. Allergen-specific IgG1⁺ memory B cells were undetected in naïve mice, and peanut-specific populations were not observed in mice sensitized to OVA and vice versa (Figure 4A). Allergen-specific IgG1⁺ memory B cells were rare (~0.002% of total cells). The frequency of OVA-specific from IgG1⁺ memory B cells was significantly higher in OVA-sensitized mice (16.2%) than in cosensitized mice (5.4%), while the frequency of peanut-specific from IgG1⁺ memory B cells was comparable between peanut (6.4%) and cosensitized mice (5.8%). Thus, the addition of OVA in the cosensitized mice did not significantly alter the relative availability of Ara h 1 and 2, which were used to measure peanut-specific IgG1⁺ B cells, whereas the presence of peanut did significantly reduce the relative availability of OVA compared to just the OVA-sensitized mice.

We examined the identity and functionality of allergen-specific IgG1⁺ memory B cells from epicutaneously sensitized mice by

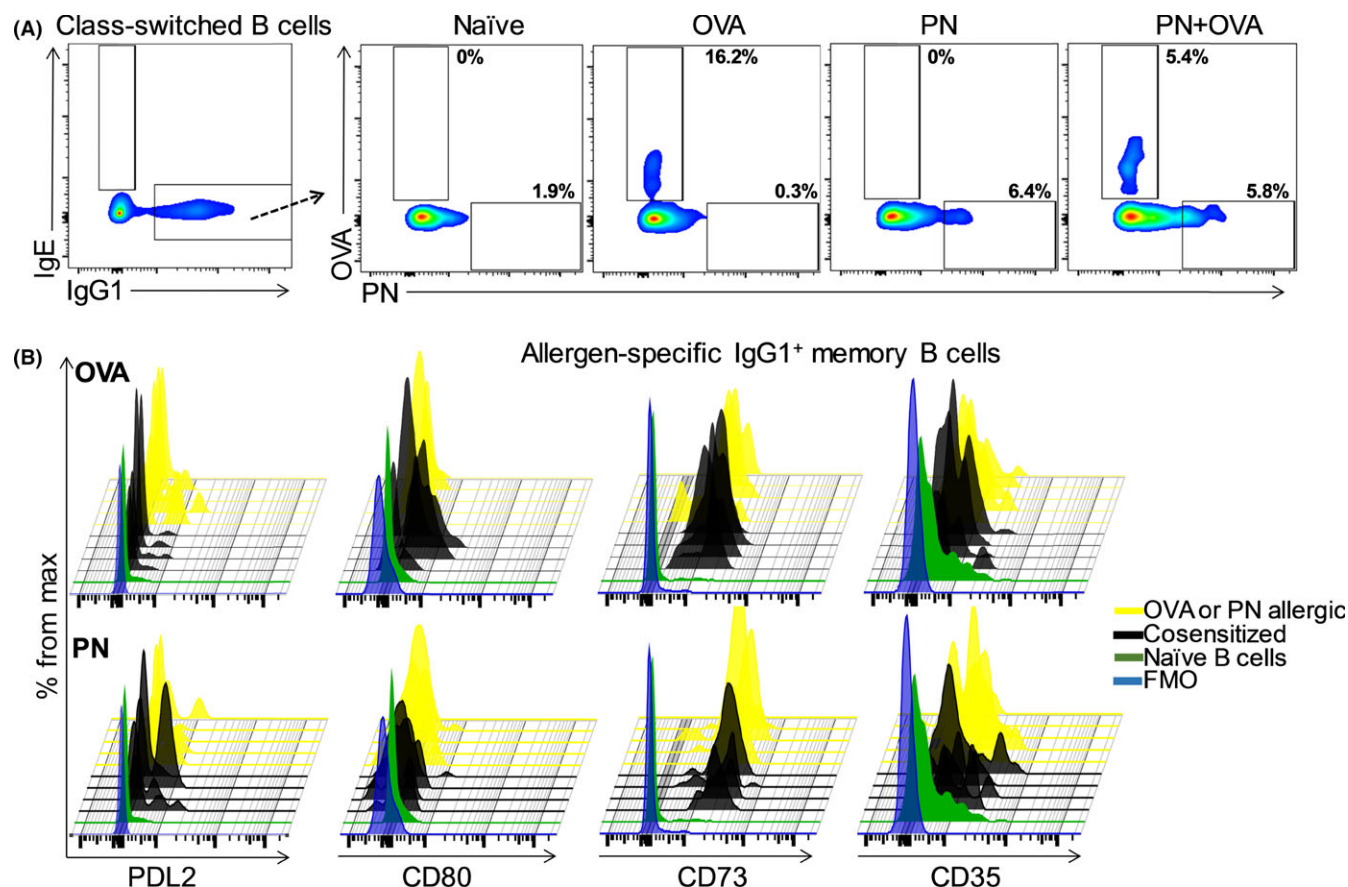


FIGURE 4 Phenotypic characterization of allergen-specific IgG1⁺ memory B cells. Separate groups of C57BL/6 mice were epicutaneously sensitized to peanut (PN), OVA, or both, and rested for 3–4 wk. ILNs were collected and stained for flow cytometry analysis