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B cells establish, but do not maintain, long-lived murine anti-peanut IgE¹

Daphne M. Moutsoglou^{a,*} and Stephen C. Dreskin^a

^aDivision of Allergy and Clinical Immunology and Departments of Medicine and Immunology, University of Colorado Denver, Aurora, CO

Abstract

Background—Peanut allergy has been reported to be transferred to tolerant recipients through organ and bone marrow transplantation. The roles T and B cells play in establishing, and the roles B cell subsets play in maintaining lifelong anti-peanut IgE levels are unknown.

Objectives—To determine the cellular requirements for the transfer of murine peanut allergy and to determine the role CD20⁺ cells play in maintaining long-lived anti-peanut IgE levels.

Methods—We developed a novel adoptive transfer model to investigate the cellular requirements for transferring murine peanut allergy. We also treated peanut-allergic mice with anti-CD20 antibody and measured IgE levels throughout treatment.

Results—Purified B220⁺ cells from peanut-allergic splenocytes and purified CD4⁺ cells from naïve splenocytes are the minimal requirements for the adoptive transfer of peanut allergy. Prolonged treatment of allergic mice with anti-CD20 antibody results in significant depletion of B cell subsets but does not affect anti-peanut IgE levels, symptoms, or numbers of IgE antibody secreting cells in the bone marrow. Adoptive transfer of bone marrow and spleen cells from allergic donors treated with anti-CD20 antibody does not result in the transfer of peanut allergy in naïve recipients, demonstrating that anti-CD20 antibody treatment depletes B cells capable of differentiating into peanut-specific IgE antibody secreting cells.

Conclusions and Clinical Relevance—Peanut allergy can be established in a naïve hosts with B220⁺ cells from peanut-allergic donors and CD4⁺ cells from peanut-naïve donors. However, long-term depletion of B220⁺ cells with anti-CD20 antibody does not affect anti-peanut IgE levels. These results highlight a novel role for B cells in the development of peanut allergy and provide evidence that long-lived anti-peanut IgE levels may be maintained by long-lived antibody secreting cells.

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*Corresponding author: University of Colorado Denver, Division of Allergy and Clinical Immunology, Campus Box B164, Research Complex 2, Room 10002, 12700 E. 19th Ave., Aurora, CO 80045. Telephone: 605-695-8979; Fax: 303-724-7212. daphne.moutsoglou@ucdenver.edu.

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Keywords

adoptive transfer; IgE; anaphylaxis; memory B cell; antibody secreting cell

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Introduction

IgE-mediated food allergy affects 4-6% of children in the United States [1], and 2% of children in the United States are allergic to peanut (PN) protein [2]. Food allergy has been transferred from peanut-allergic (PA) donors to tolerant recipients through transplantation of the liver [3-7], lung [8-10], combined liver and kidney [11], combined kidney and pancreas [12], and following bone marrow (BM) transplantation from related [13-16] and non-related [15, 17] donor-recipient pairs. This has not been studied in a murine model, and the specific cellular components responsible for transfer are unknown.

Eighty percent of PA children have lifelong peanut allergy (PNA) [18], treated by avoiding dietary peanut (PN). Although environmental exposure [19, 20] and accidental exposure [21-23] to PN may contribute to production of anti-PN IgE, overall, there is little overt antigenic stimulation throughout life, suggesting that long-lived IgE⁺ plasma cells (PCs) maintain life-long anti-PN IgE. Alternatively, short-lived IgE⁺ PCs may be generated by ongoing memory B cell differentiation due to exposure to a related antigen, persistent antigen depot [24], or polyclonal activation [25]. It is unclear if persistent PNA in humans is antigen-dependent or antigen-independent.

To address how PNA is established, we developed a novel adoptive transfer model of PNA in C3H/HeJ mice, which predominantly undergo IgE-mediated anaphylaxis [26-28], unlike C57BL/6 mice that can undergo IgG- as well as IgE-mediated anaphylaxis [26-30]. We hypothesized that B cells are both necessary and sufficient for the adoptive transfer of PNA, and that CD20⁺ B cells are central for maintaining long-lived anti-PN IgE. To determine if CD20⁺ B cells maintain anti-PN IgE, we treated PA mice with anti-CD20 antibody (Ab), measured anti-PN IgE for a prolonged period, and tested the ability of splenocytes (SPL) from donors treated with anti-CD20 Ab to adoptively transfer PNA.

Materials and methods

Crude peanut extract, Ara h 2 and Ara h 6 preparation

Crude peanut extract (CPE) and Ara h 2 and Ara h 6 proteins (97% pure without lectins) were purified as previously described [31].

