Impact of CD40 Ligand, B Cells, and Mast Cells in Peanut-Induced Anaphylactic Responses

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The effector immune mechanisms underlying peanut-induced anaphylaxis remain to be fully elucidated. We investigated the relative contribution of Igs, mast cells (MCs), and FcεRI in the elicitation of anaphylaxis in a murine model. Assessment of peanut hypersensitivity reactions was performed clinically and biologically. Our data show that wild-type (WT; C57BL/6 strain) mice consistently developed severe anaphylaxis (median clinical score: 3.5/5), an ~8°C drop in core body temperature, and significantly increased plasma levels of histamine and leukotrienes. CD40 ligand- and B cell-deficient mice presented evidence of allergic sensitization as demonstrated by production of Th2-associated cytokines by splenocytes and a late-phase inflammatory response that were both indistinguishable to those detected in WT mice. However, CD40 ligand- and B cell-deficient mice did not exhibit a late-phase inflammatory response upon airway and skin mucosal involvement of key immunological components in the elicitation of peanut hypersensitivity reactions. Our data show that oral sensitization to peanut in C57BL/6 mice generated peanut-specific Th1- and Th2-associated Igs and cytokines and provoked Th2-effector responses, namely systemic anaphylactic shock and late-phase allergic responses upon in vivo peanut recall. Examination of secondary lymphoid organs provided evidence of pervasive immune activation that was associated with the development of late-phase respiratory and cutaneous responses upon airway and skin re-exposure to peanut, respectively. In addition, our data demonstrate that CD40 ligand (CD40L)-, B cell- or mast cell (MC)-deficient mice failed to mount measurable anaphylactic responses, indicating that the presence of peanut-specific Igs along with functional MCs is necessary and sufficient for the elicitation of a peanut-induced anaphylactic event.


Materials and Methods

Animals

Female C57BL/6 mice (6–8 wk old) were purchased from Charles River Laboratories. B cell-deficient (B6.129S2-Igδ-6m1Cgn/J), CD40L-deficient (B6.129S2-CD40Lgααm1Cgn/J), MC-deficient (WBB6F1/J-kitW/v), and
Table I. Anaphylactic symptom score table

<table>
<thead>
<tr>
<th>Score</th>
<th>Symptoms</th>
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<tbody>
<tr>
<td>0</td>
<td>No clinical symptoms</td>
</tr>
<tr>
<td>1</td>
<td>Repetitive mouth/ear scratching and ear canal digging with hind legs</td>
</tr>
<tr>
<td>2</td>
<td>Decreased activity; self-isolation; puffiness around eyes and/or mouth</td>
</tr>
<tr>
<td>3</td>
<td>Periods of motionless for more than 1 min; lying prone on stomach</td>
</tr>
<tr>
<td>4</td>
<td>No response to whisker stimuli; reduced or no response to prodding</td>
</tr>
<tr>
<td>5</td>
<td>Endpoint: tremor; convulsion; death</td>
</tr>
</tbody>
</table>

FcεRI α-chain-deficient mice (C.129S2-FcεR1<sup>α-KO</sup>/J), along with the age-matched congenic normal mice, were purchased from The Jackson Laboratory. The mice were housed under a specific pathogen-free environment and maintained on a 12-h light-dark cycle. All experiments described in this study were approved by the Animal Research Ethics Board of McMaster University.

Model of PIA

Sensitization protocol. Mice were sensitized with oral gavage. To this end, mice were held in the supine position and orally administered 1 mg of peanut protein along with 10 μg of cholera toxin (CT; List Biological Laboratories) in 100 μl of sterile water by gavage once a week for 4 wk. Oral gavages were performed using intragastric feeding needles (01-290-2B; Fisher Scientific). We used peanut butter (Kraft) as a source of peanut protein (PP). In an initial series of experiments, four separate groups of sensitized mice were gavaged once a week for 1, 2, 3, or 4 wk.

Intraperitoneal challenge. Sensitized mice were challenged by injecting 5 mg of crude peanut extract (Greer Laboratories) in 500 μl of PBS into the peritoneal cavity 2 wk after the last sensitization. The mice were carefully observed for 40 min immediately following challenge. Anaphylactic responses during this lapse of time were assessed as follows:

1) Symptom score. Clinical symptoms were evaluated using the scoring system shown in Table I.

