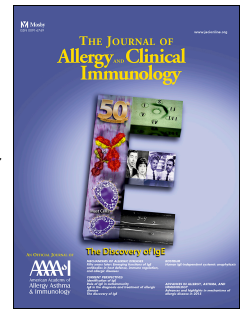


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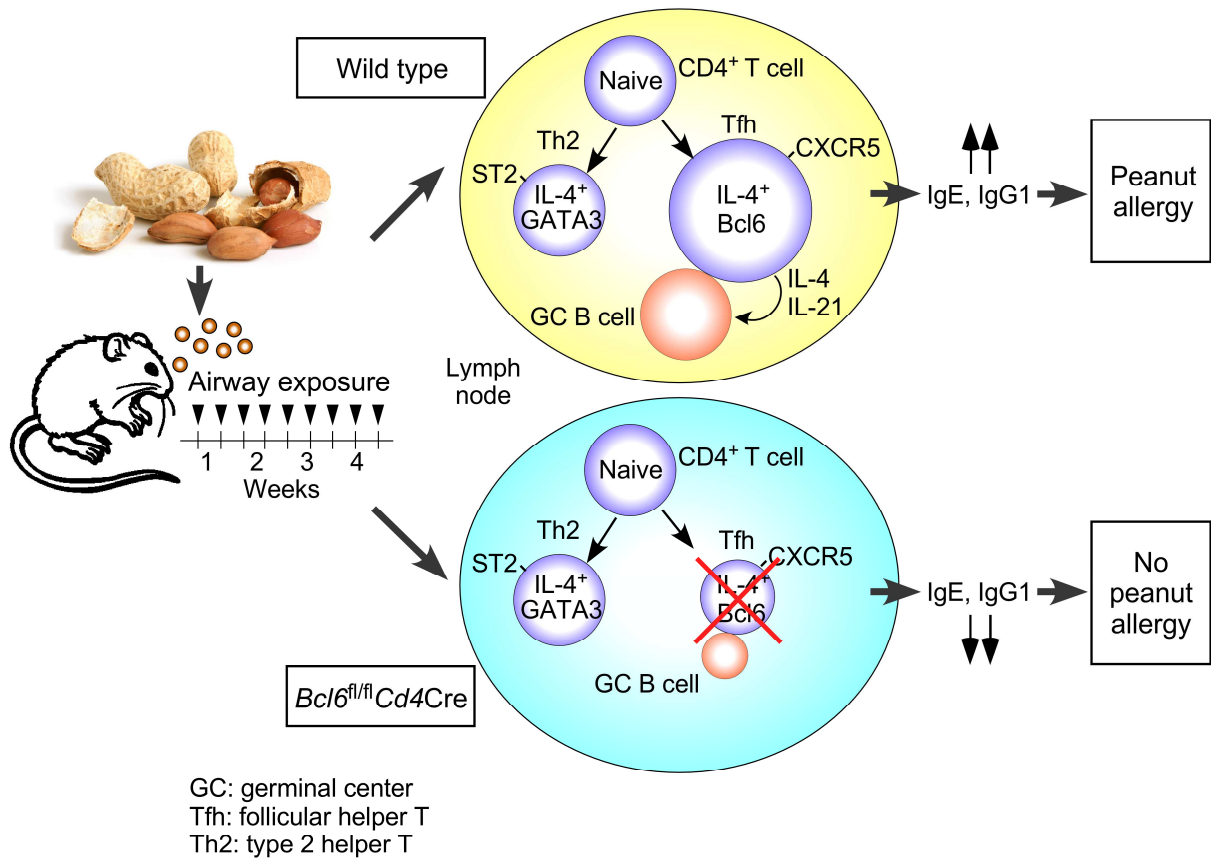
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Airway exposure initiates peanut allergy through follicular helper T cells in mice



Graphical abstract

**Airway exposure initiates peanut allergy by involving the IL-1 pathway and T follicular
helper cells in mice**

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ABSTRACT

Background: Little is currently known regarding the immunological mechanism(s) that initiate peanut allergy. Notably, peanut proteins have been detected in house dust, and their levels correlate with peanut allergy prevalence.

Objective: This study aimed to develop a new mouse model for peanut allergy and to investigate the immunological mechanisms involved in peanut allergen sensitization.

Methods: To mimic environmental exposure, naïve mice were exposed to peanut flour by inhalation for up to 4 weeks. We then analyzed serum levels of IgE antibody and challenged mice with peanut proteins. Immunological mechanisms involved in sensitization were analyzed using cytokine reporter mice, an adoptive cell transfer model, and gene knockout mice.

Results: When exposed to peanut flour by inhalation, both BALB/c and C57BL/6 mice developed peanut allergy, as demonstrated by the presence of peanut-specific IgE antibodies and manifestation of acute anaphylaxis upon challenge. A large number of follicular helper T (Tfh) cells were also detected in draining lymph nodes of allergic mice. These cells produced IL-4 and IL-21, and more robustly promoted peanut-specific IgE production than Th2 cells. Genetic depletion of Tfh cells decreased IgE antibody levels and protected mice from anaphylaxis, without affecting Th2 cells. Furthermore, peanut flour exposure increased lung levels of IL-1 α and IL-1 β , and mice deficient in the receptor for these cytokines showed a significant decrease in Tfh cells compared to wild-type mice.

Conclusion: Tfh cells play a key role in peanut allergy, and the IL-1 pathway is involved in the Tfh response to peanut allergen exposure.

Key Messages

- Inhalation of peanut flour induces naïve mice to develop clinical responses consistent with peanut allergy in humans.
- IL-4-producing Tfh cells develop in draining lymph nodes when mice are exposed to peanut flour.
- Tfh cells are essential for IgE antibody production and peanut allergy symptoms.
- IL-1 plays key roles in the Tfh response to peanut allergen exposure.

Capsule Summary

Using mouse models, Dolence *et al.* demonstrate that inhalation of peanut flour induces peanut allergy, and both Tfh cells and IL-1 are critical for disease development.

Keywords

Follicular T cells, IL-4, IgE, allergy, peanut, IL-1, IL-33

Abbreviations

BAL: bronchoalveolar lavage; BSA: bovine serum albumin; CTX: cholera toxin; eGFP: enhanced green fluorescent protein; FACS: fluorescence-activated cell sorting; GC: germinal center; HDM: house dust mite; IL: interleukin; IL-1RAcP: IL-1R-associated protein; i.n.: intranasally; i.p.: intraperitoneal; i.v.: intravenously; MCPT-1: mast cell protease-1; mLN: mediastinal lymph node; LEAP: Learning Early About Peanut Allergy; LNs: lymph nodes; OVA: ovalbumin; PD-1: programmed cell death protein 1; PN: peanut; PBS: phosphate-buffered saline; RT: room temperature; SEB: staphylococcal enterotoxin B; SEM: standard error of the mean; SNPs: single nucleotide polymorphisms; SPF: specific pathogen-free; Tfh: follicular

64 helper T; Th2: type 2 helper T; TLR4: toll-like receptor 4; TSLP: thymic stromal lymphopoietin;

65 WT: wild-type

INTRODUCTION

Food allergy is a growing public health concern that impacts approximately 4% of adults and 8% of children in the U.S.¹, and frequencies of food allergies are on the rise worldwide². In particular, the incidence of peanut allergy has more than tripled in U.S. children, increasing from 0.4% in 1997 to 1.4% in 2008³. In contrast to milk or egg allergies that are commonly outgrown in childhood, peanut allergy is often life-long². The majority of fatal food-induced anaphylaxis is associated with peanut allergy⁴, and avoidance of peanut exposure is difficult to achieve due to the popularity of peanut in our society⁵. As a result, peanut allergy can negatively impact quality of life, as well as the psychosocial status of patients and their families⁵. Therefore, it is critical to better understand the immunologic mechanisms involved in development and persistence of peanut allergy and to identify novel strategies to prevent and/or to effectively treat this disease.

Although mice and humans differ in many ways, mouse models provide robust tools to elucidate the immunological mechanisms of human diseases. In particular, both the skin and oral sensitization models for peanut allergy implicate type 2 helper T (Th2) cells in driving the allergic response to peanut⁶⁻⁸. An IL-1-family cytokine, IL-33, has further been shown to play an important role in the development of Th2 responses in these models^{6,7}, and long-lived peanut-specific memory B cells that replenish IgE⁺ plasma cells likely sustain clinical reactivity in mice⁹. Nevertheless, the molecules and cell types that drive the development of peanut allergy in humans are not fully understood. Furthermore, a majority of earlier mouse models used mucosal adjuvants, such as cholera toxin (CTX)⁷ and Staphylococcal enterotoxin B (SEB)^{10,11}, or required genetic alteration of toll-like receptor 4 (TLR4)¹² or IL-4 receptor¹³, making it difficult to determine the precise immunological mechanisms involved in the initiation of peanut allergy.

Peanut allergen sensitization has previously been thought to occur as a consequence of

the ingestion of dietary peanut products. However, ingestion of innocuous antigens generally results in oral tolerance¹⁴, and the majority of children with peanut allergy experience their first allergic reaction upon their first ingestion of peanut¹⁵. Additionally, the recent Learning Early About Peanut Allergy (LEAP) study found that early dietary introduction of peanut prevented development of peanut allergy, whereas a greater proportion of children who avoided dietary peanut developed peanut allergy¹⁶. These data suggest that patients have been sensitized to peanut proteins in their environment. Indeed, peanut proteins are readily detectable in house dust at levels comparable to those of inhaled allergens, such as house dust mite (HDM)^{17, 18}, and a dose-response relationship between environmental peanut exposure and the risk of peanut allergy has been observed^{19, 20}. A number of clinical studies have further demonstrated an association between atopic dermatitis and peanut allergy in children²⁰, suggesting that allergic sensitization to peanut is mediated through impaired skin. Consistent with this, epicutaneous exposure to crude peanut extract was found to promote Th2-type sensitization to peanut proteins in mice⁶.

Despite these studies, and the well-established evidence for the presence of peanut proteins in house dust, it remains unclear whether and how non-oral exposure initiates peanut allergy. In order to address this question, we developed a mouse model for inhalation-based peanut allergen sensitization. To mimic natural environmental exposure, we exposed naïve mice to peanut flour by inhalation and found that these animals develop anti-peanut IgE antibodies and clinical symptoms resembling peanut allergy in humans. We further determined that follicular helper T (T_{fh}) cells that produce elevated levels of IL-4 and IL-21 are generated in draining lymph nodes and that they promote production of peanut-specific IgE. Thus, T_{fh} cells are likely to be critical for the development of peanut allergy in our model.

METHODS

See the Methods section of this article's Online Repository for more details.

Mice

BALB/c, C57BL/6, C.C3-*Tlr4*^{Lps-d}/J (*Tlr4*^d), C.129-*Il4*^{tm1Lky}/J (4get), Tg(Cd4-cre)1Cwi/BfluJ (*Cd4*-Cre), B6.129P2-*Tcrb*^{tm1Mom}/J (*Tcrb*^{-/-}), B6Cr.129S4-*Tnfsf4*^{tm1Sug}/Pgn (*Tnfsf4*^{-/-}), B6;129S1-*Il1rap*^{tm1Roml}/J (*Il1rap*^{-/-}), and B6.129S7-*Il1r1*^{tm1Imx}/J (*Il1r1*^{-/-}) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). *Il1rap*^{-/-} mice were subsequently backcrossed onto the BALB/c background for 10 generations. *Il1r1*^{-/-} (*ST2*^{-/-}) mice and *Cr1f2*^{-/-} (*Tslpr*^{-/-}) mice (both BALB/c background) were kindly provided by Dr. Andrew McKenzie (MRC Laboratory of Molecular Biology, Cambridge, UK) and Dr. Steven F. Ziegler (Benaroya Research Institute, Seattle, WA, USA), respectively, and were bred under specific pathogen-free (SPF) conditions at the Mayo Clinic (Rochester, MN, USA)^{21, 22}. *Bcl6*^{fl/fl} mice were previously described²³. Animals used in this study were female and ranged from 6-12 weeks of age. All protocols and procedures for handling of the mice were reviewed and approved by the Mayo Institutional Animal Care and Use Committee, Mayo Clinic.

Allergens

Peanut flour (14.4% protein) was purchased from the Golden Peanut Company (Alpharetta, GA, USA) as a bulk raw material; endotoxin was undetectable (<0.5 EU/mg flour) in the product by Limulus Amebocyte Lysate assay (Lonza, Walkersville, MD, USA). Crude peanut extract (70.2% protein) and *Alternaria alternata* extract (20.0% protein) were purchased from Greer Laboratories (Lenoir, NC, USA). Corn flour and rice flour were purchased from Bob's Red Mill Natural Foods (Milwaukie, OR, USA).