Murine model of peanut allergy

Murine experiments were approved by the University of Colorado Denver Institutional Animal Care and Use Committee. Female, three-week old C3H/HeJ mice (Jackson Laboratories) were maintained on a PN- and soy-free diet. Mice were sensitized at five weeks of age with 1 mg of CPE plus 20 µg of cholera toxin (List Biological Laboratories, Inc.) given by oral gavage once per week for four weeks. Challenge occurred 2 weeks later with 250 µg of CPE through intraperitoneal (i.p.) injection. Symptom scores and changes in

body temperatures were measured as previously described [32] 30 minutes post-i.p. injection.

Adoptive transfer experiments

Naïve (NA), female C3H/HeJ recipients (8 weeks of age) were irradiated with 8 gray one day prior to intravenous reconstitution. Donors did not receive PN for 8 weeks prior to sacrifice for adoptive transfer. Pan B220⁺ and CD4⁺ cells were negatively selected from SPL (Miltenyi). SPL depleted of CD3⁺, CD19⁺, or B220⁺ cells were generated using positive selection (Miltenyi). Serum anti-PN IgE was obtained on days 7 and 17, and i.p. challenge with 500 µg of CPE occurred on days 10 and 18. Mice were scored in an independent, blinded manner.

In vivo depletion of B cells and adoptive transfer of cells from donors treated with anti-CD20 or isotype control antibody

PA mice were intravenously treated every 3 weeks for 18 weeks with 250 µg of anti-CD20 [33] or isotype control (IC) Ab (IgG2a) (Genentech Inc.). PA donors were not re-challenged prior to sacrifice for transplant. NA recipients were irradiated, reconstituted, and challenged (as described above).

Flow cytometry

Cells were stained with the following Ab: anti-mouse CD16/CD32 FcR Block (2.4G2), PECy7 anti-mouse B220 (RA3-6B2), APC anti-mouse CD19 (1D3), PE anti-mouse CD138 (281-2), and FITC and BV421 anti-mouse kappa light chain (187.1) (BD Pharmingen); PE anti-mouse CD4 (GK1.5), eFluor450 anti-mouse CD11c (N418), and APC-eFluor780 anti-mouse CD8a (53-6.7) (Ebioscience); and FITC anti-IgG1 (RMG1-1) and FITC anti-IgM (RMM-1) (Biolegend). Fixation Buffer and Perm/Wash Buffer (BD) were used for intracellular staining. Dead cells were excluded from analysis using the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Molecular Probes, Life Technologies), and cells were gated on singlets. Fluorescence minus one controls were used for analysis of gate placement rather than isotype control Ab since these resulted better approximation of correct gate placement when analyzing B cell subsets.

Anti-peanut IgE and mouse mast cell protease-1

Anti-PN IgE was measured as previously described [34]. Mouse mast cell protease-1 (MMCP-1) was measured using the MMCP-1 Ebioscience Affymetrix ELISA Kit.

Enumeration of antibody secreting cells

Ninety-six-well PVDF membrane ELISPOT plates (Millipore) were coated with CPE, goat anti-mouse IgG (Sigma), or rat anti-mouse IgE (R35-72, BD Biosciences). To enumerate 1) IgG antibody secreting cells (ASCs), 0.5×10^5 cells per well were incubated for 4 hours at 37°C; and 2) IgE ASCs, 1×10^6 cells per well were incubated overnight for 18 hours at 37°C. The following Ab were incubated sequentially for one hour at 37°C: biotin goat-anti-mouse IgG (BioLegend) and avidin-peroxidase for IgG ASCs; or sheep anti-mouse IgE (The Binding Site Group, Ltd), rabbit anti-sheep IgG and peroxidase goat anti-rabbit IgG (both

from Jackson ImmunoResearch Laboratories Inc.) for IgE ASCs. Spots were visualized using AEC substrate (BD Biosciences) and were subtracted from background wells (RPMI 10% FCS alone). C.T.L. ImmunoSpot Analyzer and Software 4 (Cellular Technology) were used to read and analyze plates.

Statistics

GraphPad Prism 5 was used to conduct all statistical analyses. The Mann-Whitney test was used to analyze symptom scores, and unpaired two-tailed t-tests were used for all other comparisons. P-values less than 0.05 were considered significant.