2) Core body temperature. Rectal temperature readings were performed every 10 min with a rectal probe digital thermometer (VWR).

3) Vascular permeability. Vascular leakage was determined by measuring albumin levels in the peritoneal lavage (PL) fluid 40 min after challenge. Briefly, the peritoneal cavity of anesthetized mice was injected with 3 ml of PBS, 10 mM EDTA. After a 1-min abdominal massage, a small incision was made on the peritoneal membrane and the PL fluid was gently aspirated. The aspirate was centrifuged at 600 rpm for 6 min at 4°C and the supernatant was collected and frozen at −70°C until used. The content of albumin was quantified in the supernatants using a commercial Bicinchoninic Acid kit (Sigma-Aldrich). Albumin levels of naive mice challenged with the same amount of crude peanut extract (CPE) were used as background levels. The concentration of albumin was calculated by comparison with a BSA standard curve.

4) MC mediator release. Histamine and leukotriene levels were determined in the plasma 40 min following challenge using enzyme immunoassay kits (Beckman Coulter and Cedarlane Laboratories, respectively) according to the manufacturer’s specifications. Plasma was obtained using K<sub>2</sub>-EDTA-containing tubes (BD Biosciences).

Intranasal challenge. Sensitized mice were lightly anesthetized and a 10-μl suspension containing 500 μg of CPE was delivered intranasally once daily for 5 consecutive days. Mice were sacrificed 2 days after the last challenge, and bronchoalveolar lavage (BAL) fluid was performed as previously described (19). Briefly, the lungs were dissected and the trachea was cannulated with a polyethylene tube (BD Biosciences). The lungs were lavaged twice with PBS (0.25 ml, followed by 0.2 ml); ~0.3 ml of the instilled fluid was consistently recovered. Total cell counts were determined in a blinded manner using a hemocytometer. The cell pellet was resuspended in PBS, and smears were prepared by cytocentrifugation (Thermo Shandon) at 300 rpm for 2 min. BAL smears were stained with the protocol Hema 3 stain set (Fisher Scientific). Differential cell counts were performed in a blinded manner. Plates were washed and serum samples were incubated overnight at 4°C.

Measurement of Ag-specific IgG

Peripheral blood was collected by retro-orbital bleeding 1 wk after the last sensitization. Serum peanut-specific IgG1 and IgG2a were measured by ELISA. Briefly, 96-well plate Maxi-Sorp plates (Nunc; VWR) were coated with 2 μg/ml CPE or with purified rat anti-mouse IgE Ab (BD Pharmingen) in 50 nM carbonate-bicarbonate buffer (pH 9.6; Sigma-Aldrich) overnight at 4°C.

Coated plates were blocked with 1% BSA/PBS for 2 h at room temperature. Plates were washed and serum samples were incubated overnight at 4°C. Each test sample was diluted as follows: for IgG1 detection 1/20, 1/200, 1/2,000, and 1/20,000 and for IgG2a detection 1/2, 1/20, 1/100, and 1/500. The reactions were developed with biotinylated goat anti-mouse IgG1, IgG2a, and IgE (Southern Biotechnology Associates), respectively, for 2 h at room temperature. Plates were washed and incubated with alcalase-phosphatase streptavidin (Sigma-Aldrich) for 1 h at room temperature. The color reaction was developed with p-nitrophenyl phosphate tablets and stopped with 2 M H<sub>2</sub>SO<sub>4</sub>. Ab titers were calculated using the formula 1/(x × ODX) × 0.05, where x equals the dilution factor closest to but