Inhalation peanut allergy model

Naïve BALB/c and C57BL/6 mice were exposed intranasally (i.n.) to 100 µg peanut flour in 50 µl sterile phosphate-buffered saline (PBS) or PBS alone twice/week for up to 4 weeks. Three days after the last exposure (i.e., on day 27), serum was collected via retroorbital bleeding under isoflurane anesthesia to determine levels of peanut-specific immunoglobulins. One day later (i.e., on day 28), mice were challenged systemically by intraperitoneal (i.p.) injection of 1.0 or 2.5 mg crude peanut extract in 500 µl sterile PBS. Immediately before challenge (0 min), and every 10 minutes afterward for one hour, rectal temperature was monitored by an electronic thermometer (Oakton Instruments, Vernon Hills, IL, USA) equipped with a RET-3 rectal probe (Physitemp Instruments, Clifton, NJ, USA). Clinical symptoms based on published criteria were scored as follows²⁴: 0, no symptoms; 1, scratching of ear and mouth; 2, puffiness around eyes and mouth, pilar erection, labored breathing; 3, prolonged period of motionlessness; 4, severely reduced motility, tremors, severe respiratory distress; 5, death. At 60 minutes, blood was obtained to measure plasma levels of mast cell protease-1 (MCPT-1).

ELISA for peanut-specific antibodies, MCPT-1, and cytokines

Serum levels of peanut-specific IgE antibodies were measured by enzyme-linked immunosorbent assay (ELISA). Plasma levels of MCPT-1 were determined using a commercial mouse MCPT-1 ELISA Kit (eBioscience, San Diego, CA, USA) according to the manufacturer instructions. The concentrations of IL-4, IL-5, IL-13, IL-21, TSLP, IL-1 α , and IL-1 β in supernatants from *in vitro* T cell cultures, BAL fluids, or lung homogenates were determined

using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) according to manufacturer instructions.

FACS analyses

T and B cell populations in mLNs were measured after peanut flour exposure by FACS analysis, as described²⁵. Flow cytometric analysis was performed on a FACSCalibur or Canto X cytometer (BD Biosciences, San Jose, CA), and data were analyzed with FlowJo software (Tree Star, Ashland, Oregon).

Statistical analysis

The statistical significance for differences between the various treatment groups was calculated using a Student's *t*-test, with $P < 0.05$ considered statistically significant. All numerical data are presented as the mean $\pm$ standard error of the mean (SEM).

RESULTS

Airway exposure to peanut flour induces peanut allergy in mice

To mimic exposure to peanut proteins in environmental house dust, naïve BALB/c mice were exposed to peanut flour via i.n. administration twice per week (Fig 1, A). Endotoxin was undetectable in the flour (<0.5 EU/mg flour), and no exogenous adjuvants were used throughout this study. Mice exposed i.n. to peanut flour (100 μ g/dose) developed peanut-specific IgE antibodies in their sera, as evidenced by a titration curve on individual samples (Fig 1, B) and plots from individual mice (Fig 1, C). These animals also developed IgG1 and IgG2a isotypes of peanut-specific antibodies. Time-course studies further demonstrate that anti-peanut antibodies are detectable in sera as early as 1-2 weeks after initiation of peanut flour exposure (Fig 1, D).

Peanut-sensitized mice were then challenged systemically by i.p. injection of crude peanut extract, and we found that they develop clinical symptoms consistent with acute anaphylaxis. Specifically, core body temperature began to drop 10 minutes after challenge and reached a nadir at 60 minutes (Fig 1, E). Mice also developed other clinical symptoms of anaphylaxis, including scratching of ears, puffiness around eyes and mouth, labored breathing, and reduced activity, which peaked 40 minutes after challenge. Notably, clinical symptoms were more severe in mice challenged with a higher dose of crude peanut extract (Fig 1, E). We further observed a significant increase in serum levels of MCPT-1 in mice challenged with peanut extract (Fig 1, F). Collectively, these findings demonstrate that inhalation of peanut flour induces symptoms consistent with peanut allergy in mice, resulting in an acute anaphylactic response when these animals are challenged systemically with peanut proteins.

To assess the reproducibility of these observations among different strains of mice, we exposed naïve C57BL/6 mice i.n. to peanut flour using the same protocol as described above and

in Fig 1, A. These animals also developed peanut-specific IgE, IgG1, and IgG2a antibodies (Supplemental Fig E1, A), at levels roughly comparable to BALB/c mice. Further, C57BL/6 mice challenged by i.p. injection of crude peanut extract, underwent an acute anaphylactic response, including a decrease in core body temperature and manifestation of clinical symptoms (Fig E1, B). We note, however, that some strain differences were observed, as C57BL/6 mice developed a more modest anaphylaxis than BALB/C mice (i.e., a 3.5°C vs. 7.0°C decrease in core body temperature, respectively). Although endotoxin was undetectable in peanut flour, we examined the potential involvement of contaminating endotoxin using a genetic model. We found that *Tlr4*^d BALB/c mice, which are deficient in TLR4 signaling, develop serum levels of peanut-specific IgE antibodies comparable to those of wild-type (WT) mice (Supplemental Fig E2), suggesting that TLR4, and consequently endotoxin, are unlikely to be involved in this response.

Peanut flour inhalation induces IL-4-competent follicular helper T (T_{fh}) cells

Using this mouse model, we next sought to investigate the immunological mechanism(s) involved in the initiation of peanut allergy. Because peanut-specific IgE was detectable in sera as early as 2 weeks after exposure, we focused on cellular events occurring in the first 2 weeks. IL-4 is indispensable for IgE class switch of B cells, and among the CD4⁺ T cell subsets, both prototypic Th2 cells, as well as T_{fh} cells, are capable of producing this cytokine²⁶⁻²⁸. Th2 cells have long been known to function in allergic immune responses²⁹, and T_{fh} cells have been shown to promote B cell differentiation into memory B and plasma cells^{26, 27}.

Therefore, to identify IL-4-producing cells in sensitized mice, we exposed naïve 4get IL-4 reporter mice³⁰ i.n. to peanut flour on days 0 and 7 and analyzed mLN cells on day 11 by

FACS analysis (Fig 2, A). We found that IL-4eGFP⁺CD4⁺ T cells were present in mLNs from mice exposed to peanut flour (Fig 2, B). The IL-33 receptor ST2 and the chemokine receptor CXCR5 are considered reliable markers for differentiated Th2 cells³¹ and Tfh cells²⁷, respectively. Approximately 35% of CD4⁺IL-4eGFP⁺ cells were CXCR5⁺ and ST2⁻, suggesting that a large proportion of them consist of differentiated Tfh cells. A smaller proportion of Th2 cells (i.e. CXCR5⁻ST2⁺) were also identified. In contrast, minimal expression of ST2 or CXCR5 was observed in CD4⁺IL-4eGFP⁺ cells in PBS-exposed mice; ST2⁺ cells and CXCR5⁺ cells were 6.0±1.3 and 7.4±1.0 % of CD4⁺IL-4eGFP⁺ cells, respectively (mean ± SEM, n=5). Quantification of cell populations revealed that peanut flour exposure induces a 6-fold increase in the total number of lymphocytes and a 20-fold increase in IL-4eGFP⁺CD4⁺ T cells in mLNs, as compared to levels found PBS-exposed mice (Fig 2, C). We further see that IL-4⁺ Tfh cells outnumber IL-4⁺ Th2 cells by 5.5-fold (Fig 2, D, $P<0.01$). These data suggest that inhalation of peanut flour induces Tfh cells, and a smaller number of Th2 cells, which are both capable of producing IL-4 in draining LNs.

Tfh cells mediate a robust peanut-specific IgE antibody response

We next compared the immunological functions of peanut exposure-induced Tfh and Th2 cells using both *in vitro* and *in vivo* approaches. First, we isolated the CD3⁺CD4⁺ST2⁺CXCR5⁻ (Th2) and CD3⁺CD4⁺ST2⁻CXCR5⁺ (Tfh) cell populations by FACS sorting, and cultured them with B cells isolated from the same mLNs in the presence of crude peanut extract for 7 days (Fig 3, A, Supplemental Fig E3 for the gating strategy). Double-negative CD3⁺CD4⁺ST2⁻CXCR5⁻ cells were used as a control. Whereas both Tfh and Th2 cells exhibit robust and comparable production of IL-4, Th2 cells produce significantly larger amounts of IL-5 and IL-13, as

compared to Tfh cells (Fig 3, B, $P<0.01$ or 0.05). In contrast, Tfh cells produce significantly more IL-21 than Th2 cells ($P<0.01$). Double-negative cells produce small amounts of these cytokines than either population.

We then compared the capacity of Tfh and Th2 cells to support IgE antibody production *in vivo*, using an adoptive transfer approach. Tfh and Th2 cells were isolated by FACS sorting from C57BL/6 mice that had been previously exposed to peanut flour, and an equal number of each population (i.e., 1.25×10^5 cells) was i.v. transferred to naïve T cell-deficient *Tcr β ^{-/-}* mice (C57BL/6 background), which have an intact B compartment³² (Fig 3, C). In the absence of T cell transfer, *Tcr β ^{-/-}* mice do not produce detectable levels of peanut-specific IgE antibodies, even when they are subsequently exposed to peanut flour (Fig 3, D). In contrast, after transfer of Tfh cells, *Tcr β ^{-/-}* mice produce a high titer of peanut-specific IgE antibodies. Transfer of Th2 cells also induces IgE antibody production, although the titer is approximately one-fortieth of that induced by Tfh cells. Transfer of Tfh cells further induces production of IgG1 antibodies at levels 8× higher than is induced by Th2 cells. No apparent differences were observed in levels of the IgG2 isotype. When challenged by i.p. injection of crude peanut extract, mice that received either Tfh cells or Th2 cells developed clinical symptoms of acute system anaphylaxis (Supplemental Fig E4). From these observations, we conclude that Tfh cells produce IL-4 and IL-21 and are superior to Th2 cells in supporting production of peanut-specific IgE and potentially IgG1 antibodies *in vivo*.

Tfh cells are indispensable for peanut allergy

To investigate the role of Tfh cells in peanut allergy, we utilized a genetic approach. The transcriptional repressor BCL6 is considered to be the master regulator for the Tfh cell lineage²⁷,

³³. We therefore used a floxed *Bcl6* mouse model, in which *Bcl6* is conditionally depleted in CD4⁺ T cells via action of the Cre recombinase expressed from the *Cd4* promoter [*Bcl6*^{fl/fl}*Cd4*-Cre mice]²³⁻²⁵. When these animals are exposed to peanut flour using the same protocol as describe above (Fig 2, A), Tfh cell populations (ST2⁺CXCR5⁺ or PD-1⁺CXCR5⁺) are nearly eliminated, whereas the Th2 cell population is unaffected (Fig 4, A). In contrast, control mice lacking CD4-Cre (i.e. *Bcl6*^{fl/fl}) develop both ST2⁺CXCR5⁺ Th2 cells and ST2⁺CXCR5⁺ Tfh cells (Fig 4, A), and PD-1⁺CXCR5⁺ mature Tfh cells³⁴ are also clearly detectable in these mice. Quantitative analyses further demonstrate that total numbers of CD4⁺ T cells and Th2 cells are similar in *Bcl6*^{fl/fl}*Cd4*-Cre and control *Bcl6*^{fl/fl} mice, whereas the number of ST2⁺CXCR5⁺ and PD1⁺CXCR5⁺ Tfh cells are reduced by 70% and 80%, respectively, in *Bcl6*^{fl/fl}*Cd4*-Cre mice as compared to controls (Fig 4, B).

GCs are essential for production of high-affinity, class-switched antibodies³⁵. We found that the frequencies of B220⁺ B cells within mLNs were similar in *Bcl6*^{fl/fl} mice and *Bcl6*^{fl/fl}*Cd4*-Cre mice (Fig 4, C, D). However, a marked decrease in both the frequency and total number of FAS⁺PNA⁺ GC B cells was observed in *Bcl6*^{fl/fl}*Cd4*-Cre mice, as compared to control animals. Thus, deletion of *Bcl6* in CD4⁺ T cells results in striking decrease in both Tfh cells and GC B cells, but Th2 cell numbers appear to be unaffected.