Results

Peanut-allergic splenocytes, not peanut-allergic bone marrow cells, are required for the adoptive transfer of peanut allergy

Timing for sensitization, challenge, and adoptive transfer for this transplant (and all subsequent transplants) is shown in Fig. 1a. Preliminary experiments demonstrated that the transfer of BM cells alone, from PA donors did not result in the transfer PNA. In the experiment shown in Supplementary Fig. 1a-d, either 6 or 60 million BM cells were transferred from PA donors into NA recipients. Recipients given PA SPL served as a positive control. PNA was not transferred to recipients given either 6 or 60 million PA BM cells (Supplementary Fig 1a-c) even though recipients of 60 million PA BM cells were given twice as many CD19⁺ cells than positive control recipients given 10 million PA SPL. These results illustrate that CD19⁺ cells from the PA BM are not capable of transferring PNA (Supplementary Fig. 1a-d). To determine if PNA could be adoptively transferred with unfractionated SPL, NA recipients were reconstituted with either: 1) NA BM and NA SPL, 2) NA BM and PA SPL, 3) PA BM and NA SPL, or 4) PA BM and PA SPL. None of the recipients had measureable anti-PN IgE on day 7, and none displayed symptoms or hypothermia during the first challenge on day 10 (for this transplant and all subsequent transplants, data not shown). However, one week after the first exposure to CPE, both groups receiving PA SPL with either NA or PA BM had significantly elevated anti-PN IgE levels (Fig. 1b). Upon the second challenge on day 18, both groups given PA SPL had significantly elevated symptom scores and hypothermia (Fig. 1c and 1d). Thus, we conclude that B-lineage cells from the PA BM do not transfer PNA, and the cell population responsible for transfer resides in the SPL.

It is possible that radiation one day prior to reconstitution results in destruction of niches within the BM that would be important for engraftment and survival of transferred ASCs. We found that irradiating recipient mice was necessary for a robust IgE response. In an additional set of experiments, we adoptively transferred whole PA SPL (10 million cells) into non-irradiated recipient mice and found that only 40% of recipient mice developed symptoms, which were relatively mild compared to their irradiated counterparts (data not shown). In order to focus on the role of SPL cells in the adoptive transfer of PNA, we used NA BM for the remainder of the cell transfer experiments.

In vitro depletion of either splenic B220⁺ or CD19⁺ cells abrogates the adoptive transfer of murine peanut allergy

Since PNA is an IgE-dependent condition, and only one challenge after adoptive transfer is needed to generate an IgE response, we hypothesized that B220⁺ cells were required. To test this hypothesis, we independently depleted B220⁺ or CD19⁺ cells from PA SPL *in vitro* and added back B220⁺ cells purified from NA donor SPL to control for the number of B cells (purity, Supplementary Fig. 2a). As a positive control, a group of recipients was given PA SPL.

Mice that received PA SPL depleted of either B220⁺ or CD19⁺ cells plus B220⁺ cells from NA SPL did not develop anti-PN IgE on day 17 in contrast to recipients given PA SPL (Fig. 2a). In addition, groups that received PA SPL depleted of either B220⁺ or CD19⁺ cells plus NA B220⁺ cells also did not develop symptoms or hypothermia upon the second challenge (Fig. 2b and 2c). Mice receiving PA SPL had significantly elevated MMCP-1 levels compared to recipients given PA SPL depleted of B220⁺ or CD19⁺ cells (Fig. 2d). Thus, SPL B220⁺ and CD19⁺ cells are required for the adoptive transfer of PNA.

B220⁺ cells from peanut-allergic spleens are not sufficient for the adoptive transfer of peanut allergy

To determine if B cells were sufficient for the adoptive transfer of PNA, B220⁺ cells purified from PA SPL were transferred alone or in combination with NA SPL (purity, Supplementary Fig. 2b). Control groups were given B220⁺ cells purified from NA SPL plus NA SPL, or B220⁺ cells from PA SPL. On day 17, mice given the combination of B220⁺ cells from PA SPL with the addition of NA SPL developed significantly elevated anti-PN IgE levels compared to controls (Fig. 3a).

Upon the second challenge, mice receiving B220⁺ cells from PA SPL with added NA SPL also displayed significantly elevated symptom scores and hypothermia compared to control groups (Fig. 3b and 3c). These results suggest that a cell population(s) within the NA SPL, following a single exposure to CPE, is capable of helping B220⁺ cells rapidly develop into IgE ASCs.

In vitro depletion of CD3⁺ cells from peanut-allergic splenocytes abrogates the adoptive transfer of peanut allergy, which can be restored by the addition of CD4⁺ cells purified from naïve splenocytes

Given that sensitization to PN is T cell-dependent [35], we hypothesized that cells within the NA SPL helping PA B cells were CD4⁺ T cells. Thus, we depleted PA SPL of CD3⁺ cells and added CD4⁺ cells purified from NA SPL (purity, Supplementary Fig. 2c). Recipients were given either: 1) PA SPL depleted of CD3⁺ cells, 2) PA SPL depleted of CD3⁺ cells plus CD4⁺ cells purified from NA SPL, 3) PA SPL depleted of CD3⁺ cells plus CD4⁺ cells purified from PA SPL, or 4) PA SPL.