of the frequency (A) and phenotype (B) of allergen-specific IgG1⁺ memory B cells (every histogram represents a single mouse). Representative data from 3 to 4 independent experiments; $n = 4$ –6 per experiment [Colour figure can be viewed at wileyonlinelibrary.com]

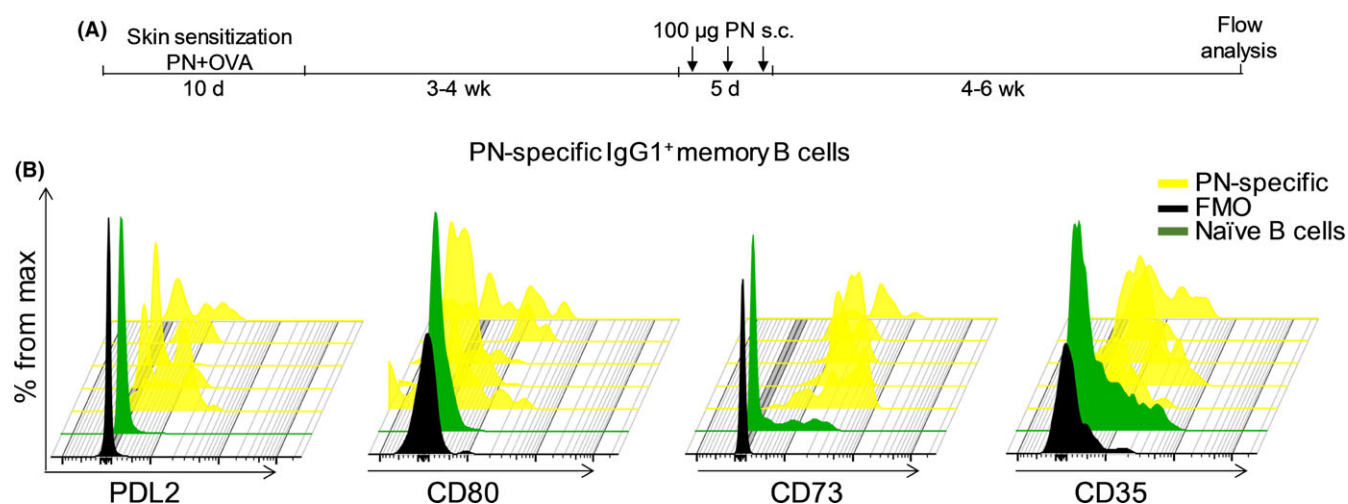


FIGURE 5 Phenotypic changes of allergen-specific IgG1⁺ memory B cells following allergen exposures. Separate groups of C57BL/6 mice were epicutaneously sensitized to peanut (PN) and OVA, rested for 3–4 wk, and subcutaneously (s.c.) exposed to subclinical doses of PN. Then, 4–6 wk later, the iLNs were collected (A) and stained for phenotypic analysis by flow cytometry (B, every histogram represents a single mouse). Representative data from 2 independent experiments; $n = 4$ –5 per experiment [Colour figure can be viewed at wileyonlinelibrary.com]

assessing PDL-2, CD80, CD73, and CD35 expression^{43–46} (Figure 4B). Regardless of allergen specificity (peanut vs OVA) and diversity (single allergen vs complex mixture), the phenotype of the allergen-

specific IgG1 memory compartment was consistently the same, dominated by cells negative for PDL-2 and CD80, and positive for CD73 and CD35. However, additional exposures to the allergen, under