To further investigate the role of Tfh cells in peanut allergy development, *Bcl6*^{fl/fl}*Cd4*-Cre mice and littermate controls (*Bcl6*^{fl/fl}) were exposed i.n. to peanut flour for 4 weeks. We have previously performed a comprehensive analysis of Th2 cells in *Bcl6*^{fl/fl}*Cd4*-Cre mice, and found that Th2 cell function is not impaired in these mice (Figures 6 and 7 of reference²⁵). As expected, *Bcl6*^{fl/fl}*Cd4*-Cre mice treated with PBS alone did not develop IgE antibodies to peanut (Fig 4, E), whereas peanut flour-exposed *Bcl6*^{fl/fl} control mice develop serum IgE antibodies to peanut

proteins, as well as IgG1 and IgG2a isotypes. In contrast, peanut-specific IgE antibodies were nearly undetectable in *Bcl6^{fl/fl}*Cd4-Cre mice exposed to peanut flour ($P<0.01$). Similarly, a significant decrease in the levels of peanut-specific IgG1 and IgG2 antibodies was observed in *Bcl6^{fl/fl}*Cd4-Cre mice ($P<0.01$), as compared to *Bcl6^{fl/fl}* controls. Consequently, when challenged with peanut extract, *Bcl6^{fl/fl}*Cd4-Cre mice were completely protected from acute anaphylaxis, whereas *Bcl6^{fl/fl}* mice develop a robust response, including a drop in core body temperature and manifestation of clinical symptoms (Fig 4, F , $P<0.01$).

The tumor necrosis factor (TNF) family ligand, OX40 ligand (OX40L), and its receptor, OX40, are thought to play a key role in development of Th2 cells³⁶. Further, a role for OX40L in peanut allergy was found in mice that were sensitized by oral administration of peanut proteins in conjunction with the mucosal adjuvant, CTX³⁷. Conversely, in our model, we found that mice deficient in OX40L (i.e. *Tnfsf4^{-/-}* mice) develop serum levels of peanut-specific IgE and IgG1 antibodies comparable to those of control animals (Supplemental Fig E5). Collectively, our data suggest that Tfh cells play a critical role in the development of peanut allergy in response to i.n. peanut flour exposure in mice, although OX40L are not required for this process.

IL-1-family cytokines are rapidly induced in the airway by peanut flour exposure

Although the immunological mechanisms for development of Th2-type immune responses have been well-studied^{38, 39}, little is currently known regarding how Tfh cells develop during allergic immune responses. Evidence suggests that several cytokines produced in the mucosal epithelium, including TSLP and an IL-1-family cytokine, IL-33, promote differentiation and proliferation of conventional Th2 cells^{38, 39}. Other IL-1-family cytokines, such as IL-1 α and IL- β , are considered prototypic innate cytokines with diverse immunological activities⁴⁰. We

therefore sought to determine whether any of these cytokines are involved in the Tfh cell response. To this end, we performed a time-course analysis of the levels of these cytokines in BAL fluid and lung tissues in mice exposed to peanut flour, using extract from an asthma-related airborne allergen, the fungus, *A. alternata*⁴¹, as a positive control.

After a single exposure to peanut flour, IL-1 α and IL-1 β were present in BAL fluids from naïve BALB/c mice, with levels peaking at 6 hours post-exposure and declining thereafter (Fig 5, A); conversely, neither IL-33 nor TSLP was detectable. When these mice were exposed to *Alternaria* extract, a rapid increase in IL-33 was detected in BAL fluids within 1 hour; however, the levels of IL-1 α or IL-1 β were lower, as compared to those induced by peanut flour. Further, IL-5 was detectable only in *Alternaria*-exposed mice. In lung tissues, levels of IL-1 α , IL-1 β , IL-33, and TSLP increased after challenge in mice exposed to either peanut flour or *Alternaria*; expression peaks at 6 hours post-exposure and declines by 24 hours. However, cytokine levels induced by *Alternaria* were generally higher than those induced by peanut (Fig 5, B).

We next examined whether the ability of peanut flour to induce IL-1 α is unique among food products. We observed a significant increase in lung levels of IL-1 α at both 6 and 24 hours in naïve BALB/c mice exposed to peanut flour (Fig 5, C). In contrast, elevated IL-1 α was not detected in mice exposed to corn or rice flour. Similarly, crude peanut extract did not induce IL-1 α , although it contains more peanut proteins than peanut flour (70.2% vs. 14.4% protein content, respectively). Consistent with this observation, peanut flour promoted stronger IgE antibody production to the model antigen ovalbumin (OVA) than did crude peanut extract (Supplemental Fig E6). Overall, these data demonstrate that although numerous innate cytokines are elevated in lungs after inhalation exposure to peanut flour or *Alternaria* extract, induction of IL-1 α /IL-1 β in BAL fluid is unique to peanut flour, whereas IL-33/IL-5 is specific to *Alternaria*.

IL-1-family cytokines are involved in production of peanut-specific IgE

To determine the roles of IL-1 and IL-33 in peanut allergy development, we utilized a series of mice that are deficient in their receptors, including *Il1r1*^{-/-} (i.e. IL-1 α and IL-1 β receptor deficient), *ST2*^{-/-} (i.e. IL-33 receptor deficient), and *Tslpr*^{-/-} mice (TSLP receptor deficient). WT or receptor deficient mice were then exposed to peanut flour for 4 weeks. As compared to WT animals, *Il1r1*^{-/-} mice showed a 45% reduction in serum levels of peanut-specific IgE antibodies ($P<0.01$, Fig 6, A), whereas *ST2*^{-/-} mice displayed a slight (i.e. 27%), but significant, decrease in anti-peanut IgE ($P<0.05$, Fig 6, B). Conversely, there was no difference in anti-peanut IgE levels in *Tslpr*^{-/-} vs. WT control mice (Fig 6, C). Likewise, *Il1r1*^{-/-} and *ST2*^{-/-} mice showed a 51% and 64% decrease, respectively, in serum levels of anti-peanut IgG1 antibodies, vs. WT, whereas a paradoxical increase in IgG1 antibodies was observed in *Tslpr*^{-/-} mice ($P<0.01$).

Based on these observations, we hypothesized that IL-1-family cytokines, such as IL-1 α , IL-1 β , and IL-33, but not TSLP, play key roles in peanut allergy development. Further, because IL-1R1- and ST2-deficient mice exhibit partial phenotypes, we suspected these molecules might function complementally to promote peanut allergy. IL-1-family cytokine receptors are heterodimers, consisting of a specific receptor (e.g., IL-1R1 and ST2) and a co-receptor, such as IL-1R-associated protein (IL-1RAcP), which together form a high-affinity receptor complex⁴². Because IL-1RAcP is shared by IL-1R1 and ST2, we speculated that this co-receptor might be critical for peanut allergy formation. Indeed, mice deficient for IL-1RAcP (i.e. *Il1rap*^{-/-}) showed approximate reductions in anti-peanut IgE and IgG1 antibodies of 82% and 93%, respectively (Fig 6, D).

IL-1 is involved in the Tfh cell response in mice exposed to peanut

To determine whether IL-1RAcP is involved in development and/or expansion of Tfh cells, we exposed *Il1rap*^{-/-} mice i.n. to peanut flour and examined CD3⁺CD4⁺ T cell populations in mLN (Fig 7, A, Supplemental Fig E7). WT BALB/c control mice generated CD3⁺CD4⁺ST2⁺ Th2 cells, as well as CD3⁺CD4⁺CXCR5⁺ and PD-1⁺CXCR5⁺ Tfh cells (Fig 7, B). However, in *Il1rap*^{-/-} mice, the proportion of Tfh cells was decreased, whereas Th2 cells were unaffected (Fig 7, B). By cell number, the total number of CD4⁺ T cell decreased by 50% in *Il1rap*^{-/-} mice as did Th2 cells (Fig 7, C). Strikingly, in *Il1rap*^{-/-} mice, the numbers of CD3⁺CD4⁺ST2⁻CXCR5⁺ and PD1⁺CXCR5⁺ Tfh cells were reduced more by 80% and 95%, respectively, as compared to WT ($P<0.05$ and $P<0.01$). We also found that both the proportion and total numbers of FAS⁺PNA⁺ GC B cells was decreased by 95% in *Il1rap*^{-/-} mice (Fig 7, D, E, $P<0.05$).

To further dissect the roles for IL-1 and IL-33 in the Tfh response, we exposed naïve *Il1r1*^{-/-} and *ST2*^{-/-} mice i.n. to peanut flour using the same protocol as described in Figure 7A. *Il1r1*^{-/-} mice showed a striking decrease in the total numbers of PD1⁺CXCR5⁺ Tfh cells as well as FAS⁺PNA⁺ GC B cells ($p<0.01$, Fig 7, F, G), similar to observations in *Il1rap*^{-/-} mice. In contrast, *ST2*^{-/-} mice showed no difference in the numbers of Tfh cells or GC B cells compared to WT (Fig 7, H, I). From these observations, we conclude that IL-1-family cytokines, in particular IL-1 α and/or IL-1 β , are critical for development and/or expansion of Tfh cells and GC B cells in response to peanut exposure, and consequently for the initiation of peanut allergy in mice.

DISCUSSION

The LEAP study¹⁶ has suggested that oral consumption of peanut is tolerogenic in humans. Further, peanut proteins are readily detectable in household dust^{17, 18}, and their levels correlate with peanut allergy prevalence^{19, 20}. Based on this information, we took a straightforward approach to establish a mouse model for peanut allergy development in which we exposed naïve animals to peanut flour by inhalation without exogenous adjuvants. These mice produced anti-peanut IgE antibodies, and when challenged systemically with peanut proteins, they developed acute anaphylaxis and increased serum levels of MCPT-1. Several studies have investigated the immunological mechanisms associated with development of peanut allergy in mouse models of disease. However, a majority used either mucosal adjuvants, such as CTX^{7, 10, 37} and SEB¹⁰, or genetically-modified mice deficient in TLR4 signaling (e.g., C3H/HeJ mice)¹² or carrying a disinhibited form of the IL-4 receptor¹³. More recently, a humanized mouse model was developed by engrafting human hematopoietic stem cells into immunodeficient mice⁴³. Our model is unique in that WT mice were utilized, and no adjuvants were provided; therefore, we predict it will be particularly useful for elucidating the immunological mechanisms involved in peanut allergy. Furthermore, our results also suggest that although epicutaneous exposure is generally considered to be the route of sensitization⁴⁴, airway inhalation may need to be considered as an alternative or additional route(s) of exposure in peanut allergy development.

IL-4 was initially identified as a prototypic Th2 cytokine⁴⁵, and for this reason, Th2 cells have been assumed to be the main facilitator of IgE antibody production by B cells. Our study suggests, however, that Tfh cells, rather than Th2 cells, play a key role in peanut allergy based on the following lines of evidence; 1) Tfh cells outnumber Th2 cells by >5-fold in draining LNs (Fig 2), 2) Tfh cells produce both IL-4 and IL-21 *in vitro*, which are two major cytokines that

assist B cells³⁴, and Tfh cells were superior to Th2 cells for promoting IgE antibody production *in vivo* (Fig 3), 3) genetic depletion of Tfh cells (e.g. *Bcl6^{fl/fl}Cd4-Cre*) completely protected mice from developing peanut allergy, without affecting the Th2 cell compartment (Fig 4), and 4) depletion of OX40L, which is presumed to play an important role in development of Th2 cells³⁶, does not affect IgE antibody levels. Finally, in a previous study, *Bcl6^{fl/fl}Cd4-Cre* mice showed nearly abolished production of IgE antibody while the function of Th2 cells was not impaired²⁵.

An earlier study of epicutaneous sensitization in mice reported the development of peanut-specific Th2-type CD4⁺ T cells⁶; however, it is unknown whether these cells play any role in the induction of peanut-specific IgE antibodies. During allergic immune responses, IL-4-producing Th2 cells localize to lung tissues, whereas IL-4-producing Tfh cells reside in draining LNs^{25, 46}, suggesting distinct anatomic locations for these cell subsets. Persistent IgE antibody production requires memory B cells in GCs that are supported by IL-4 producing CD4⁺ T cells in the LNs⁹. Therefore, it is reasonable to conclude that Tfh cells provide a unique niche in allergic immune responses, in particular, those involving IgE-mediated acute responses, such as peanut allergy. Indeed, a meta-analysis of genome-wide association study data identified *BCL6* and *IL21*, critical regulators of Tfh cells³⁴, as susceptibility loci for allergy⁴⁷.