By day 17, mice receiving PA SPL depleted of CD3⁺ cells did not develop anti-PN IgE (Fig. 4a) indicating that SPL CD3⁺ cells are required for the transfer of PNA. After one challenge,

mice receiving PA SPL depleted of CD3⁺ cells plus NA CD4⁺ cells developed significantly elevated anti-PN IgE by day 17 (Fig. 4a).

Mice receiving CD3-depleted PA SPL did not develop symptoms or hypothermia during the second challenge (Fig. 4b and 4c). However, recipients given CD3-depleted PA SPL plus CD4⁺ cells from NA SPL developed significantly elevated symptom scores, hypothermia, and elevated MMCP-1 levels compared to the group given CD3-depleted PA SPL (Fig. 4b-d). Thus, NA CD4⁺ cells help PA SPL devoid of T cells rapidly form PN-specific IgE ASCs.

B220⁺ cells purified from peanut-allergic splenocytes and CD4⁺ helper T cells purified from naïve splenocytes are the minimal requirements for the adoptive transfer of peanut allergy

To determine whether B220⁺ cells from PA SPL and CD4⁺ cells from NA SPL were solely responsible for IgE production, or if dendritic or type II innate lymphoid cells were also required, we purified CD4⁺ and B220⁺ cells from both NA and PA SPL (purity, Supplementary Fig. 2d). Recipients were given either: 1) B220⁺ cells with CD4⁺ cells both purified from NA SPL, 2) B220⁺ cells purified from PA SPL, 3) B220⁺ cells purified from PA SPL with CD4⁺ cells purified from NA SPL, 4) B220⁺ cells purified from PA SPL with NA SPL, or 5) B220⁺ cells and CD4⁺ cells both purified from PA SPL.

One week after the first challenge, recipients given B220⁺ cells from PA SPL plus CD4⁺ cells from NA SPL had significantly elevated anti-PN IgE levels compared to the negative control given B220⁺ cells with CD4⁺ cells both purified from NA SPL, and compared to the negative control given only B220⁺ cells from PA SPL (Fig. 5a). Upon the second challenge, recipients given B220⁺ cells from PA SPL plus CD4⁺ cells from NA SPL developed significantly elevated symptom scores, hypothermia, and increased serum MMCP-1 levels (Fig. 5b-d) compared to the two negative control groups.

As an additional control, one recipient group was reconstituted with CD4⁺ cells from PA SPL and B220⁺ cells from NA SPL and did not develop anti-PN IgE, symptoms, or hypothermia (Supplementary Fig. 1e-g), indicating that allergic CD4⁺ cells cannot transfer PNA. Thus, the minimal requirements for the adoptive transfer of PNA are B220⁺ cells from PA SPL and CD4⁺ cells from NA SPL.

To determine if exposure to PN protein was required on day 10 for IgE production to occur by day 17 we challenged two groups of recipients given B220⁺ cells from PA SPL and CD4⁺ cells from NA SPL with either saline (sham) or a preparation of Ara h 2 and Ara h 6 proteins (20kD fraction devoid of lectins [31]) on day 10. Both groups were challenged with CPE on day 18. Sham-challenged recipients did not develop anti-PN IgE by day 17 or symptoms upon challenge with CPE on day 18 (Supplementary Fig. 1e-g), indicating that the development of IgE by day 17 and symptoms are dependent upon prior exposure to PN (day 10). Recipients challenged with the 20kD fraction developed significantly elevated anti-PN IgE levels, symptom scores and hypothermia (Supplementary Fig. 1e-g) indicating that lectins found in CPE given on day 10 are not required to develop PN-specific IgE levels by day 17, or symptoms and hypothermia on day 18.

CD20⁺ B cells are not required for maintaining long-lived serum anti-peanut IgE levels

Since SPL B220⁺ cells previously exposed to CPE are capable of transferring murine PNA with help from PN-NA CD4⁺ cells, we hypothesized that B220⁺ cells would also be required for maintaining anti-PN IgE levels. To test this hypothesis, PA mice were divided into two groups so that there were no significant differences in anti-PN IgE levels, symptoms (all scored a 3 out of 5), or temperature drops (Supplementary Fig. 3a). Mice were treated with anti-CD20 or IC Ab, at 3 week intervals over 18 weeks, (Fig. 6a) and were re-challenged 21 weeks after their first challenge (week -3).