FIGURE 1. Induction of systemic anaphylaxis in orally sensitized mice following i.p. challenge with CPE. Mice were sensitized with peanut protein along with CT by intragastric gavage once a week for 4 wk. Two weeks after the last sensitization, mice were challenged with CPE. A, Peak anaphylactic symptom score of each individual mouse from naive (○) and sensitized (●) groups within 40 min after challenge. B, Changes in rectal temperature (mean ± SEM of naive and sensitized groups) following i.p. challenge at the indicated time points. Data are representative of three independent experiments. *, p < 0.05 compared with naive controls.
FIGURE 2. CPE-induced anaphylaxis is preceded by a polyspecific peanut-specific Ig response and accompanied by release of biological markers of anaphylaxis. A, Impact of the number of sensitizations on the production of peanut-specific IgGs. Four groups of mice were sensitized by oral route once, twice, three and four times, respectively. Figures show levels of peanut-specific IgE, IgG1, and IgG2a in serum samples collected 1 wk after the last sensitization. †, p < 0.05 compared with all other groups; *, p < 0.05 between the two linked groups. Histamine (B), Cys-leukotrienes (C) in plasma and albumin (D) in the peritoneal lavage fluid of naive and sensitized mice 40 min following CPE challenge. *, p < 0.05 compared with naive controls. Data are expressed as mean ± SEM; n = 8–12 mice/group. Results are representative of three independent experiments.

Greater than twice the background OD reading, and ODX is the OD reading of x. Sample IgG levels were expressed as units per milliliter. Serum peanut-specific IgE was measured by sandwich ELISA. Briefly, 96-well plate Maxi-Sorp plates (Nunc; VWR Canlab) were coated with 2 µg/ml purified rat anti-mouse IgE Ab (BD Pharmingen) in PBB overnight at 4°C. Coated plates were washed and blocked with 10% normal serum 1% BSA/PBS/0.5% Tween 20 for 1 h at 37°C. After washing, serum samples (four serial dilutions) were incubated for 2–3 h at room temperature. Each test sample was diluted as follows: 1/10, 1/50, 1/250, and 1/500. Subsequently, a CPE-digoxigenin (DIG) conjugate solution was added (1 h at 37°C). The coupling of DIG to CPE was performed according to the manufacturer’s instructions (Roche). After incubation (1 h at 37°C) with peroxidase-conjugated anti-DIG fragments (Roche), a tetramethylbenzidine substrate (0.1 mg/ml) solution was used and the color reaction was stopped with 2 M H2SO4. Absorbance was measured at 450 nm and results expressed as nanograms of DIG-CPE bound times the dilution factor.

Splenocytes and lymph node (LN) cell cultures
Spleens or LNs (inguinal, mediastinal, and mesenteric) were excised from naive and sensitized mice 1 wk after the last sensitization and placed into sterile tubes containing sterile HBSS on ice. Spleens/LNs were triturated between the ends of sterile frosted slides and the resulting cell suspension was filtered through a 70-µm nylon cell strainer (Falcon; BD Biosciences), then washed at 1200 rpm for 10 min at 4°C. RBC were lysed from spleens by adding 1 ml of ACK lysis buffer (0.5 M NH4Cl, 10 mM KHCO3, and 0.1 mM Na2EDTA (pH 7.2–7.4)) for 1 min. Splenocytes and dispersed LN cells were washed once with HBSS and then resuspended in RPMI 1640 medium supplemented with 10% FBS (Sigma-Aldrich), 1% l-glutamine, 1% penicillin/streptomycin (Invitrogen Life Technologies), and 0.1% 2-ME (Invitrogen Life Technologies). Cells were seeded at 8 × 106 cells/well (spleen) or 5 × 106 cells/well (LNs) in a flat-bottom, 96-well plate (BD Biosciences) and cultured in medium alone or with 50 µg/ml CPE/well. Following 120 h of culture incubation, supernatants were harvested and stored at −20°C for cytokine measurement.

Cytokine measurements
Levels of IL-4, IL-5, IL-10, IL-13, and IFN-γ were determined using Beadlyte mouse multicytokine fluorescent bead-based FLEX assays (Upstate Biotechnology) as previously described, and quantified using a Luminex 100 instrument according to the manufacturer’s instructions. In some instances, cytokine levels were determined using ELISA kits for murine IL-4, IL-5, and IL-13 purchased from R&D Systems. Each of these essays has a threshold of detection between 1.5 and 5 pg/ml.

Data analysis
Data are expressed as mean ± SEM. Statistical analysis was performed using SigmaStat software (SPSS). When applicable, results were analyzed using one- or two-way ANOVA with repeated measures followed by Tukey post-hoc test. In cases of raw data failing normality test, the ANOVA test on ranking was performed followed by pairwise comparisons using Dunn’s methods. An unpaired Student t test (two tailed) was used when only two sets of data were compared. A p value of <0.05 was considered statistically significant.