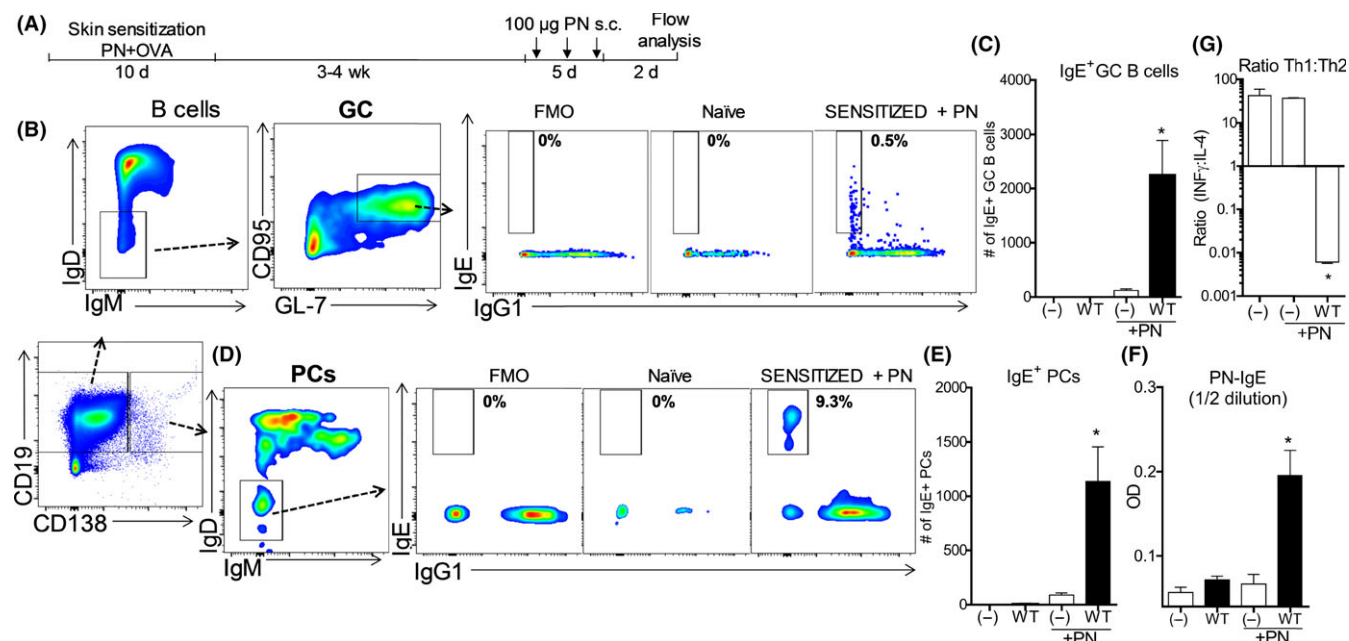


FIGURE 6 Allergen re-exposures generate IgE⁺ GC B cells and humoral IgE in epicutaneously sensitized mice. Separate groups of C57BL/6 mice were epicutaneously sensitized (WT) to peanut (PN) and OVA, and rested for 3–4 wk. Some groups were subcutaneously (s.c.) exposed to 100 µg of PN extract, 3 times (A). Two days after the last exposure, we assessed IgE⁺ GC B cells (B,C) and PCs (D,E) by flow cytometry, serum levels of allergen-specific IgE by ELISA (F), and cytokine secretion in supernatants from splenocyte cultures by MAGPIX (G). Representative (B, D,G) or pooled (C,E,F) data from 2–3 independent experiments represented as mean ± SEM, n = 6–15. *P < .05 vs naïve [Colour figure can be viewed at wileyonlinelibrary.com]

subclinical conditions (Figure S3), modified the phenotype of this population, which partially increased PDL-2 and CD80 expression and decreased CD35, while remained positive for CD73 (Figure 5). Collectively, these data show that the incipient stages of epicutaneous sensitization to foods generate a memory population of IgG1⁺ B cells with an immature and pro-GC phenotype (PDL-2⁺CD80⁺CD35⁺CD73⁺).