Similar to other mucosal organs, respiratory exposure to innocuous proteins in the absence of adjuvants generally induces immune tolerance⁴⁸. In spite of this, mice that inhaled peanut flour without any adjuvants developed Tfh cells and peanut allergy in our model. Epithelium-derived cytokines, such as IL-25, IL-33 and TSLP, are involved in antigen-specific Th2-type CD4⁺ T cell development^{38, 39, 49}. However, little information is currently available on how Tfh cells form, and the mechanisms for Tfh cell development appear to be rather complex. For example, *Il6^{-/-}*, *Il21^{-/-}*, and *Il21r^{-/-}* mice, as well as *Stat3*-deficient mice, exhibit minimal to no

defects in Tfh cell development *in vivo*^{50, 51}, while blockage of IL-6 in *Il21*^{-/-} mice or *Il6/Il21* double-knockout mice display a partial decrease in Tfh cells⁵². This suggests that IL-6 and IL-21 work together to drive Tfh cell formation. Our study demonstrates that, in response to inhaled peanut flour, IL-1-family cytokines, in particular IL-1 α and/or IL-1 β , likely function to promote allergy development, as *Il1rap*^{-/-} mice and *Il1rl*^{-/-} mice show a marked reduction in Tfh cell number (Fig 7). These observations are consistent with the previous finding that IL-1 β alone serves as an effective airway adjuvant to promote development of Tfh cells and IgE antibodies against the innocuous ovalbumin (OVA) protein in mice²⁵. Further, cord blood cells from patients with food allergy produce IL-1 β , which could promote generation of IL-4-expressing and GATA3-negative CD4⁺ T cells⁵³. A more recent study also suggests that IL-1 plays a key role in activation of Tfh cells in mice immunized systemically by intraperitoneal injection of OVA plus alum adjuvant⁵⁴. It remains to be determined whether IL-1 acts directly on Tfh cells, or if its effects are through activation of other cell types, such as airway epithelial cells, which may provide signals (e.g. IL-6) required for development and/or expansion of Tfh cells. Notably, microarray and NanoString assays showed mRNA expression of the IL-1 receptor (i.e. *Il1r1*) by Tfh cells^{25, 54}, suggesting a possible direct action of IL-1 α or IL-1 β on Tfh cells. Selective knockout of IL-1R1 in Tfh cells should help address these remaining questions in the future.

In this study, IL-1 α and IL-1 β were clearly detectable in BAL fluids from mice exposed to peanut flour (Fig 5, A). In particular, the IL-1 α response was unique in that an asthma-related airborne allergen *Alternaria* failed to induce this cytokine in BAL fluids. A recent study showed that alveolar macrophages produce IL-1 α in response to aluminum hydroxide and silica⁵⁵, suggesting a role for inhaled fine particles in promoting IL-1 α production in the airway. Here, however, neither rice nor corn flour was able to induce IL-1 α in the lungs (Fig 5, C), suggesting

that the physical presence of particles alone is insufficient. Indeed, glycan structures of a major peanut allergen, Ara h1, have been implicated in activation of C-type lectin dendritic cell-specific ICAM-grabbing nonintegrin (DC-SIGN) on dendritic cells⁵⁶. In contrast, peanut flour was superior to peanut extract in inducing IL-1 α production in the lungs (Fig 5, C) and promoting allergy sensitization to a bystander antigen, OVA, (Supplemental Fig E6), suggesting both the physical properties and biochemical composition of peanut flour are likely to be crucial. Therefore, in future studies, it will be important to elucidate the cellular and molecular mechanisms involved in recognition of peanut flour and in the production of cytokines by immune cells in the airway.

A potential caveat of this study is the noted immunologic differences between mice and humans⁵⁷. We were unable to demonstrate acute systemic anaphylaxis when peanut was administered orally to the sensitized animals (data not shown). Clinical evidence directly demonstrating that patients with peanut allergy have been sensitized by inhalation of peanut proteins in house dust is also scarce, although it has been speculated that environmental peanut protein levels are sufficient to induce sensitization in humans¹⁸. Furthermore, the dose of the peanut flour used for this study (100 μ g/day) may exceed the levels of human exposure. We note, however, that our pilot study also found that a 10-fold smaller dose of peanut flour (e.g. 10 μ g peanut flour/day or 2.1 μ g peanut protein/day) is sufficient to sensitize some animals (data not shown). Finally, the perinatal alveolization period (~3 years in humans)⁵⁸ provides a vulnerable time for the lungs to develop long-lasting allergen sensitization due to activated CD11b⁺ dendritic cells⁵⁹. These points need to be taken into consideration when translating the information of this study to peanut allergy in humans.

In summary, Tfh cells have been implicated in autoimmune diseases, such as systemic lupus erythematosus, arthritis, and type I diabetes⁶⁰⁻⁶². This study adds to this field by demonstrating that Tfh cells play a critical role in peanut allergy formation. A vast majority of Tfh cells are found within the B cell follicles of secondary lymphoid tissue⁶³, making it difficult to investigate the roles of Tfh cells in human diseases. However, there is increasing evidence suggesting the presence of circulating CXCR5⁺ CD4⁺ memory T cells in peripheral blood cells that are committed to the Tfh lineage^{64, 65}. Therefore, we believe it will be feasible to study Tfh cells in patients with peanut allergy. We propose that enhanced understanding of the molecular and immunological mechanisms involved in differentiation and function of Tfh cells, as well as methods to disrupt these pathways, will be of central importance in the development of new treatments and preventive strategies for the potentially life-threatening disease of peanut allergy.

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FIGURE LEGENDS

Figure 1. Airway exposure to peanut flour induces peanut allergy phenotypes in mice. (A) Experimental model. (B) Titers of anti-peanut antibodies in sera were determined on day 27. $**P<0.01$ compared to mice exposed to PBS. Data are a pool from three experiments and are presented as the mean $\pm$ SEM (n = 12-15 in each group). (C) Levels of anti-peanut antibodies in each mouse are shown. (D) Kinetic changes in levels of anti-peanut antibodies are shown. $**P<0.01$ compared to mice exposed to PBS. Data are presented as the mean $\pm$ SEM (n = 6 in each group). (E) Changes in rectal temperature (left) and clinical scores (right) in mice challenged by intraperitoneal (i.p) injection of peanut extract are shown. Data are presented as the mean $\pm$ SEM (n = 9-15 in each group) and are a pool of three experiments. $*P<0.05$ and $**P<0.01$ compared to mice exposed to PBS. (F) MCPT-1 levels in plasma are presented as the mean $\pm$ SEM (n = 9-12 in each group). Data are a pool of three experiments. $*P<0.05$ and $**P<0.01$ between groups indicated by horizontal lines.

Figure 2. Airway exposure to peanut flour promotes development of follicular helper T (Tfh) cells in draining lymph nodes. (A) Experimental model: naïve 4get mice (BALB/c background) were exposed i.n. to peanut flour or PBS. On day 11, mediastinal lymph nodes (mLNs) were analyzed by FACS. (B) mLN cells were gated on lymphocytes, and expression of CD4, IL-4eGFP, ST2, and CXCR5 was measured. Representative scattergrams showing expression of CD4 and IL-4eGFP in the total lymphocyte population (left panels), and the expression of ST2 and CXCR5 in CD4⁺IL-4eGFP⁺ lymphocytes (right panels). (C) Proportions and numbers of IL-4eGFP⁺CD4⁺ T cells are presented as the mean $\pm$ SEM (n = 5-6 in each group, pool of two experiments). $*P<0.05$ and $**P<0.01$ between the groups indicated by horizontal lines. (D)

Proportions and numbers of IL-4eGFP⁺ Th2 and Tfh cells are presented as the mean $\pm$ SEM (n = 8 in each group, pool of 2 experiments). ** P <0.01 between groups indicated by horizontal lines.

Figure 3. Tfh cells are superior to Th2 cells for promoting production of peanut-specific IgE antibodies. (A) Experimental model: naïve BALB/c mice were exposed i.n. to peanut flour. On day 11, mLN cells were sorted into CD3⁺CD4⁺ST2⁻CXCR5⁻ (control), CD3⁺CD4⁺ST2⁺CXCR5⁻ (Th2), and CD3⁺CD4⁺ST2⁻CXCR5⁺ (Tfh) populations and cultured with B cells isolated from the same mLNs in the presence of crude peanut extract. (B) Supernatant cytokine levels were analyzed by ELISA. Data are presented as the mean $\pm$ range from duplicate cultures. Figure is representative of three experiments. * P <0.05 and ** P <0.01 between groups indicated by horizontal lines. (C) Experimental model: naïve C57BL/6 mice were exposed i.n. to peanut flour on day 0 and day 7, and Tfh and Th2 cells were sorted from day 11 mLNs. Cells (1.25×10^5) were i.v. transferred to *Tcr β ^{-/-}* (C57BL/6 background) mice, and animals were exposed i.n. to peanut flour for 4 weeks. (D) Titers of anti-peanut antibodies in sera were determined by analyzing serial dilutions of the specimens by ELISA. Results are the mean $\pm$ SEM (n = 3 in each group) and are representative of two experiments. ** P <0.01 and * P <0.05 compared to mice that received Th2 cells.

Figure 4. Tfh cells are critical for peanut allergy formation. (A) Mice were exposed i.n. to peanut flour as described in Fig 2, A. Representative scattergrams of the CD3⁺CD4⁺ T cell population are shown. (B) Cell numbers in each population are presented as the mean $\pm$ SEM (n = 3 in each group). Data are a pool of two separate experiments. * P <0.05 compared to *Bcl6^{fl/fl}* mice. (C) Representative scattergrams of the B220⁺ B cell population are shown. (D) Cell

numbers in each population are presented as the mean $\pm$ SEM ($n = 3$ in each group). Data are a pool of two separate experiments. $**P < 0.01$ compared to *Bcl6^{fl/fl}* mice. (E) Mice were exposed i.n. to peanut flour as described in Fig 1, A. Titers of anti-peanut antibodies are shown. (F) Changes in rectal temperature (left) and clinical scores (right) in mice challenged by i.p. injection of peanut extract are shown. (E and F) Data are presented as the mean $\pm$ SEM ($n = 3-7$ in each group) and are representative of two experiments. $**P < 0.01$ and $*P < 0.05$ compared to *Bcl6^{fl/fl}* mice exposed to peanut flour and challenged with peanut extract.

Figure 5. The BAL and lung levels of IL-1 family cytokines increase rapidly following airway exposure to peanut flour. Naïve BALB/c mice were exposed i.n. to single dose of peanut flour (100 μ g/dose), *Alternaria* extract (100 μ g/dose), or PBS. Cytokine levels in supernatants of BAL fluids (A) and lung homogenates (B) were analyzed. Results are the mean $\pm$ SEM ($n = 3$ in each group) and are representative of two experiments. $*P < 0.05$ and $**P < 0.01$ compared to mice exposed to PBS. (C) Naïve BALB/c mice were exposed i.n. to a single dose of PBS, peanut flour, peanut extract, corn flour, or rice flour (each at 100 μ g/dose). At 6 or 24 hours, levels of IL-1 α in the supernatants of lung homogenates were analyzed. Results are the mean $\pm$ SEM ($n = 3$ in each group) and are representative of two experiments. $*P < 0.05$ and $**P < 0.01$ compared to mice exposed to PBS.

Figure 6. IL-1 family cytokines are involved in the production of peanut-specific IgE and IgG1 antibodies in mice exposed to peanut flour. Naïve wild-type (WT) mice (BALB/c or C57BL/6) or mice deficient in specific cytokine receptors were exposed i.n. to PBS or peanut flour for 4 weeks. Titers of anti-peanut antibodies in sera were determined by analyzing serial dilutions of

the specimens by ELISA. (A, B, C, and D) Data are presented as the mean $\pm$ SEM (n = 4 or 5 in each group). (A, B, and D) Data are a representative of two experiments. * P <0.05 and ** P <0.01 compared to WT mice exposed to peanut flour.

Figure 7. IL-1 plays a critical role in the Tfh response in mice exposed to peanut flour. (A) Experimental model. (B) Representative scattergrams of the CD3⁺CD4⁺ T cell population are shown. (C) Cell numbers in each population are presented as the mean $\pm$ SEM (n = 8 in each group). Data are a pool of two separate experiments. * P <0.05 and ** P <0.01 compared to WT BALB/c mice. (D) Representative scattergrams of the B220⁺ B cell population are shown. (E) Cell numbers in each population are presented as the mean $\pm$ SEM (n = 4 in each group). * P <0.05 compared to WT BALB/c mice. (F) Representative scattergrams of the CD3⁺CD4⁺ T cell population and the B220⁺ B cell population are shown. (G) Cell numbers in each population are presented as the mean $\pm$ SEM (n = 4 in each group). ** P <0.01 compared to WT BALB/c mice. (H) Representative scattergrams of the CD3⁺CD4⁺ T cell population and the B220⁺ B cell population are shown. (G) Cell numbers in each population are presented as the mean $\pm$ SEM (n = 4 in each group).