Results
Induction of acute systemic anaphylaxis by peanut challenge in sensitized wild-type (WT) mice
To assess the development of systemic anaphylaxis, clinical and biological responses were carefully monitored in sensitized and challenged mice. We observed clinical symptoms within a few minutes after challenge (Fig. 1A). Starting with continuous muzzle scratching and ear canal digging with hind legs (score 1/5, see Table I), symptoms rapidly progressed to puffiness around eyes and/or mouth and self-isolation (score 2/5). Most mice experienced prolonged periods of motionless that gradually worsened to no response to whisker stimuli and only slight reaction to prodding (average score 3.5/5). The most severe reactions provoked loss of consciousness, tremor, and death. The kinetics and severity of the anaphylactic reactions were consistent among mice. Naive mice did not develop any clinical symptoms upon CPE challenge. As shown in Fig. 1, core body temperature readings were inversely correlated with symptom scores. Sensitized mice, but not naive

FIGURE 3. Intraperitoneal CPE challenge evokes a late-phase inflammatory response in the peritoneal cavity of orally sensitized mice. A, Representative photomicrographs of slide-mounted inflammatory cells collected from the peritoneal lavage fluid of naive and sensitized mice 72 h following i.p. challenge and stained with H&E. B, Total inflammatory and differential cell counts at the indicated time points following i.p. challenge. Data represent mean ± SEM; n = 4–6 mice/group. *, †, and ‡, p < 0.05 compared with 0 min, 40 min, and 24 h, respectively. Results are representative of three independent experiments.
controls, experienced a marked drop (up to 9°C) in body temperature during the 40-min observation period following i.p. challenge (Fig. 1B). Mice sensitized to peanut but challenged with saline did not manifest any anaphylactic reactions (data not shown).

To investigate the impact of oral sensitization on humoral responses, we evaluated serum peanut-specific IgGs generated in response to increased number of oral sensitizations. To this end, we sensitized four groups of mice with one, two, three or four oral administrations of PP and CT. Serum samples were obtained by retroorbital bleeding one day before i.p. challenge with CPE. We observed that repeated delivery of PP and CT significantly increased the levels of peanut-specific IgE, IgG1, and IgG2a with the highest Ig titers achieved following four oral gavages (Fig. 2A). Importantly, the number of oral sensitizations and, thus, the level of peanut-specific IgGs closely correlated with the severity of the anaphylactic reactions. Peanut-specific Abs were not detected in naive mice.

The development of clinical signs and symptoms of anaphylaxis was accompanied by a marked increase in biological markers of anaphylaxis. We detected significantly greater histamine (≈50-fold increase) and Cys Leukotriene C (LTc) (≈10-fold increase) levels in the plasma of peanut-sensitized mice following CPE challenge compared with naive mice (Fig. 2, B and C). Moreover, increased vascular permeability was demonstrated by a near 20-fold increase in the content of albumin in the PL fluid (Fig. 2D) of sensitized challenged mice compared with naive challenged mice.

Late-phase inflammatory responses at the site of immunological challenge

Next, we examined late-phase cellular responses in the peritoneal cavity at various time points following i.p. challenge. We observed a marked and time-dependent cellular inflammatory response in the PL fluid of sensitized, but not naive, mice following CPE challenge (Fig. 3). Peak cellular infiltration occurred 72 h after challenge, with a near 8-fold increase in total cell number compared with naive mice. As shown in Fig. 3B, inflammation was initially characterized by a considerable, albeit transient, accumulation of neutrophils (before challenge: 0.2% vs 24 h after challenge: 25% ± 2%) followed by a marked increase in the proportion of both lymphocytes (before challenge: 3 ± 1% vs 72 h after challenge: 18 ± 2%) and eosinophils (before challenge: 0.4 ± 0.2% vs 72 h after challenge: 12 ± 2%). The increase in the proportion of inflammatory cells consistently correlated with the increase in the overall number of cells in the peritoneal cavity (data not shown).