Anti-CD20 treatment significantly depleted the peripheral blood of B220⁺CD19⁺ cells by 95% and IgG1⁺ cells by 98%; SPL B220⁺ cells by 98%; SPL and BM IgG1⁺ cells by 96% and 77%, respectively; and peritoneal CD19⁺IgM⁺ cells by 94% (Supplementary Fig. 3b-3e). In contrast, B220⁺ cells in the BM were not affected (Fig. 6b and Supplementary Fig. 3c). However, when CD19⁺ BM cells were analyzed for surface and intracellular Ig kappa light chain (sIgκ and iIgκ, respectively) expression, there was approximately a 60% decrease in CD19⁺sIgκ⁺iIgκ⁺ cells in anti-CD20 treated mice (Fig. 6c and Supplementary Fig. 3f), indicating that the majority of residual CD19⁺ cells in the BM are early B-lineage cells not expressing κ light chain. This depletion in the BM and SPL was observed as early as 3 weeks of anti-CD20 Ab treatment (one injection) in a separate group of mice (Supplementary Fig. 2e).

Strikingly, despite this prolonged and severe depletion of mature B cells in the blood, peritoneum, SPL, and BM, serum anti-PN IgE levels did not significantly change compared to those in mice treated with the IC (Fig. 6d). After 18 weeks of treatment, mice receiving either anti-CD20 or IC Ab were challenged and did not have significant differences in symptoms or hypothermia (Fig. 6e-f) demonstrating that prolonged B cell depletion does not affect symptoms of PNA.

Immediately after the final challenge (week 18), mice were sacrificed and BM and SPL cells were isolated for FACS analysis and ASC enumeration. Although total IgG levels in the serum were unaffected by anti-CD20 Ab (Fig. 7a), unexpectedly, PN-specific IgG and total IgG ASCs in the SPL were significantly decreased in anti-CD20 treated mice (Fig. 7b). In contrast, PN-specific IgG ASCs in the BM were significantly elevated, and total IgG ASCs in the BM were unaffected by treatment with anti-CD20 Ab (Fig. 7c). The decrease of SPL ASCs suggests that these are short-lived, CD20-expressing plasmablasts depleted by anti-CD20 Ab [36, 37].

SPL, total IgE ASCs were significantly decreased in anti-CD20 treated mice (Fig. 7d). However, total IgE ASCs in the BM were not affected by anti-CD20 Ab treatment (Fig. 7e). CD138⁺ cells were also not affected in the BM but were significantly decreased in the SPL (Supplementary Fig. 3g). Additionally, CD3⁺ cells in the BM and SPL were unaffected by anti-CD20 Ab treatment (Supplementary Fig. 3h). These results suggest that total IgE ASCs in the SPL are CD20-expressing plasmablasts that are depleted by anti-CD20 Ab, whereas total IgE ASCs in the BM are long-lived and are responsible for maintaining serum IgE levels. Taken together these data show that anti-CD20 treatment leads to profound depletion

of peripheral B cells and SPL ASCs, but not BM ASCs, serum total IgG Ab titers, or PN-specific IgE Ab titers.

Prolonged in vivo depletion of B cells with anti-CD20 antibody abrogates the adoptive transfer of peanut allergy

To determine if B cells in the BM or SPL surviving anti-CD20 depletion were capable of maintaining serum IgE levels, BM and SPL cells from PA donors treated with either anti-CD20 or IC Ab were adoptively transferred into recipients. Donors were not re-challenged prior to transplant and, thus, did not receive any PN protein for 21 weeks prior to sacrifice (Fig. 8a). As previously seen, treatment with anti-CD20 Ab did not affect anti-PN IgE levels in donor groups (Fig. 8b).

FACS analysis of cells transferred for expression of CD19⁺, CD19⁺sIgκ⁺iIgκ⁺, IgG1⁺, CD138⁺, CD3⁺, and CD11c⁺ is shown (Supplementary Fig. 4a-f). As expected, recipients given cells from donors treated with anti-CD20 Ab received significantly fewer CD19⁺, CD19⁺sIgκ⁺iIgκ⁺ cells, and IgG1⁺ cells in the BM and SPL compared with those receiving cells from donors treated with the IC (Supplementary Fig. 4a-c). Recipients also received significantly increased numbers of CD138⁺ cells from the BM and significantly decreased numbers of CD138⁺ cells from the SPL of donors treated with anti-CD20 Ab compared to controls (Supplementary Fig. 4d).