3.5 | Allergen re-exposures in epicutaneously sensitized mice generate IgE⁺ GC B cells and humoral IgE

Lastly, we sought to evaluate the functional impact of allergen re-exposures after skin sensitization. To do this, mice were epicutaneously cosensitized to peanut and OVA, and then rested for 3–4 weeks prior to being re-exposed to the allergen 3 times at subclinical doses (Figure S3). The B-cell compartment was analyzed 2 days after the last exposure (Figure 6A). We only detected significant peanut-specific GC activity in the iLNs of epicutaneously sensitized mice that had been subclinically re-exposed to the allergen (Figure S4A). Interestingly, the subclinical exposures to peanut—in mice cosensitized to peanut and OVA—induced bystander OVA-specific GC activity in a nonspecific manner (Figure S4B). We employed a well-established flow cytometry method to detect *bona fide*, IgE⁺ GC B cells and PCs.^{31,47} A clear population of IgE⁺ GC B cells (Figure 6B,C) and PCs (Figure 6D,E) was exclusively detected in the iLNs in sensitized mice subjected to allergen re-exposure. Accordingly, peanut-specific IgE levels in circulation were detected in these mice

compared to sensitized but not re-exposed mice. Furthermore, splenocyte cultures with peanut extract from allergic mice exposed to the allergen, but not those from naïve mice, exhibited a Th1:Th2 ratio marked by IL-4 production (Figure 6G). In a similar fashion, we found that oral exposures to allergen after epicutaneous sensitization induced allergen-specific IgE responses in ~40% of the mice (Figure S5A). Furthermore, these mice underwent anaphylaxis despite macrophage depletion (Figure S5B), which suggests activation of the classical pathway. Collectively, these data show that subclinical exposures to allergen in epicutaneously sensitized mice induce allergen-specific GC activity, generation of IgE⁺ GC B cells and PCs, and allergen-specific IgE, thus likely enabling classical IgE-mediated food allergy.

4 | DISCUSSION

Defining the evolution of IgE-mediated food allergy in humans is exceedingly difficult because the immunological events underlying this process are clinically silent. The proposition that the skin epithelium can be a relevant site for Th2 sensitization to foods in humans^{9–12} has prompted the development of experimental models of epicutaneous sensitization to investigate the underlying mechanisms leading to the generation of IgE responses.^{13–19} While the role of a number of cytokines^{13,19,48} and innate cells^{18,49} in epicutaneous sensitization has been reported, the events pertaining to the generation of humoral immunity and memory remain unexplored. Here, we investigated the nature of the allergen-specific B-

cell response and its evolution from the initial stages of food sensitization.

We found that, as previously reported, tape-stripping of the skin induced damage and inflammation.^{50,51} The generation of damage (via mechanical, chemical, or biological stress) and subsequent release of alarmins, such as uric acid crystals, IL-25 or TSLP,^{17,26,48,52} that precondition DCs to initiate Th2 immunity is a common feature of allergic sensitization regardless of the route of allergen exposure.⁵³ Our data showed that the application of food allergens onto the tape-stripped skin induced allergen-specific GC activity. Its duration was shorter than that reported in models of Th2 immunity against helminths (eg, *Nippostrongylus brasiliensis*),^{44,54,55} likely because these models involve extended antigen availability.⁵⁶ We found that practically the entire GC B-cell compartment was expressing IgG1 throughout all stages of sensitization. This resulted in an allergen-specific IgG1 humoral response, likely maintained by long-lived PCs as the titers were sustained for, at least, 6 weeks following sensitization.