Impact of intranasal and cutaneous Ag challenge, in sensitized mice

We next investigated whether oral sensitization would also give rise to allergic inflammatory responses in distant compartments (i.e., lungs and skin) upon Ag re-exposure in vivo. Specifically, we examined the impact of respiratory and cutaneous exposure to CPE...
following oral peanut sensitization. To this end, mice were sensitized to peanut via oral gavage and exposed to CPE intranasally once daily for 5 consecutive days. Histological analysis of lung tissues demonstrated that intranasal (i.n.) challenge of orally sensitized mice resulted in marked peribronchial and perivascular infiltrates. These mice demonstrated that intranasal (i.n.) challenge of orally sensitized mice 72 h after i.p. CPE challenge. C. Percentage of eosinophils among total peritoneal inflammatory cells. Data represent mean ± SEM; n = 4–6/group, and are representative of two independent experiments; ∗, p < 0.05 compared with naive mice.

Impact of CD40L and B cell deficiencies on PIA

To evaluate the role of humoral immunity in the elicitation of PIA, we used a system where Ig responses are impaired due to selective gene-targeted mutations, namely CD40L- and B cell-deficient mice. First, we ascertained that both genetically deficient mice were unable to produce peanut-specific Igs (Fig. 6A). Importantly, when challenged with CPE, these mice failed to show any overt indication of anaphylaxis (Fig. 6B). To address the impact of CD40L and B cell deficiencies on the induction of peanut-specific cellular responses, we harvested splenocytes from sensitized WT, CD40L−/−, and B cell−/− mice and subsequently measured the production of peanut-specific cytokines following in vitro stimulation with either CPE or medium alone. We found that oral sensitization resulted in similar production of IL-4, IL-5, and IL-13 by splenocytes cultures from both transgenic and WT mice (Fig. 7A). Of interest, CPE stimulation led to significantly higher IFN-γ production in splenocytes cultures from B cell−/− mice compared with WT or CD40L−/− mice. Thus, sensitized CD40L−/− and B cell−/− mice, albeit clinically unresponsive to i.p. CPE challenge, still exhibited clear evidence of peanut sensitization. Furthermore, the late-phase inflammatory response in the peritoneal

A

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<th>TNF-α (pg/ml)</th>
<th>IL-12 (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
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<tr>
<td>WT</td>
<td>40 ± 2</td>
<td>50 ± 3</td>
</tr>
<tr>
<td>CD40L−/−</td>
<td>25 ± 1</td>
<td>40 ± 2</td>
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<td>B cell−/−</td>
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C

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<th>TNF-α (pg/ml)</th>
<th>IL-12 (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
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<td>B cell−/−</td>
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between the two groups. 

Importantly, we detected almost anaphylactic reactions. In addition, no histamine was detected in systemic anaphylaxis, MC-deficient mice experienced no measurable whereas littermate controls exhibited signs and symptoms of systemic anaphylaxis, MC-deficient mice experienced no measurable anaphylactic reactions. In addition, no histamine was detected in the plasma of KitW/Kit W-v mice. Importantly, ultrastructural analysis demonstrated that MCs are significantly higher eosinophils (−10%).

Impact of MC deficiency on PIA

That murine studies have previously reported the induction of active systemic anaphylaxis in the absence of MCs prompted us to investigate the role of MC activity as an effector mechanism in PIA. To this end, genetically MC-deficient (WBB6F1/J-KitW/ KitW−/−) and littermate control (FcerRI−/−, □) mice were sensitized and challenged through the oral route has been previously reported (12); however, the responses documented are, compared with the responses that we observed, relatively modest and considerably more variable. Indeed, our data show that 80% of mice reached a clinical score of ≥3.5 and a very robust −8°C drop in core body temperature. We did perform a number of detailed experiments using previously reported protocols and explored a series of methodological variations including: an increase in the number of oral sensitizations to up to eight, executing two oral challenges rather than one (17, 21), and increasing gut permeability and absorption of Ag with ethanol (22) at the time of challenge. However, we were not able to elicit consistent anaphylactic reactions not only in C57BL/6 mice but also in C3H/HeJ mice. Thus, we currently do not have an explanation for our inability to generate systemic anaphylaxis in orally sensitized mice upon oral exposure to peanut. Despite this, the robustness and consistency of systemic anaphylactic responses that are elicited with i.p. challenge represent a notable experimental advantage. Moreover, because the peritoneal cavity is a closed anatomical compartment, it provides a most useful site for the evaluation of both immediate and late-phase biomarkers of anaphylactic responses. In this respect, the assessment of albumin and late-phase inflammatory responses after challenge that we furnish here illustrate this point. Of particular interest, that this model was established in C57BL/6 mice, the most commonly used background to breed gene-targeted mice, facilitates the use of knockout mice to decisively investigate the contribution of molecular pathways involved in peanut hypersensitivity.