One week after the first challenge, mice receiving both BM and SPL from anti-CD20 treated donors did not develop anti-PN IgE, whereas recipients given BM and SPL from PA donors treated with the IC developed significantly elevated levels of anti-PN IgE (Fig. 8c). Thus, the residual CD19⁺ cells in the PA BM (Supplementary Fig. 3f) were not able to transfer PNA. Upon the second challenge on day 18, those given cells from donors treated with anti-CD20 Ab did not develop symptoms or hypothermia (Fig. 8d and 8e). These results also show that although some B cells survive depletion with anti-CD20 Ab, particularly in the BM, they are not capable of adoptively transferring PNA and thus, do not form IgE ASCs. These results also show that the population of CD20⁺ cells in the SPL in IC-treated donors are long-lived, since they produce IgE upon transfer and challenge in recipients. A previous study has shown that IgE⁺ B cells transferred from *Nippostrongylus brasiliensis*-inoculated donor mice can establish high IgE levels in NA recipients [38]. In our model, we were not able to distinguish whether IgE⁺ or IgG1⁺ B cells are responsible for adoptively transferring PNA due to a lack of transgenic strains on the C3H/HeJ background that would allow us to distinguish between these two subgroups of B cells. However, we were able to detect significant depletion of IgG1⁺ cells in the BM and SPL of anti-CD20 treated mice (Supplementary Fig. 3d), showing that IgG1⁺ cells likely do not function in maintaining persistently elevated anti-PN IgE levels.

Discussion

In this study, we developed a novel adoptive transfer model coupled with depletion of CD20⁺ cells to investigate the role of B220⁺ cells to establish and maintain murine PNA. We chose the i.p. challenge route since doses of CPE in the µg range elicit symptoms. In contrast, intragastric challenge requires adjuvants like ethanol to increase mucosal

permeability [39] (resulting in systemic absorption of PN) and a larger concentration of CPE (200 mg given in multiple doses) [40].

Transfer of PNA in humans through BM transplantation has been reported [13-17]; however, it is unknown which cell population(s) is responsible. The transfer of Ab-mediated immunity through human BM transplantation has been reported following vaccination of the donor with *Haemophilus influenza* type B capsular polysaccharide and booster in the recipient 9 and 11 months post-transplant [41]. Clonal B cells specific for *H. influenza* type B capsular polysaccharide detected in the donor were also present in the recipient following a post-transplant booster of the vaccine, showing that memory B cells can be transferred through BM transplantation and can result in the production of Ab [41]. We observed that splenic B cells were required for the adoptive transfer of PNA, and that transfer of PA BM did not result in established PNA even when twice as many B cells are transferred from PA BM compared to the number transferred through PA SPL (Supplementary Fig. 1a-d). Higher numbers of B cells with a rapid recall response to PN protein may occur in the SPL compared to the BM, likely due to production of memory B cells through germinal center reactions [42], although, this was not directly tested in these experiments. In the intragastric gavage model, our experiments confirm that there is systemic maintenance of PNA by B cells found within SPL. It is also possible that local maintenance in the gut within gut-associated lymphoid tissue such as the Peyer's patches may also be important in maintaining this response.

B220⁺ cells from PA SPL were not sufficient for the adoptive transfer of PNA, requiring help from PN-NA CD4⁺ T cells for the production of IgE. Our finding is compatible with a previous study using CD4⁺ cells (purified from NA DO11.10 mice) that helped OVA-immunized B cells almost as well as OVA peptide-primed (memory) CD4⁺ cells form anti-OVA IgG in NA recipients [43].

In our model, CD4⁺ cells purified from NA SPL are PN-NA, since they have not encountered CPE prior to challenge on day 10. Priming of these NA CD4⁺ cells with PN-allergen may occur in recipients following day 10 challenge with CPE. Adoptively transferred B220⁺ cells could potentially directly present antigen through IgE-facilitated antigen presentation [44-49], which may explain the rapid IgE production (7 days) in response to challenge. It is also possible that T cell-derived cytokines, such as IL-4 formed from these transferred NA CD4⁺ cells, are required for production of PN-specific IgE, as previously found with formation of 'natural' IgE in mice [50]. Because both CD4⁺ cells from either NA or PA donors were capable of helping transferred B cells produce IgE, it is also possible that B cells with a memory phenotype only require CD40 ligand interaction that could be supplied by activated CD4⁺ cells found in PN-NA SPL. Additionally, it is unknown if the CD4⁺ cells were polarized by transferred B cells from PA SPL at the time of adoptive transfer, or if CD4⁺ cells require priming with PN to help B cells.

Similar to PN-specific IgE titers that persist for decades in PA humans, we show that the C3H/HeJ model of PNA is similarly characterized by high anti-PN IgE levels that persist for at least 21 weeks after the last introduction of CPE. Given that the half-life of free IgE in murine plasma ranges from 2 to 14 hours [51-53], persistent IgE levels found in PA mice are

likely perpetuated by either long-lived IgE PC, or by IgG1⁺ or IgE⁺ B cells that provide a constant source of short-lived IgE⁺ PC.