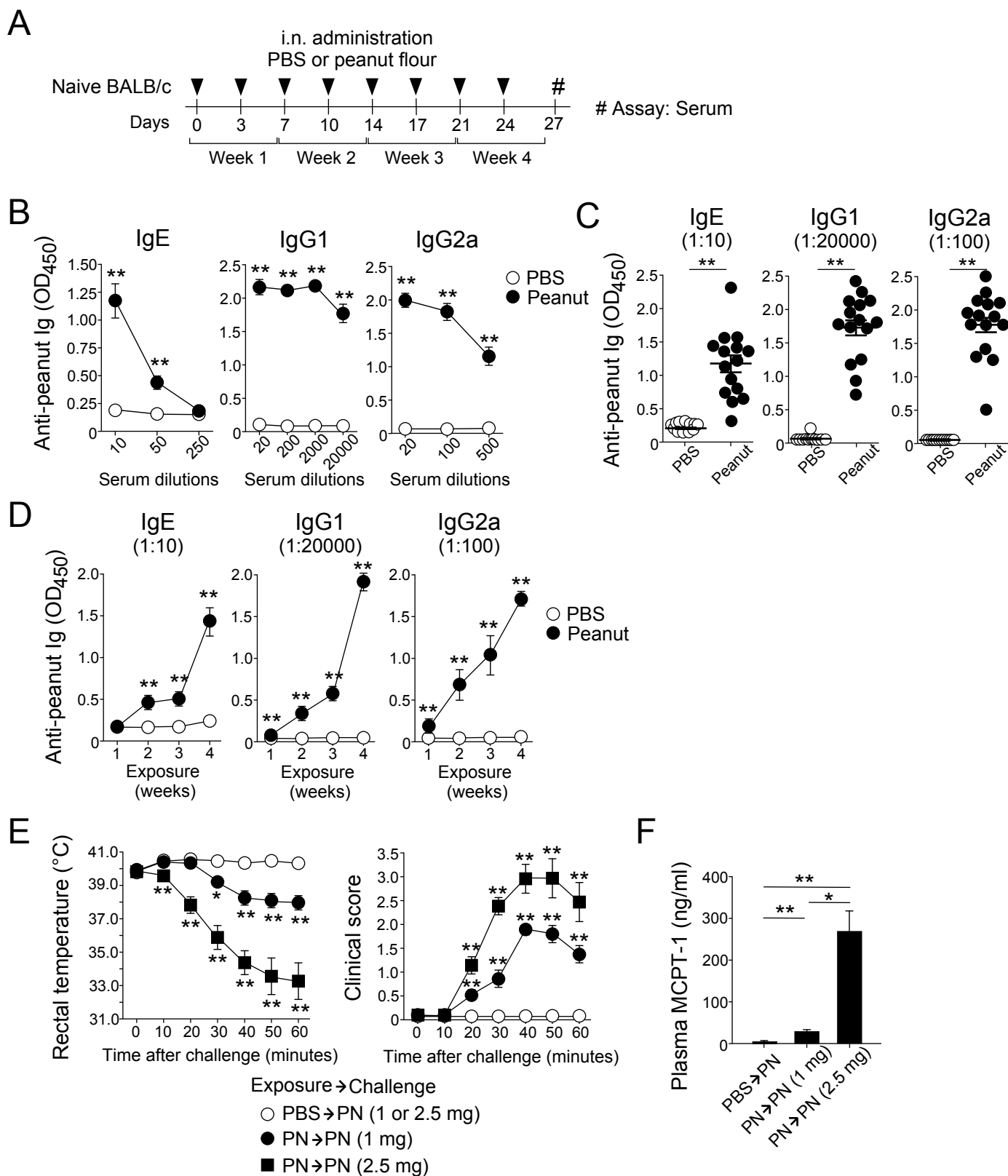
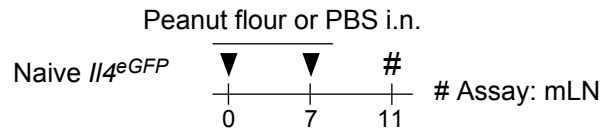
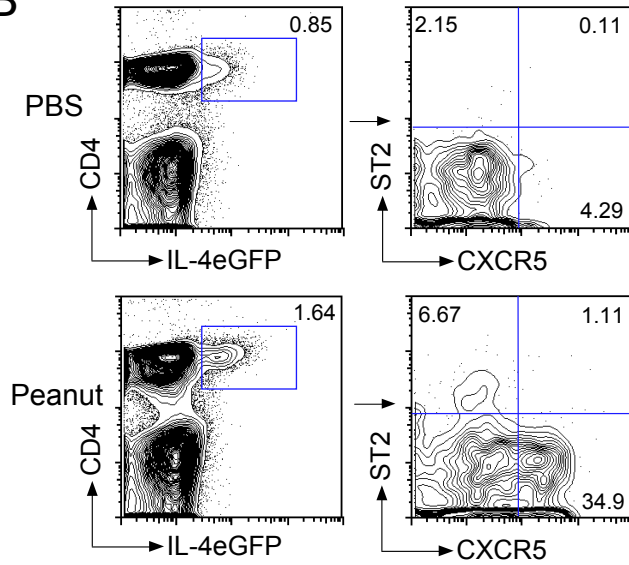


Figure 1

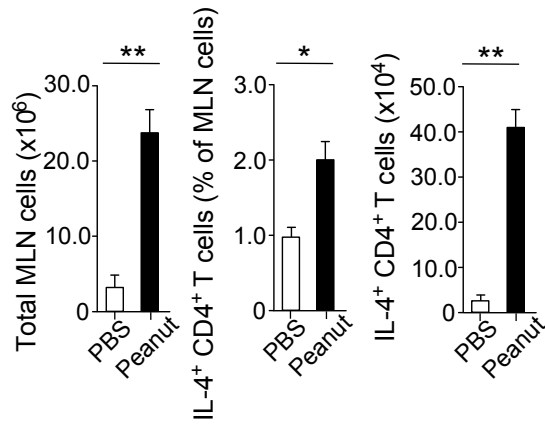
A



B



C



D

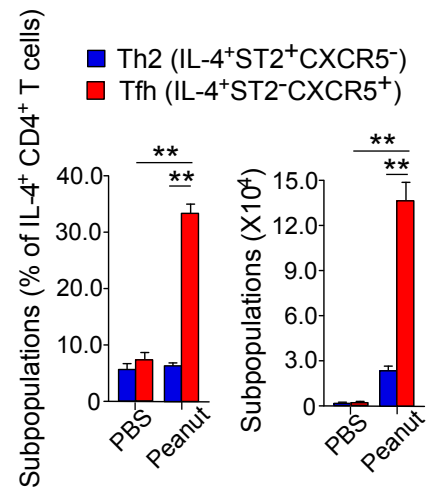


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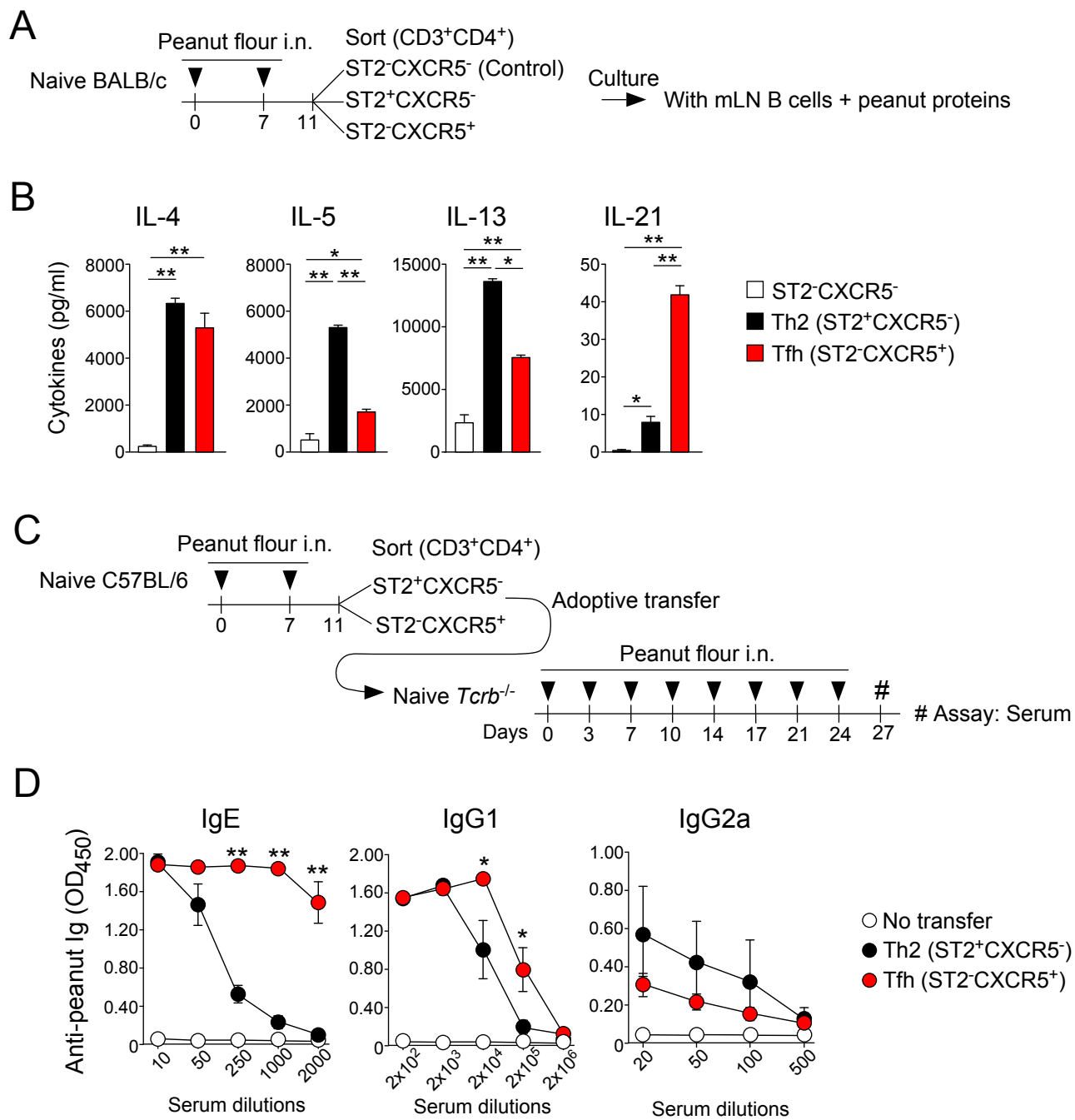