Mice underwent anaphylaxis on allergen challenge, and clinical reactivity was primarily effected by the alternative pathway—IgG, macrophages, and PAF. However, subsequent subclinical, nonsensitizing allergen exposures facilitated the evolution of the humoral response from an IgG1 (at the incipient stages of sensitization) to an IgE-dominated response (established allergy) thus likely enabling the classical pathway to mediate clinical reactivity. While the ability of the alternative pathway to mediate anaphylaxis in mice is well established,^{6,57} its contribution to human anaphylaxis is debatable and appears to be restricted to patients treated parentally with large quantities of biologics, drugs, or transfusions (eg, dextran, aprotinin, Willebrand factor, and immunoglobulin treatments).⁵⁸ Here, we show that IgG1 responses developed at the incipient stages of food sensitization, as it has been reported in birch-sensitized children,^{59,60} but that they may remain clinically undetectable.

Allergen-specific IgG1⁺ memory B cells established at the incipient stages of food sensitization were predominantly PDL-2[−]CD80[−]CD73⁺CD35⁺. This intimates that its generation (ie, GC history, and maturity), regardless of the allergen used for sensitization (peanut and/or OVA), was comparable. Recently, Weisel et al⁴⁶ described a population of early memory B cells that were PDL-2[−]CD80[−]CD73⁺ in a model of systemic immunization to a hapten (ie, NP) suggesting that the memory compartment originated during epicutaneous sensitization is rather immature. Anderson et al⁴⁵ reported a population of CD80[−]CD35⁺ memory B cells that were unmutated in a model of NP-alum, which is consistent with the brief GC reaction following epicutaneous sensitization. In experiments in which mice were cosensitized to peanut and OVA, we observed that the number of allergens at the time of sensitization—Ara h 1-17 for peanut vs OVA—limited the magnitude of the immune response (ie, clonal expansion, and GC activity) to each individual allergen, intimating the existence of mechanisms that regulate clonal expansion and diversity in the GC.^{37,61} It is known that allergens differ in their innate immunostimulatory properties^{15,62}; however, the features of the memory compartment that we identified were consistent regardless of the allergen specificity (ie, OVA, peanut, or cosensitization),

which may indicate that immunological features intrinsic to the skin epithelium govern the nature of the B-cell response.

Following nonsensitizing, subclinical allergen exposures, memory B cells generated allergen-specific GCs, in agreement with the functionality attributed to PDL-2[−]CD80[−] memory B cells.⁴³ Interestingly, secondary responses to peanut caused bystander OVA-specific GC activity in mice that were sensitized to both allergens. This might constitute a mechanism that perpetuates Th2 immunity in a non-specific manner, as reported by Bernasconi et al,⁶³ in the context of human vaccination. We employed a well-established flow cytometry method to detect *bona fide*, IgE⁺ GC B cells and PCs.^{31,47} The formation of IgE⁺ GC B and PCs, and production of IgE following allergen exposure indicate that IgG1⁺ B-cell immunity predates IgE responses in food allergy; a process that we show that is dominated by IL-4, and that is likely dependent on CD4 T-cell help and sequential class-switching.^{31,44,54,64} Importantly, a recent analysis of Ig heavy regions in peripheral blood of allergic patients demonstrated that a majority of IgE⁺ cells arise from antigen-experienced, somatically hypermutated, IgG1-expressing cells⁶⁵ suggesting that the IgG1 memory compartment matures likely due to allergen stimulation. This is supported by our observation that the predominantly immature, pro-GC, IgG1⁺ memory B-cell compartment (PDL-2[−]CD80[−]CD73⁺CD35⁺) that exists in the preclinical stages of epicutaneous sensitization partially shifted to a more mature PDL2⁺CD73⁺CD80⁺ phenotype on further allergen exposures.

Our findings indicate that IgG1⁺ B-cell immunity against food allergens dominates the initial stages of epicutaneous sensitization. This process results in the generation of an immature IgG1⁺ memory B-cell compartment that on subclinical, repeated allergen exposure predates IgE responses. The assessment of allergen-specific IgG⁺ memory B cells and humoral IgG may help to predict the risk for developing clinical allergy, thus providing a window for immunological interventions to prevent disease progression.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

RJS designed and conducted experiments, analyzed data, and wrote the manuscript. YE, JFEK, MEG, TDW, DR, PS, KB, and JW helped with experiments. JK and KF performed and analyzed data from experiments that involved telemetry measurements and IL-33 KO mice, respectively.