In addition to investigating systemic anaphylaxis, we examined the consequences of re-exposing orally sensitized mice to peanut in compartments other than the peritoneum, namely lung and skin.

FIGURE 9. FcerRI deficiency partially disrupts PIA. A, The graph depicts serum levels of peanut-specific IgE, IgG1, and IgG2a from FcerRI-deficient (FcerRI−/−, □) and littermate control (FcerRI+/+, ■) mice 4 wk following four oral sensitizations. B, Peak anaphylactic symptom score and rectal temperature in FcerRI−/− and FcerRI+/+ mice 40 min following i.p. CPE challenge. Data show mean ± SEM and are representative of two independent experiments; n = 8 mice/group; *, p < 0.05 compared between the two groups.

cavity of CD40L−/− and B cell−/− mice was statistically indistinguishable to that observed in WT mice (Fig. 7B), with an ~8-fold increase in total cells compared with naive mice, and significantly higher eosinophils (~10%).

Impact of FcerRI deficiency on PIA

To investigate the relative contribution of the high-affinity IgE receptor, FcerRI, in PIA, we used mice with a targeted mutation that results in failure of production of the α-chain of the FcerRI. As shown in Fig. 9A, both FcerRI α-chain −/− mice and FcerRI α-chain +/+ mice (WT) controls produced identical levels of peanut-specific IgE following peanut oral sensitization. Despite the presence of peanut-specific Abs, clinical markers of systemic anaphylactic responses were partially abrogated in FcerRI α-chain −/− mice compared with those observed in FcerRI α-chain +/+ mice.

Discussion

The precise mechanisms underlying the pathogenesis of PIA remain to be fully elucidated. The objective of this study was to evaluate the relative contribution of humoral and MC responses in the elicitation of this event. To this end, we developed a murine model in C57BL/6 mice that involved oral sensitization with peanut Ag along with CT followed by an i.p. challenge with CPE. This protocol consistently evoked the rapid onset of severe anaphylactic responses as indicated by severe clinical symptoms, a significant drop in core body temperature, marked MC mediator release (i.e., histamine and leukotrienes) and pronounced vascular leakage. Moreover, we examined immune-inflammatory responses in the peritoneal cavity at various time points following CPE challenge. We observed a marked and time-dependent cellular inflammatory response that was initially characterized by a considerable, albeit transient, accumulation of neutrophils followed by a significant increase in the number of both lymphocytes and eosinophils. Of note, i.p. challenge resulted in a rapid and sustained decrease in the number of MCs detected within the peritoneal cavity. In this regard, Claman et al. (20) showed that immunological processes in chronic graft-vs-host disease cause a slow release of MC granules that leads to an apparent disappearance of dermal MCs, as assessed by toluidine blue staining. As this stain is a marker for mature MC granules, the authors suggested that the loss of granule substances may account for the failure to identify MCs by toluidine blue. Importantly, ultrastructural analysis demonstrated that MCs are present in graft-vs-host disease skin and showed granule depletion and expansion, the first steps in degranulation. These MCs, invisible by toluidine blue stain but visible with the electron microscope, were referred to as “phantom MCs” (20). We used H&E staining to evaluate inflammatory responses including MC responses. However, we confirmed mast numbers with toluidine blue staining. Thus, we suggest that a phenomenon similar to that proposed by Claman et al. (20) may very likely explain the apparent decrease in the number of MCs following peanut challenge.