Recent studies using transgenic strains to track IgE⁺ B cells [38, 54-56] have predominantly found IgE⁺ PC to be short-lived. Although the evidence for short-lived IgE⁺ PC is very strong, and *N. brasiliensis* generates robust levels of serum IgE, the IgE in this model is not long-lived and dissipates within 4 weeks of inoculation after the infection has been cleared [38, 57]. Several other rodent models of long-lived serum IgE titers exist and have resulted in long-lived serum IgE levels [58-61]. Rodent serum IgE has been shown to persist even after total body irradiation [62, 63].

Previous reports investigating the effects of anti-CD20 Ab treatment on humoral responses found that anti-CD20 Ab did not affect established basal Ig titers, including IgE titers in rituximab-treated patients [64], but did affect the generation of new Ab [33], compatible with the independent observation that long-lived PC down-regulate CD20 expression and are not depleted by anti-CD20 treatment [65]. Similarly, we observed that after prolonged depletion of B cells in the blood, peritoneum, and SPL, and depletion of the majority of mature B cells in the BM, total IgE ASCs and CD138⁺ cells in the BM were unaffected by anti-CD20 Ab treatment (Fig. 7e and Supplementary Fig. 3g).

Given the short half-life of plasma IgE and that anti-CD20 therapy depletes B cells capable of differentiating into IgE ASCs, and IgE levels are persistently elevated following prolonged treatment with anti-CD20 Ab, it is possible that long-lived IgE⁺ PC (not requiring a functional B cell pool) are responsible for the persistent serum IgE levels in PA mice.

Immunologic memory capable of producing PN-specific IgE ASCs after one challenge resides in PA B cells that require help from NA CD4⁺ cells. IgE levels persist for a prolonged time, even in the absence of mature B cells in the blood, SPL, and peritoneum indicating BM IgE ASCs could be long-lived in murine PNA.

Our data and that of others [64] suggest that therapies targeted only against memory B cells are unlikely to be useful to treat food allergy. Our data demonstrate that serum IgE is long-lived (even in the absence of B cells), and B cells from PA mice are able to develop into IgE-producing cells. Thus, we predict that a successful, B-lineage focused approach to treating food allergies will likely require targeting both antigen-specific IgE⁺ memory B cells and IgE-producing cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations in this article

Ab	antibody
ASC	antibody secreting cell
CPE	crude peanut extract
FACS	fluorescence-activated cell sorting
Ig	immunoglobulin
i.p	intraperitoneal

IC	isotype control
iIgκ	intracellular immunoglobulin kappa light chain
MMCP-1	mouse mast cell protease-1
NA	naïve
PN	peanut
PA	peanut-allergic
PNA	peanut allergy
PC	plasma cells
SPL	splenocytes/spleen/splenic
sIgκ	surface immunoglobulin kappa light chain

Fig. 1.

Peanut-allergic splenocytes, but not peanut-allergic bone marrow cells, are required for the adoptive transfer of peanut allergy. (a) Timing of sensitization and challenge in donors and timing of adoptive transfer and challenge in recipients. (b) Serum anti-PN IgE levels in recipients on day 17. (c) Symptom scores and (d) changes in body temperature in recipients upon the second challenge on day 18. All recipients were given 6×10^6 BM cells and 10×10^6 SPL from either NA or PA donors. In a cell titration experiment, 60% of recipients given 6×10^6 PA SPL developed anaphylaxis by day 18 versus 100% of recipients given 10×10^6 PA

SPL (data not shown). Data represent one of three independent experiments with 5-14 mice per group. Error bars denote mean $\pm$ SD.

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Fig. 2.

B cells are required for the adoptive transfer of peanut allergy. **(a)** Serum anti-PN IgE levels in recipients on day 17. **(b)** Symptom scores, **(c)** changes in body temperature, and **(d)** serum MMCP-1 levels in recipients upon the second challenge on day 18. All recipients were given 6×10^6 cells from NA BM. Other groups received a combination of 5.5×10^6 cells from PA SPL depleted of either B220⁺ or CD19⁺ cells, with the addition of 4.5×10^6 B220⁺ cells purified from NA SPL. Another group received 10×10^6 PA SPL. Data represent one of two independent experiments with 10 mice per group. Similar results were obtained in two additional experiments in which both B220⁺ and CD19⁺ cells were depleted from PA SPL. Error bars denote mean $\pm$ SD.