EIT, RK, and DKC provided reagents and/or input. SW and MJ obtained funding. MJ oversaw the project and edited the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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SUPPLEMENTARY DATA

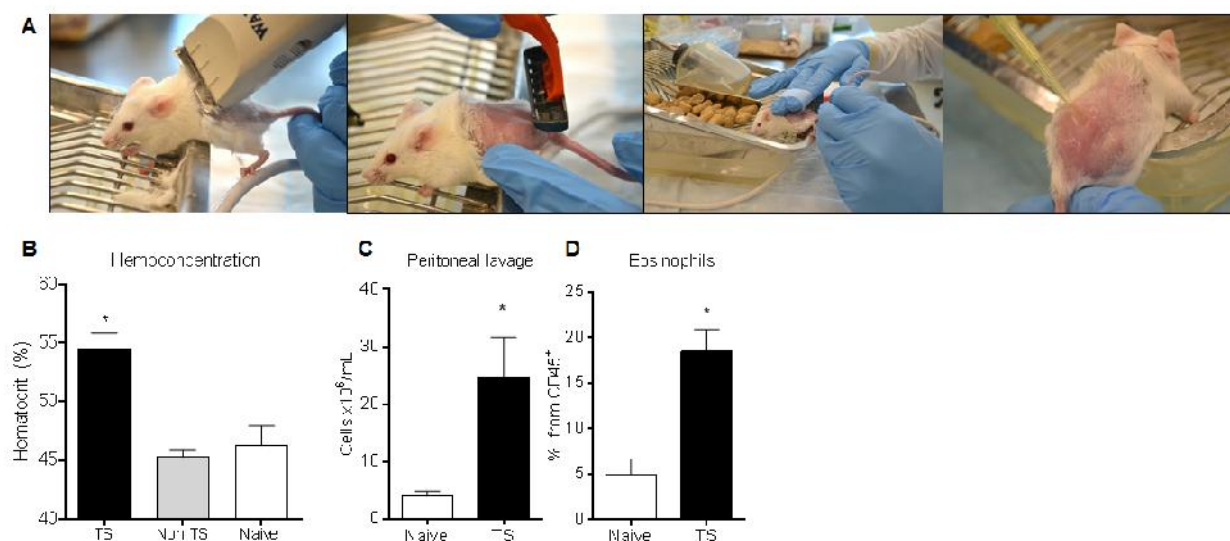


Figure S1. Characterization of the model of epicutaneous sensitization to food allergens. Separate groups of C57BL/6 and BALB/c mice were epicutaneously sensitized to peanut and/or OVA (TS) and representative photos of the process were taken (A). Some groups were shaved but not tape-stripped (Non-TS) followed by allergen application, and clinical reactivity on challenge was assessed by measuring hematocrit levels (B). Inflammation (C) and eosinophilia (D) in the peritoneal cavity 72 hours post-allergen challenge were assessed by performing total cell counts and flow cytometry analysis respectively. Pooled data from 2-3 independent experiments represented as mean $\pm$ SEM, $n=6-15$. * $P<0.05$ vs naïve.