Figure 3

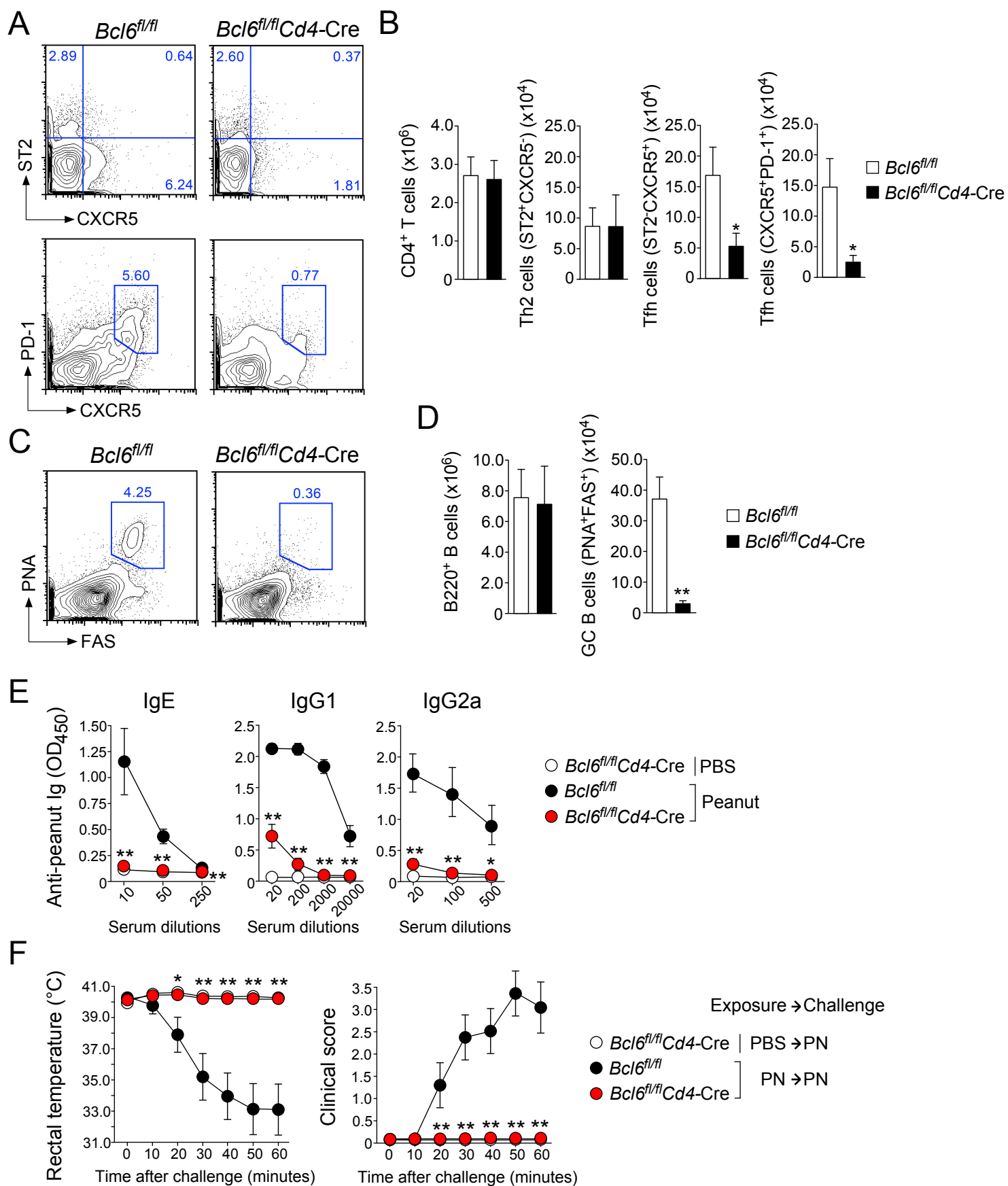


Figure 4

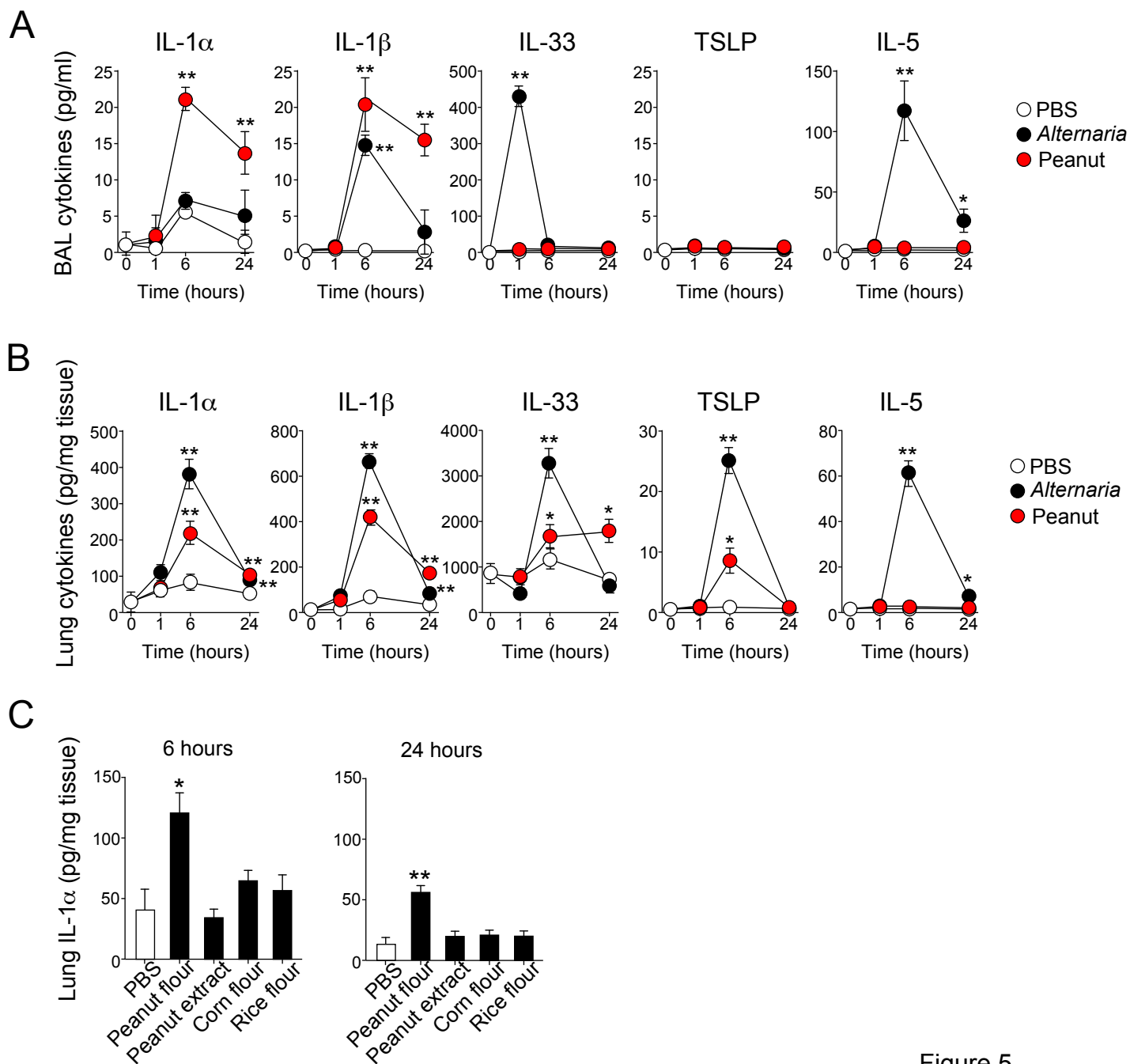


Figure 5

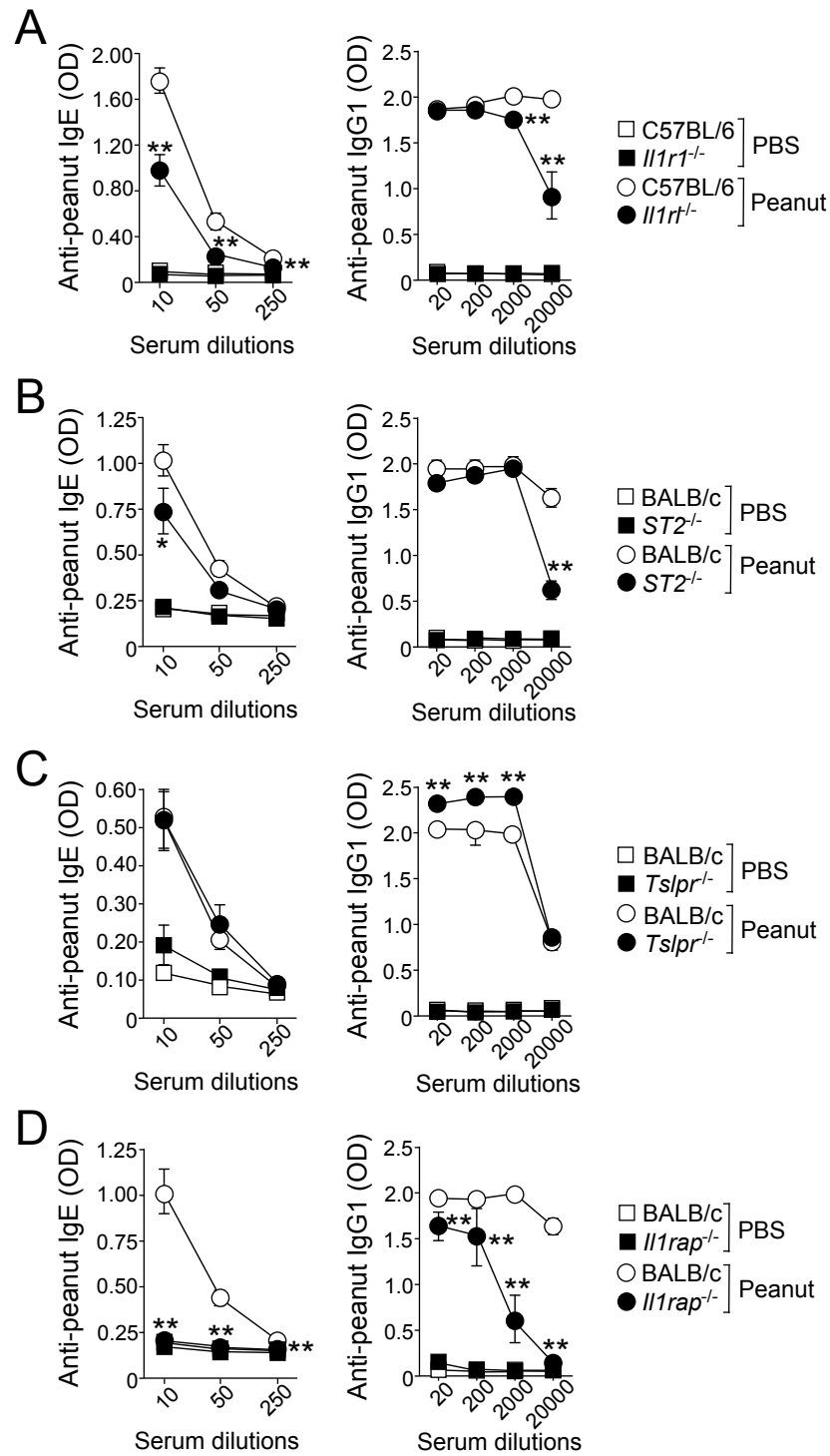


Figure 6

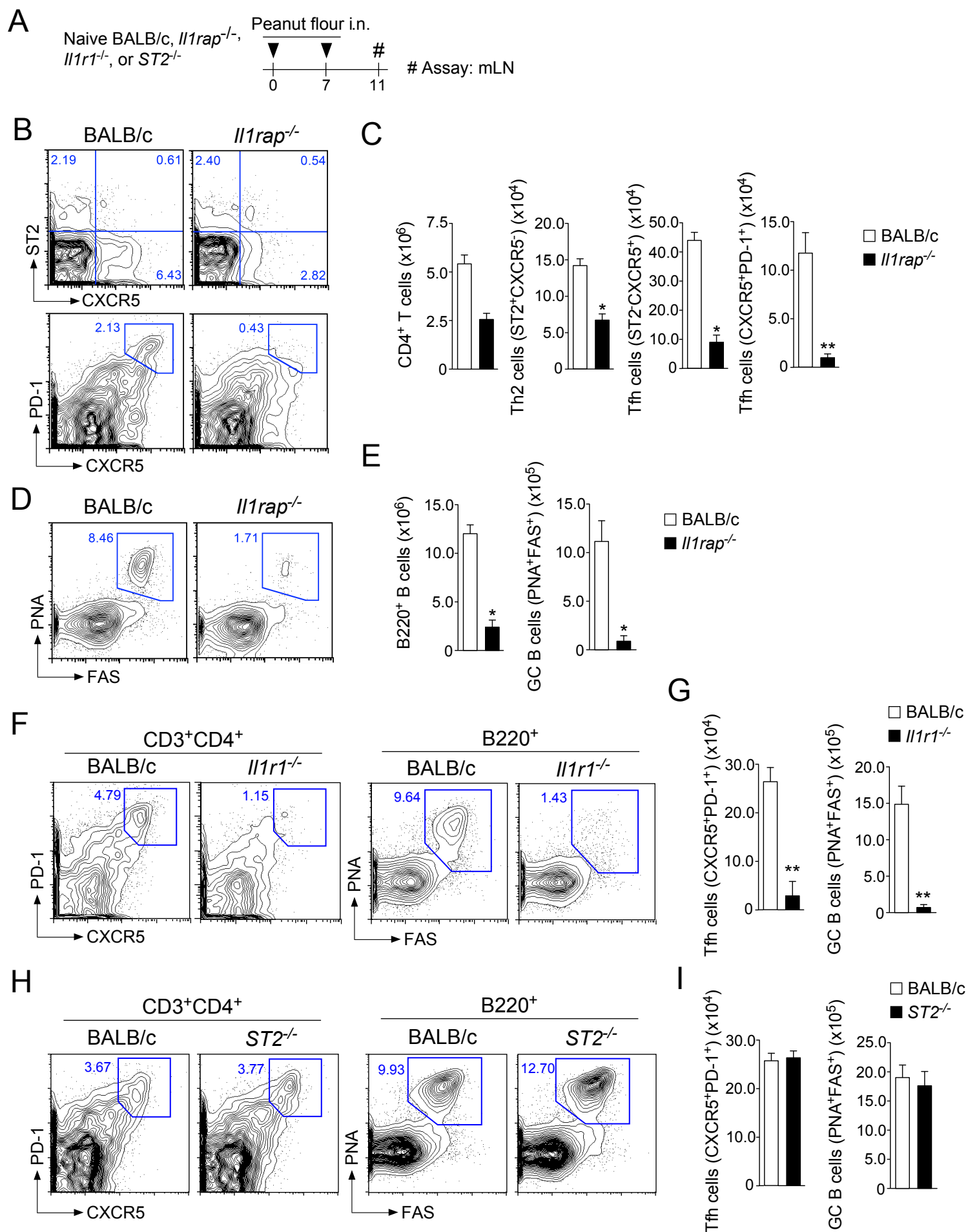


Figure 7

1 **ONLINE REPOSITORY**

2
3 **Airway exposure initiates peanut allergy by involving the IL-1 pathway and T follicular**
4 **helper (Tfh) cells in mice**