It may be of significance to note that induction of systemic anaphylaxis in mice sensitized orally to peanut and subsequently challenged through the oral route has been previously reported (12); however, the responses documented are, compared with the responses that we observed, relatively modest and considerably more variable. Indeed, our data show that 80% of mice reached a clinical score of ≥3.5 and a very robust −8°C drop in core body temperature. We did perform a number of detailed experiments using previously reported protocols and explored a series of methodological variations including: an increase in the number of oral sensitizations to up to eight, executing two oral challenges rather than one (17, 21), and increasing gut permeability and absorption of Ag with ethanol (22) at the time of challenge. However, we were not able to elicit consistent anaphylactic reactions not only in C57BL/6 mice but also in C3H/HeJ mice. Thus, we currently do not have an explanation for our inability to generate systemic anaphylaxis in orally sensitized mice upon oral exposure to peanut. Despite this, the robustness and consistency of systemic anaphylactic responses that are elicited with i.p. challenge represent a notable experimental advantage. Moreover, because the peritoneal cavity is a closed anatomical compartment, it provides a most useful site for the evaluation of both immediate and late-phase biomarkers of anaphylactic responses. In this respect, the assessment of albumin and late-phase inflammatory responses after challenge that we furnish here illustrate this point. Of particular interest, that this model was established in C57BL/6 mice, the most commonly used background to breed gene-targeted mice, facilitates the use of knockout mice to decisively investigate the contribution of molecular pathways involved in peanut hypersensitivity.

In addition to investigating systemic anaphylaxis, we examined the consequences of re-exposing orally sensitized mice to peanut in compartments other than the peritoneum, namely lung and skin.
Our data demonstrate that CPE re-exposure via the respiratory mucosa elicited pronounced late-phase peribronchial and perivascular inflammation characterized by marked eosinophilic infiltration. Similarly, exposure to peanut in the skin by way of i.d. ear injection of CPE evoked late-phase cutaneous responses, as demonstrated by ear thickness with a concomitant influx of eosinophils into the ear tissue. The current understanding is that the initial site of Ag priming, i.e., the draining LNs, where dendritic cells (DCs) carrying Ag first interact with T cells, determines the future homing of memory/effectector T cells and, hence, the site at which immune-inflammatory responses will manifest upon Ag re-exposure (23–27). This notion along with the observations that we made in the lung and skin prompted us to comprehensively seek for evidence of peanut-specific immunity in a variety of LNs. Interestingly, our data demonstrate production of peanut-specific cytokines not only in GI-draining (mesenteric) but also in distant (non-GI-draining such as inguinal and thoracic) LNs following in vitro peanut stimulation, suggesting that peanut oral sensitization is, under these experimental conditions, pervasive.

The presence of eosinophilia in the peritoneum, lung, and ear after peanut challenge of sensitized mice is a hallmark of Th2 immunity. Hence, we further investigated the peanut-specific effector profile generated following oral peanut sensitization. Our data show that in vitro peanut recall induced a similar cytokine profile in splenocyte, GI-, and non-GI-draining LN cultures, which was characterized by both Th1 (IFN-γ) and Th2 (IL-4, IL-5, IL-13) associated cytokines. Moreover, sensitization led to the production of both peanut-specific Th1 (IgG2a) and Th2 (IgE and IgG1) associated Igs. The detection of both Th1 and Th2 effector biomarkers following oral priming in the model presented here in C57BL/6 mice is in agreement with previous reports in BALB/c (16), C3H/HeJ (15), and C3H/HeOuJ (28) mice. Collectively, these findings intimate that there is not a linear relationship between the mere detection of Th1 and/or Th2 markers and the expression of Th1 and/or Th2 immunity (29–34).