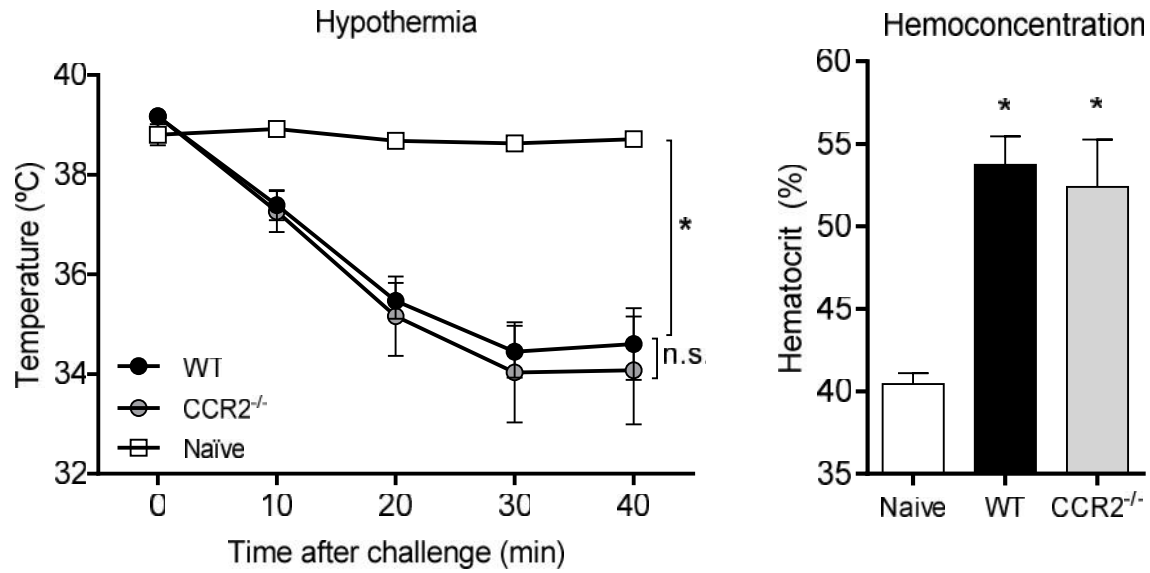


Figure S2. CCR2^{-/-} mice have unimpaired anaphylactic responses following epicutaneous sensitization. Wild type (WT) and CCR2^{-/-} mice on a C57BL/6 background were epicutaneously sensitized to peanut; 2 weeks after sensitization clinical reactivity on allergen challenge was assessed by measuring changes in core temperature (A) and hemoconcentration (B). Pooled data from 2 independent experiments represented as mean $\pm$ SEM, n=6-12. *P<0.05 vs naïve.

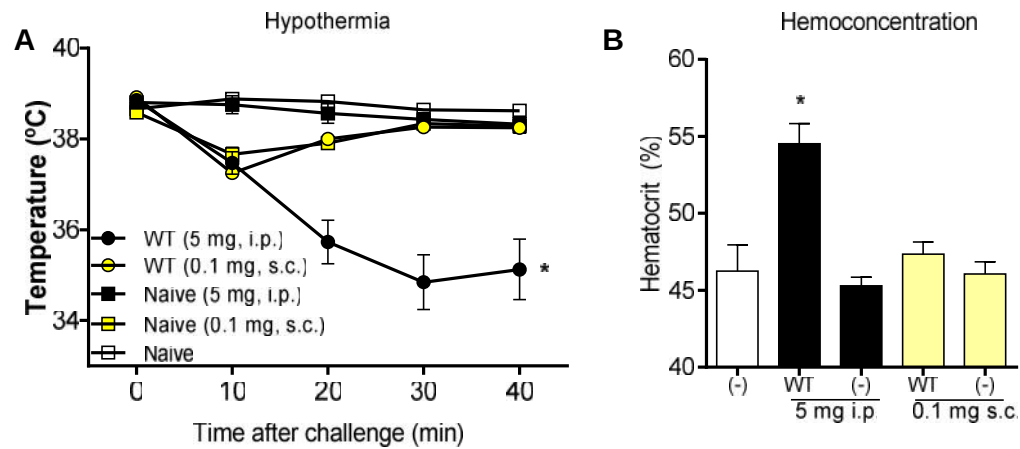


Figure S3. Subclinical exposures to allergen. Naïve (-) and peanut-allergic (WT) mice on a C57BL/6 background were anesthetized and subcutaneously (s.c.) administered 0.1 mg of peanut extract. Immediately after the mice were taken off the anesthesia, clinical reactivity was evaluated by measuring hypothermia (A) and hemoconcentration (B). Some groups of mice received 5 mg of peanut extract intraperitoneally (i.p.; without being anesthetized) as positive control. Pooled data from 3 independent experiments represented as mean $\pm$ SEM, n=10-15. *P<0.05 vs naïve.