5
6
7 Joseph J. Dolence, Ph.D., Takao Kobayashi, Ph.D., Koji Iijima, Ph.D., James Krempski, Li Y.
8 Drake, M.D., Ph.D., Alexander L. Dent, Ph.D., and Hirohito Kita, MD
9
10

METHODS

Mice

BALB/c, C57BL/6, C.C3-*Tlr4*^{Lps-d}/J (*Tlr4*^d), C.129-*Il4*^{tm1Lky}/J (4get), Tg(Cd4-cre)1Cwi/BfluJ (*Cd4*-Cre), B6.129P2-*Tcrb*^{tm1Mom}/J (*Tcrb*^{-/-}), B6Cr.129S4-*Tnfsf4*^{tm1Sug}/Pgn (*Tnfsf4*^{-/-}), B6;129S1-*Il1rap*^{tm1Roml}/J (*Il1rap*^{-/-}), and B6.129S7-*Il1r1*^{tm1Imx}/J (*Il1r1*^{-/-}) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). *Il1rap*^{-/-} mice were subsequently backcrossed onto the BALB/c background for 10 generations. *Il1rl1*^{-/-} (*ST2*^{-/-}) mice and *Crlf2*^{-/-} (*Tslpr*^{-/-}) mice (both BALB/c background) were kindly provided by Dr. Andrew McKenzie (MRC Laboratory of Molecular Biology, Cambridge, UK) and Dr. Steven F. Ziegler (Benaroya Research Institute, Seattle, WA, USA), respectively, and were bred under specific pathogen-free (SPF) conditions at the Mayo Clinic (Rochester, MN, USA)^{1, 2}. *Bcl6*^{fl/fl} mice were previously described³. Animals used in this study were female and ranged from 6-12 weeks of age. All protocols and procedures for handling of the mice were reviewed and approved by the Mayo Institutional Animal Care and Use Committee, Mayo Clinic.

Allergens

Peanut flour (14.4% protein) was purchased from the Golden Peanut Company (Alpharetta, GA, USA) as a bulk raw material; endotoxin was undetectable (<0.5 EU/mg flour) in the product by Limulus Amebocyte Lysate assay (Lonza, Walkersville, MD, USA). Crude peanut extract (70.2% protein) and *Alternaria alternata* extract (20.0% protein) were purchased from Greer Laboratories (Lenoir, NC, USA). Corn flour and rice flour were purchased from Bob's Red Mill Natural Foods (Milwaukie, OR, USA).

34 *Inhalation peanut allergy model*

35 Naïve BALB/c and C57BL/6 mice were exposed intranasally (i.n.) to 100 µg peanut flour
36 in 50 µl sterile phosphate-buffered saline (PBS) or PBS alone twice/week for up to 4 weeks.
37 Three days after the last exposure (i.e., on day 27), serum was collected via retroorbital bleeding
38 under isoflurane anesthesia to determine levels of peanut-specific immunoglobulins. One day
39 later (i.e., on day 28), mice were challenged systemically by intraperitoneal (i.p.) injection of 1.0
40 or 2.5 mg crude peanut extract in 500 µl sterile PBS. Immediately before challenge (0 min), and
41 every 10 minutes afterward for one hour, rectal temperature was monitored by an electronic
42 thermometer (Oakton Instruments, Vernon Hills, IL, USA) equipped with a RET-3 rectal probe
43 (Physitemp Instruments, Clifton, NJ, USA). Clinical symptoms based on published criteria were
44 scored as follows⁴: 0, no symptoms; 1, scratching of ear and mouth; 2, puffiness around eyes and
45 mouth, pilar erection, labored breathing; 3, prolonged period of motionlessness; 4, severely
46 reduced motility, tremors, severe respiratory distress; 5, death. At 60 minutes, blood was
47 obtained to measure plasma levels of mast cell protease-1 (MCPT-1).

48 For time-course studies, mice were exposed i.n. to 100 µg peanut flour twice/week, and
49 blood specimens were collected on days 7, 14, 21, and 27 for determining serum levels of
50 peanut-specific IgE, IgG1, and IgG2a. To measure CD4⁺ T cell and B220⁺ B cell responses in
51 draining lymph nodes (LNs), mice were exposed i.n. to 100 µg peanut flour on days 0 and 7. On
52 day 11, lung-draining mediastinal lymph nodes (mLNs) were harvested for fluorescence-
53 activated cell sorting (FACS) analysis. To examine acute innate immune responses, mice were
54 exposed i.n. to 100 µg of either peanut flour, crude peanut extract, corn flour, rice flour or
55 *Alternaria alternata* extract in 50 µl sterile PBS. Brochoalveolar lavage (BAL) fluids and lungs
56 were then collected at 1, 6, and 24 hours for measurement of cytokine levels.

ELISA for peanut-specific antibodies, MCPT-1, and cytokines

Serum levels of peanut-specific IgE antibodies were measured by enzyme-linked immunosorbent assay (ELISA). Briefly, ELISA plates (Immulon 4 HBX, Thermo Fisher, Waltham, MA, USA) were coated with 50 µl of 5 µg/ml rat anti-mouse IgE mAb (LO-ME-3) (Bio-Rad, Raleigh, NC, USA) in 0.1 M carbonate buffer, pH 9.5, overnight at 4°C. Plates were blocked with 200 µl PBS, containing 1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA), for 2 hours at room temperature (RT). After blocking, serial dilutions of serum samples were added, and plates were incubated for 2 hours at RT. The plates were then incubated with 0.24 µg/ml biotinylated crude peanut extract for 1 hour at RT, followed by streptavidin-poly-HRP (Pierce, Rockford, IL) diluted 1:5,000 for 30 minutes at RT. After the final washing, peroxidase substrate (TMB Substrate Kit, Pierce) was added, and after 30 minutes, the reaction was stopped with 2 M H₂SO₄. Absorbance at 450 nm was then read in a microplate autoreader (Thermomax, Molecular Devices, Sunnyvale, CA, USA). Between each step, the plates were repeatedly washed with PBS, containing 0.05% Tween 20.

To measure serum levels of peanut-specific IgG1 or IgG2a, ELISA plates were coated with 2 µg/ml crude peanut extract in 0.1 M carbonate buffer, pH 9.5, overnight at 4°C. Plates were blocked with BSA as described; serial dilutions of serum samples were then added, and plates were incubated for 1 hour at RT. After washing, plates were incubated with HRP-conjugated anti-mouse IgG1 or anti-mouse IgG2a (BD Pharmingen, San Diego, CA, USA) diluted 1:1,000 for 1 hour at RT. Finally, peroxidase substrate was added, and after 15 minutes, the reaction was stopped with 2 M H₂SO₄.

Plasma levels of MCPT-1 were determined using a commercial mouse MCPT-1 ELISA Kit (eBioscience, San Diego, CA, USA) according to the manufacturer instructions. Sensitivity for the MCPT-1 ELISA was 234 pg/ml. The concentrations of IL-4, IL-5, IL-13, IL-21, TSLP, IL-1 α , and IL-1 β in supernatants from *in vitro* T cell cultures, BAL fluids, or lung homogenates were determined using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) according to manufacturer instructions. To prepare lung homogenates, lungs were ground in 0.5 ml PBS, and centrifuged at 10,000 $\times g$ at 4°C for 15 minutes; protein concentrations in the supernatant were quantified with the PierceTM BCA Protein Assay Kit (Thermo Fisher).

FACS analyses

T and B cell populations in mLNs were measured after peanut flour exposure by FACS analysis, as described⁵. Briefly, mLNs were collected from IL-4 reporter 4get mice or various wild-type (WT) and gene knockout strains, and single cell suspensions were treated with Fc blockers for 30 minutes at 4°C prior to staining with combinations of the following antibodies: CD3 (clone 145-2C11; FITC), CD4 (L3T4; PerCP), ST2 (DJ8, MD Biosciences, St. Paul, MN, USA; biotin), CXCR5 (2G8; PE, biotin), and PD-1 (J43; PE). Incubation with streptavidin-APC was used to visualize biotin-conjugated antibodies. To analyze germinal center (GC) B cells, cells were stained with FITC-conjugated peanut agglutinin (PEANUTA; Vector Laboratories, Burlingame, CA), anti-B220 PerCP (RA3-6B2), and anti-CD95/FAS biotin (Jo2). Subsequent incubation with streptavidin-APC visualized the biotin-conjugated antibody. After washing, cells were resuspended in PBS containing 1% BSA and 0.1% NaN₃ and fixed with 1% paraformaldehyde. Flow cytometric analysis was performed on a FACSCalibur or Canto X

cytometer (BD Biosciences, San Jose, CA), and data were analyzed with FlowJo software (Tree Star, Ashland, Oregon). Antibodies were obtained from BD Biosciences unless otherwise noted.

T cell cytokine production in vitro

Antigen-induced cytokine production by Th2 and Tfh cells was analyzed as described previously⁵. Briefly, CD4⁺ T cell subsets (CD3⁺CD4⁺ST2⁻CXCR5⁻ double-negative cells, CD3⁺CD4⁺ST2⁺CXCR5⁻ Th2 cells, and CD3⁺CD4⁺ST2⁻CXCR5⁺ Tfh cells) were isolated by FACS sorting from day 11 mLNs obtained from mice that had been i.n. exposed to peanut flour on days 0 and 7. Prior to T cell sorting, B cells were isolated by an EasySep Mouse B Cell Isolation Kit (STEMCELL Technologies, Vancouver, Canada). Sorted T cell subsets (20,000 cells/well) were cultured with isolated B cells (100,000 cells/well) in complete RPMI medium (RPMI 1640 medium with 10% calf serum, 100 µg/ml streptomycin, and 100 U/ml penicillin; total volume 200 µl/well) with or without 100 µg/ml crude peanut extract for 7 days. Concentrations of IL-4, IL-5, IL-13, and IL-21 in cell supernatants were measured by ELISA.

T cell transfer and IgE antibody production in vivo

The ability of Tfh and Th2 cells to support IgE antibody production *in vivo* was examined as described previously, with some modifications⁵. The CD3⁺CD4⁺ST2⁺CXCR5⁻ Th2 and CD3⁺CD4⁺ST2⁻CXCR5⁺ Tfh cell populations were prepared by FACS sorting from day 11 mLNs from C57BL/6 donor mice that had been exposed i.n. to peanut flour on days 0 and 7. *Tcrβ*^{-/-} recipient mice were injected intravenously (i.v.) into the tail vein with 1.25×10⁵ sorted Th2 or Tfh cells in PBS or PBS alone, 1 day after the first exposure to peanut flour. Mice were subsequently exposed i.n. to peanut flour twice/week for 4 weeks. Serum was collected for

analysis of peanut-specific antibodies on day 27.

Mouse airway exposure and challenge model to examine the adjuvant activities of peanut flour

On days 0 and 7, naïve BALB/c mice were lightly anesthetized with isoflurane and administered with 100 µg ovalbumin (OVA) intranasally (i.n.) in the presence or absence of 100 µg crude peanut extract or peanut flour in 50 µl endotoxin-free PBS. On day 14, plasma specimens were collected for analysis of anti-OVA IgE. On days 21, 22 and 23, mice were challenged i.n. with 100 µg OVA alone, and on day 24 bronchoalveolar lavage (BAL) fluids were collected for analysis of total cell counts and differentials.

Statistical analysis

The statistical significance for differences between the various treatment groups was calculated using a Student's *t*-test, with $P < 0.05$ considered statistically significant. All numerical data are presented as the mean $\pm$ standard error of the mean (SEM).

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FIGURE LEGENDS FOR ONLINE REPOSITORY

Figure E1. Airway exposure to peanut flour induces peanut allergy phenotypes in C57BL/6 mice. (A) Naïve BALB/c mice and C57BL/6 mice were exposed intranasally (i.n.) to PBS or 100 µg peanut flour twice a week for 4 weeks using the protocol described in Figure 1A. Serum specimens were collected on day 27. The levels of anti-peanut antibodies are shown at the dilutions indicated. ** $P < 0.01$ compared to mice exposed to PBS alone. Data are presented as the mean $\pm$ SEM [$n = 12-15$ (BALB/c) and $3-5$ (C57BL/6) in each group]. (B) C57BL/6 mice that had been exposed i.n. to peanut (PN) flour or PBS were challenged by intraperitoneal (i.p.) injection of 2.5 mg crude PN extract. The kinetic changes in rectal temperature (left) and clinical scores (right) were recorded. Data are presented as the mean $\pm$ SEM ($n = 3-5$ in each group). * $P < 0.05$ and ** $P < 0.01$ compared to mice exposed to PBS.