Fig. 3. B220⁺ cells from allergic donors are not sufficient for the adoptive transfer of peanut allergy. (a) Serum anti-PN IgE levels in recipients on day 17. (b) Recipient symptom scores and (c) changes in body temperature upon the second challenge on day 18. All recipients were given 6×10^6 cells from NA BM. Select groups were given 4.5×10^6 B220⁺ cells purified from either NA or PA SPL, 5.5×10^6 NA SPL, or 10×10^6 PA SPL. Data represent one of three independent experiments with 4-10 mice per group. Error bars denote mean $\pm$ SD.

Fig. 4.

CD4⁺ cells isolated from naïve splenocytes can help allergic splenocytes depleted of CD3⁺ cells differentiate into IgE antibody secreting cells. (a) Serum anti-PN IgE levels in recipients on day 17. (b) Symptom scores, (c) changes in body temperature, and (d) serum MMCP-1 levels in recipients upon the second challenge on day 18. All recipients were given 6×10^6 cells from NA BM. Recipients were also given one the following: 10×10^6 cells from CD3⁺ depleted PA SPL, 8.5×10^6 cells from CD3⁺ depleted PA SPL with 1.5×10^6 CD4⁺ cells (isolated either from NA or PA SPL), or 10×10^6 PA SPL. Data represent one of two independent experiments with 5-10 mice per group. Error bars denote mean $\pm$ SD.

Fig. 5.

B220⁺ cells from peanut-allergic donor spleens and CD4⁺ cells isolated from naïve donor spleens are the minimal requirements for the adoptive transfer of peanut allergy. **(a)** Serum anti-PN IgE levels in recipients on day 17. **(b)** Symptom scores, **(c)** changes in body temperature, and **(d)** serum MMCP-1 levels in recipients upon the second challenge on day 18. All recipients were given 6×10^6 cells from NA BM. Recipients were also given combinations of the following: 4.5×10^6 B220⁺ cells purified from either NA or PA SPL, 1.5×10^6 CD4⁺ cells purified from NA or PA SPL, 5.5×10^6 NA SPL, or 10×10^6 PA SPL. Data represent one of three independent experiments with 5-10 mice per group. Error bars denote mean $\pm$ SD. See Supplementary Fig. 1 for additional controls.

Fig. 6.

Prolonged B cell depletion does not affect serum anti-peanut IgE levels or symptoms of peanut allergy. (a) Timing of sensitization, challenge, treatment with anti-CD20 or IC Ab, and re-challenge. (b) FACS analysis of total B cells in the BM and SPL of one mouse after 18 weeks of treatment with either Ab. (c) Analysis of sIgk⁺ and iIgk⁺ cells among CD19⁺ cells in the BM of one mouse after 18 weeks of treatment with either Ab. (d) Pre-treatment anti-PN IgE levels (week -3), and anti-PN IgE levels throughout treatment with either Ab. (e) Symptom scores and (f) changes in body temperature in mice following re-challenge

after 18 weeks of treatment with either Ab. Data represent one of three independent experiments with 10-12 mice per group. Error bars denote mean $\pm$ SD.

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Fig. 7.

Total serum IgG, peanut-specific IgG, and total IgE antibody secreting cells in peanut-allergic mice after 18 weeks of treatment with either anti-CD20 or isotype control antibody. (a) Serum total IgG levels after 18 weeks of treatment with anti-CD20 or IC Ab. Total numbers of PN-specific IgG and total IgG ASCs within the (b) SPL and (c) BM of each treated mouse. Total numbers of total IgE ASCs within the (d) SPL and (e) BM of each treated mouse. All ASC numbers have been normalized to total numbers of cells in either the BM or SPL of each mouse and were determined immediately after challenge on week 21. Data represent one of two independent experiments with 10-12 mice per group. Error bars denote mean ± SD.

Fig. 8.

B cells with a rapid recall response to peanut protein are depleted in peanut-allergic mice treated with anti-CD20 antibody. (a) Schematic of sensitization, challenge, and treatment of donors with either anti-CD20 or IC Ab followed by adoptive transfer of BM cells and SPL into NA recipients. Donors were not re-challenged after Ab treatment prior to adoptive transfer. (b) Serum anti-PN IgE levels in donors treated with either Ab one week prior to sacrifice for transplant (day -8). (c) Serum anti-PN IgE levels in recipients on day 17. (d) Symptom scores and (e) changes in body temperature in recipients upon the second challenge on day 18. All recipients were given 6×10^6 BM cells and 10×10^6 SPL purified

from peanut-allergic donors treated with either anti-CD20 or IC Ab. Data represent one of two independent experiments with 13-15 mice per group. Error bars denote mean $\pm$ SD.

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