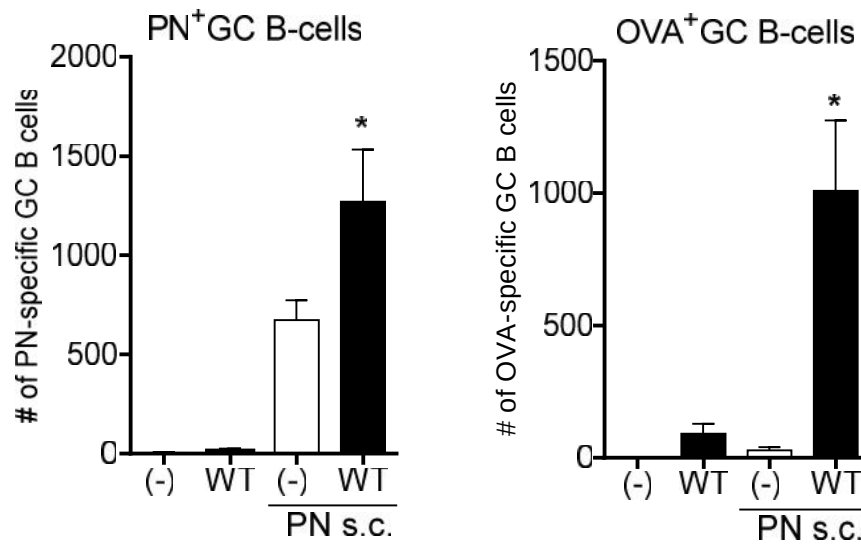


Figure S4. Allergen-specific GC activity following subclinical exposures to peanut in epicutaneously sensitized mice. Separate groups of C57BL/6 mice were epicutaneously sensitized (WT) to both peanut (PN) and OVA, and rested for 3-4 weeks. Some groups were subcutaneously (s.c.) exposed to 100 μ g of PN extract, 3 times. 2 days after the last exposure, the mice were sacrificed to assess GC B cells specific to PN (A) and OVA (B) by flow cytometry. Pooled data from 2 independent experiments represented as mean $\pm$ SEM, n=6-10. *P<0.05 vs naïve.

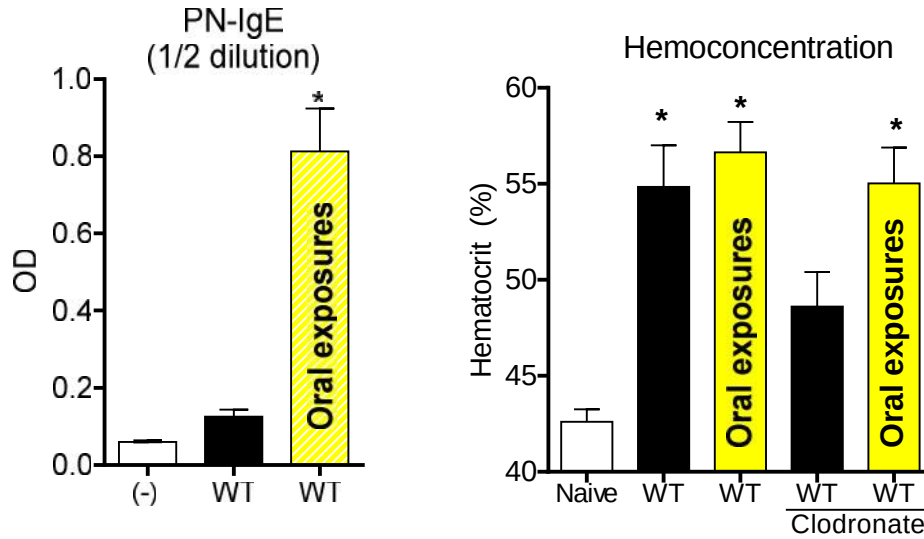


Figure S5. Oral exposures in epicutaneously sensitized mice induce allergen-specific IgE. Separate groups of C57BL/6 wild type (WT) mice were epicutaneously sensitized to peanut (PN). Some of the groups were orally exposed to allergen (3.75 mg of PN butter, ~ 1 mg of PN protein weekly for 3-4 weeks) and ~40% of them developed PN-specific IgE as measured by ELISA (A). Some of the groups that developed PN-specific IgE responses were depleted of phagocytes using clodronate-containing liposomes prior to challenge. Anaphylaxis was assessed measuring changes in hemoconcentration (B). Pooled data from 2-3 independent experiments represented as mean $\pm$ SEM, n=6-12. *P<0.05 vs naïve.