To investigate the role of Igs in PIA, we performed experiments in mice unable to generate Igs, namely CD40L- and B cell-deficient mice, both of which have a C57BL/6 background. That these mice failed to mount any measurable anaphylactic responses demonstrates that Igs are critically required in this process. Importantly, the inability to elicit anaphylaxis cannot be attributed to defective sensitization. Indeed, splenocyte cultures from WT, CD40L-/-, and B cell-/- mice produced similar levels of Th2-affiliated cytokines upon in vitro recall. Of note, peanut-specific IFN-γ was significantly elevated in B cell-/- mice compared with WT and/or CD40L-/- mice. This is in accordance with observations made in studies where B cell-deficient mice were infected with Schistosoma mansoni eggs (35). In particular, a study by Hernandez et al. (35) showed that LN cells from schistosome-infected B cell-deficient mice produce significantly more Th1-associated cytokines ( IFN-γ and IL-12) than cells from control mice following in vitro stimulation with soluble schistosomal egg Ag. Similarly, irradiated splenocytes from B cell-deficient mice elicited stronger Th1 responses in schistosomal egg Ag-specific CD4+ cells than splenocytes from normal mice (35). These findings may be explained by the ability of B cells to regulate the Th1/Th2-polarized effector function of DCs (36). In fact, it has been shown that increased production of IL-12 by DCs from B cell-deficient mice results in the induction of Th1-polarized responses (37). Further evidence of sensitization in CD40L-/- and B cell-/- mice is indicated by the development of a late-phase allergic inflammatory response, including eosinophilia, in the peritoneal cavity following peanut challenge that is comparable to that observed in WT mice. The generation of late-phase responses observed in CD40L-/- mice is in agreement with studies showing a comparable degree of pulmonary inflammation in presensitized CD40L-/- and WT mice following i.n. challenge with OVA (38) and Aspergillus fumigatus Ag (39). Thus, our findings provide evidence of systemic sensitization in CD40L-/- and B cell-/- mice and suggest that while Th2-associated cytokines are an inherent marker of allergic sensitization, they are not directly required to elicit the anaphylactic event.

Presently, two distinct mechanisms have been identified to induce systemic anaphylaxis in the mouse (40). The classical pathway is mediated by Ag cross-linking of IgE bound to the high-affinity receptor (FceRI) on MCs. Activation of MCs results in the rapid release of preformed granule-associated molecules such as histamine and serotonin, and newly synthesized lipid-derived mediators such as PGD2, platelet-activating factor, and leukotrienes. The alternative pathway is thought to involve macrophages, FcyIII, IgG Abs, and platelet-activating factor (41). The finding that treatment of peanut allergic individuals with a humanized IgG1 mAb against the Fc portion of IgE increases the threshold of sensitivity only partially (42) suggests the potential contribution of both pathways in PIA. However, the relative contribution of the individual components involved in the elicitation of food-induced anaphylaxis, and specifically PIA, remains to be explored. To investigate the role of MCs in this process, we evaluated clinical and biological markers of systemic anaphylaxis in orally sensitized MC-deficient (WBB6F1/J-Kit+/Kit-W/-) mice following peanut challenge. Our data show that despite elevated levels of peanut-specific Igs, MC-deficient mice failed to develop peanut-induced anaphylactic responses, hence establishing a preeminence effector role for MCs in this experimental model. The absence of measurable anaphylactic responses in MC-deficient mice was very likely due to lack of MCs; however, this was not formally proven via reconstitution.

To investigate the relative contribution of FceRI in this model, we evaluated clinical and biological markers of systemic anaphylaxis in orally sensitized FcεRIα-chain-deficient mice following peanut challenge. It is noteworthy that, in these mice, the genes encoding the common γ-chain of the FceRI and the FcγRIII are intact. Previous studies have demonstrated that the absence of FcεRI α-chain leads to up-regulation of FcγRIII-dependent MC degranulation (43). This is relevant to our findings because we observed that absence of the FcεRI α-chain did not entirely abrogate anaphylactic responses. It has been reported that mouse macrophages do not express FceRI (40, 44, 45) and, in addition, there is evidence that both IgE and IgG1 can bind to the FcγRIII on MCs (46–49). Hence, the partial protection observed in FcεRI-deficient mice may suggest that the remaining anaphylactic response may be mediated via IgG1-dependent activation of FcγRIII on MCs. It is important to note, however, that the partial responses observed in FcεRI-deficient mice do not demonstrate that the FcγRIII pathway is important in the native animal. This notion may be of therapeutic relevance given that, in humans, the use of Abs that block IgE binding to FcεRI has been shown to be of clear but partial benefit to abrogate anaphylactic responses; strategies to additionally impair MC degranulation might be necessary to increase the efficacy of this therapy.

In short, our data provide direct experimental evidence that peanut-specific Igs along with functional MCs constitute the necessary and sufficient effector requirements for the elicitation of peanut-induced systemic anaphylaxis. In addition, our findings encourage further investigation into the role of FcRs as it may contribute to a better understanding of the mechanisms underlying PIA and, hence, to the design of novel therapeutic approaches.
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Disclosures

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