Figure E2. Production of anti-peanut antibodies is independent of TLR4. (A) Naïve BALB/c or TLR4-deficient mice (*Tlr4*^d) were exposed i.n. to PBS or 100 µg peanut flour for 4 weeks using the protocol described in Figure 1A. The levels of anti-peanut antibodies are shown at the dilutions indicated. Data are presented as the mean $\pm$ SEM ($n = 3-5$ in each group). * $P < 0.05$ compared to mice exposed to PBS. n.s., not significant.

Figure E3. Gating strategy for sorting of the Th2 and Tfh cell populations that were used for cell culture and adoptive transfer experiments. Naive WT C57BL/6 mice were exposed intranasally to peanut flour on days 0 and 7. Day 11 mLN cells were collected, enriched for CD4⁺ T cells, and sorted into CD3⁺CD4⁺ST2⁻CXCR5⁻ (controls), CD3⁺CD4⁺ST2⁺CXCR5⁻ (Th2) and CD3⁺CD4⁺ST2⁻CXCR5⁺ (Tfh) subpopulations. Representative FACS scattergrams are shown.

Figure E4. Mice that received Tfh cells or Th2 cells develop acute anaphylaxis upon peanut challenge. As described in Fig 3, Panel C, naïve C57BL/6 mice were exposed i.n. to peanut flour on day 0 and day 7, and Tfh and Th2 cells were sorted from day 11 mLNs. Cells (1.25×10^5) were i.v. transferred to *Tcr β ^{-/-}* (C57BL/6 background) mice, and animals were exposed i.n. to peanut flour for 4 weeks. Mice were then challenged by i.p. injection of 2.5 mg crude peanut extract. Changes in rectal temperature (left) and clinical scores (right) over time were recorded, as described in Materials and Methods. Results are the mean $\pm$ SEM (n = 3 in each group) and are representative of two experiments. **P* < 0.05 compared to mice that received no Tfh or Th2 transfer.

Figure E5. OX40L deficiency does not affect IgE antibody production in response to peanut. Naïve C57BL/6 (WT) and OX40L-deficient (*Tnfsf4^{-/-}*) mice were exposed i.n. to peanut flour or PBS for 4 weeks. Titers of anti-peanut antibodies in sera were determined by analyzing serial dilutions of the specimens by ELISA. Results are the mean $\pm$ SEM (n = 3 in each group).

Figure E6. Peanut flour, but not peanut extract, demonstrates adjuvant activity that promotes allergic sensitization to the model antigen OVA. (A) A schematic representation of the mouse airway sensitization and challenge model to examine the adjuvant activities of peanut flour. (B) Plasma levels of anti-OVA IgE and the numbers of inflammatory cells in BAL fluids were analyzed on day 14 and day 24, respectively. Results are the mean $\pm$ SEM (n=4 in each group). **P* < 0.05 between the groups indicated by the horizontal lines.

Figure E7. FACS analysis of Th2 and Tfh cells. Naive WT BALB/c mice were exposed

202 intranasally to peanut flour on days 0 and 7. Day 11 mLN cells were collected and analyzed by
203 FACS. The lymphocyte population was gated on the CD3⁺CD4⁺ cell population, and then
204 analyzed for expression of ST2, CXCR5 and PD-1. Representative FACS scattergrams are
205 shown.
206

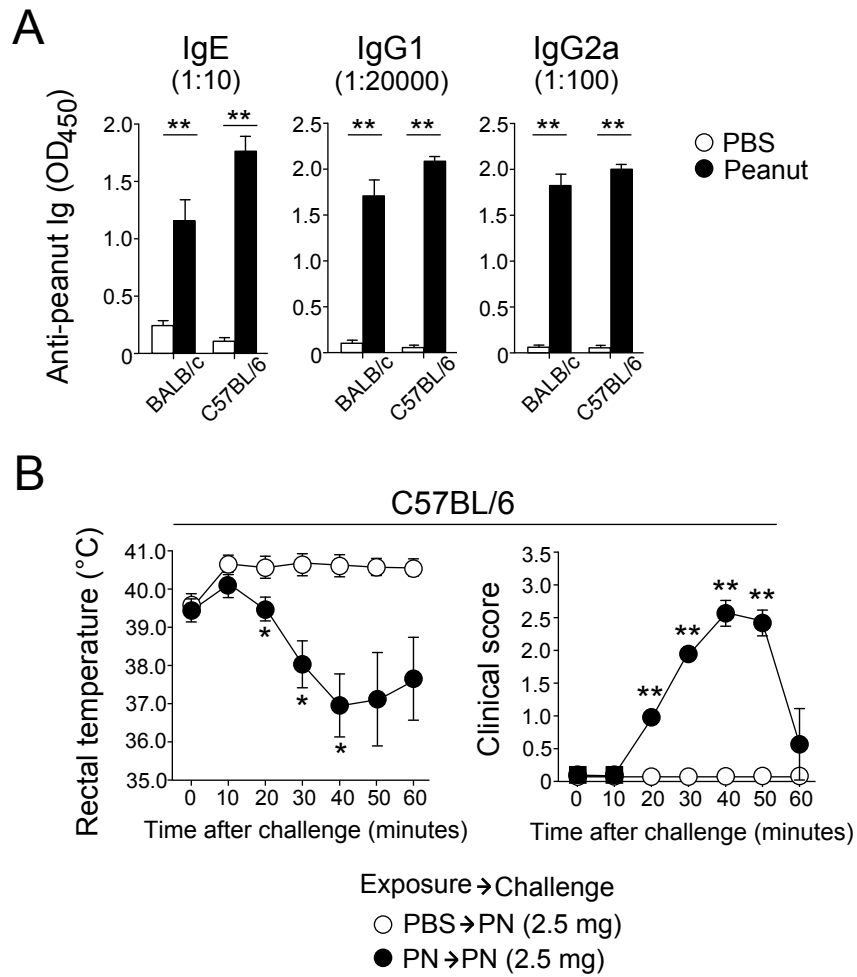


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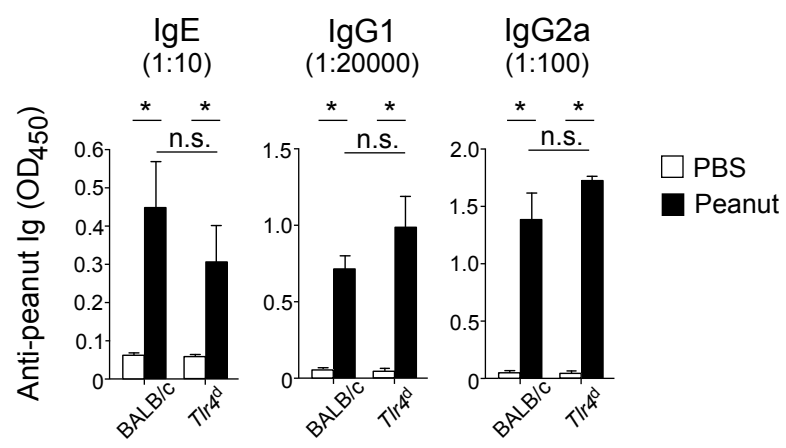


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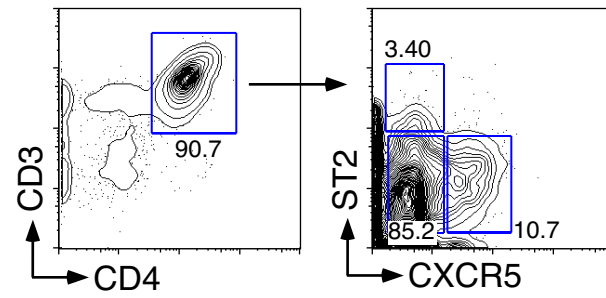


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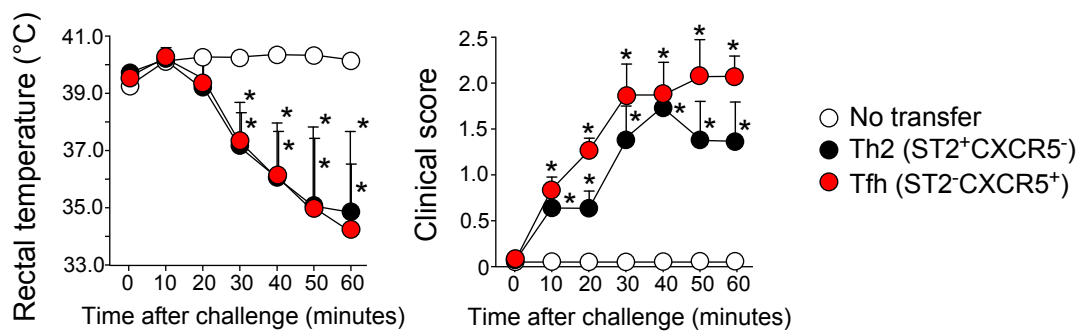


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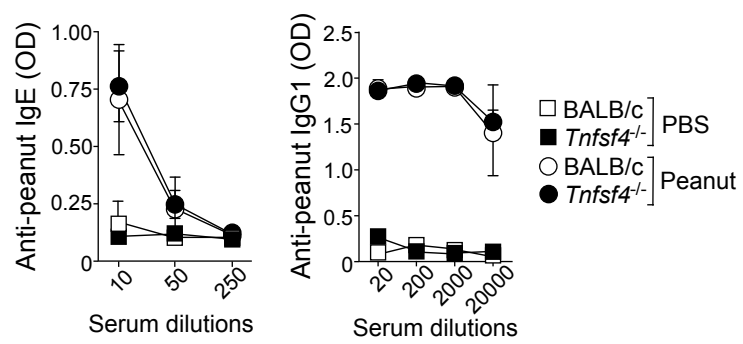
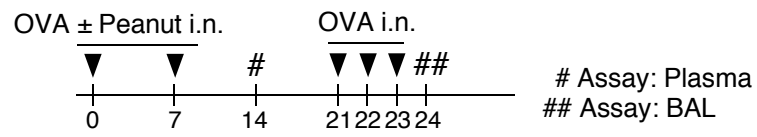


Figure E5

A



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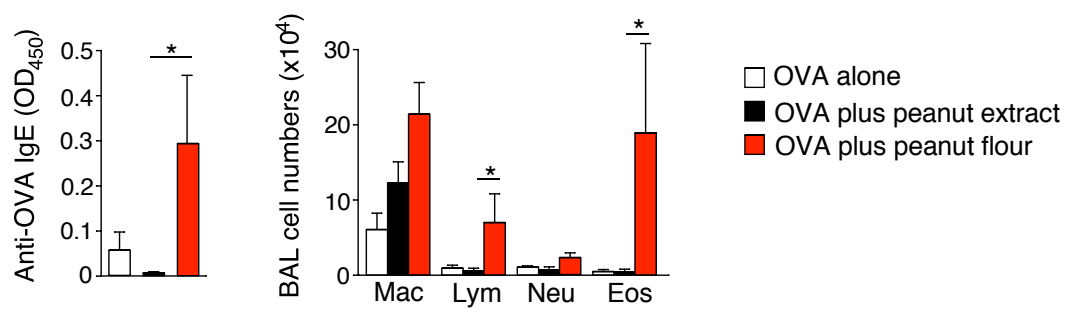


Figure E6

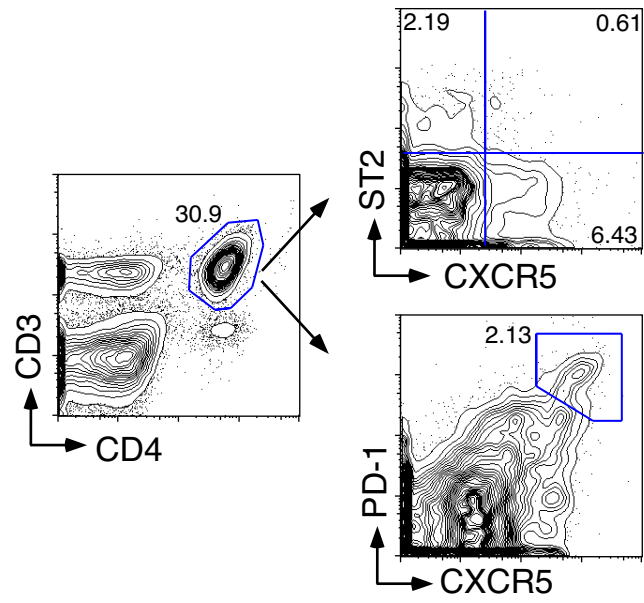


Figure E7