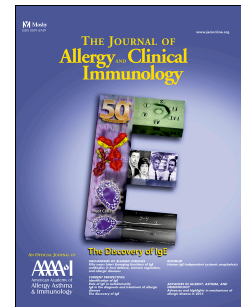


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Lifelong Memory Responses Perpetuate Humoral Th2 Immunity and Anaphylaxis in Food Allergy

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TITLE: Lifelong Memory Responses Perpetuate Humoral Th2 Immunity and Anaphylaxis in Food Allergy

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ABSTRACT

Background: A number of food allergies (e.g. fish, shellfish, nuts) are lifelong, without any disease-transforming therapies and unclear in their underlying immunology. Clinical manifestations of food allergy are largely mediated by IgE. Although persistent IgE titres have conventionally been attributed to long-lived IgE⁺ plasma cells (PCs), this has not been directly and comprehensively tested.

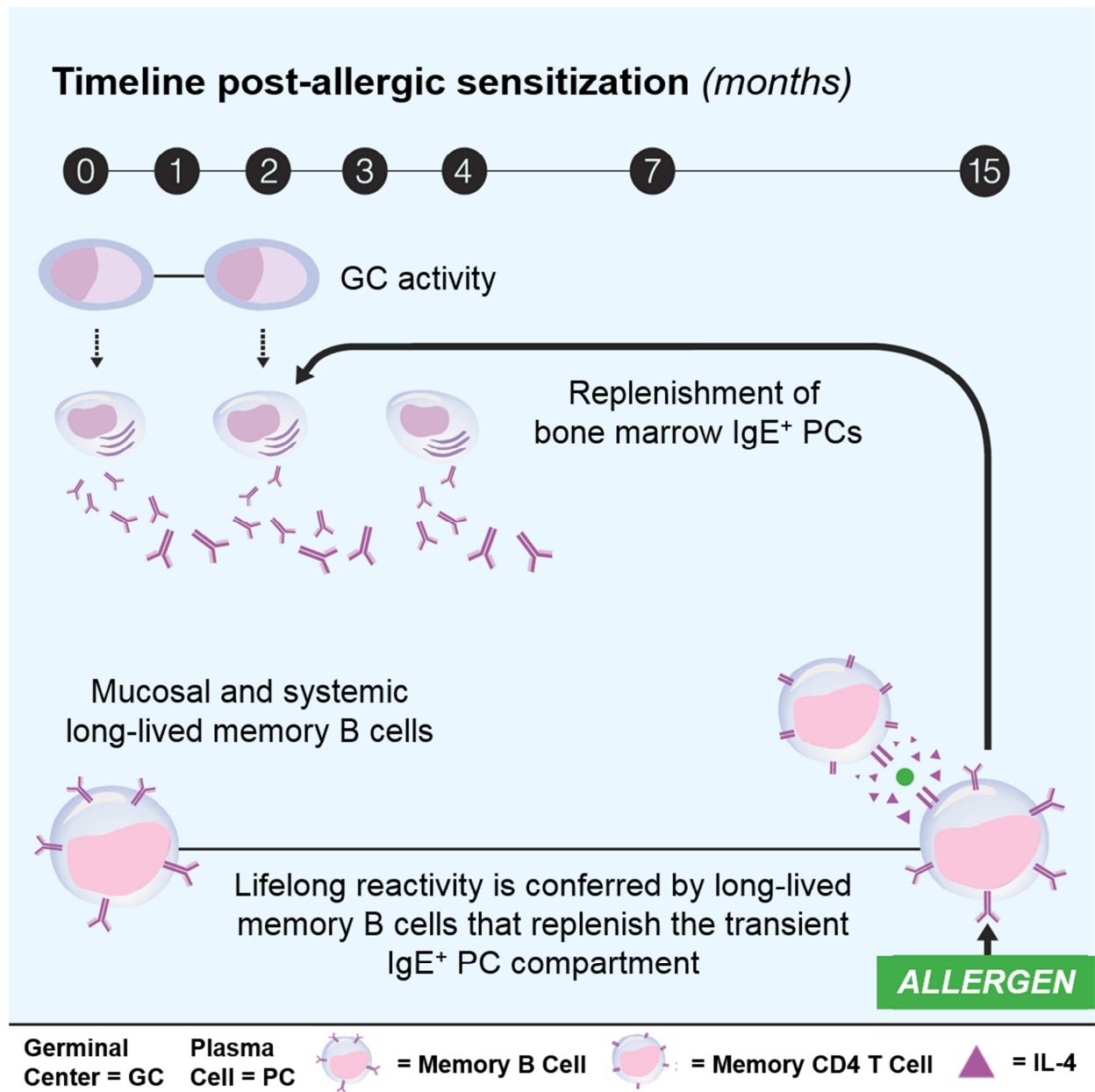
Objective: To evaluate mechanisms underlying persistent IgE and allergic responses to food allergens.

Methods: We used a model of peanut allergy and anaphylaxis, various knockout mice, adoptive transfer experiments and in vitro assays, to identify mechanisms underlying persistent IgE humoral immunity over almost the entire life-span of the mouse (18-20 months).

Results: Contrary to conventional paradigms, our data show that clinically relevant lifelong IgE titres are not sustained by long-lived IgE⁺ PCs. Instead, lifelong reactivity is conferred by allergen-specific long-lived memory B cells that replenish the IgE⁺ PC compartment. B cell re-activation requires allergen re-exposure and IL-4 production by CD4 T cells. We define the half-lives of antigen-specific: germinal centers (23.3 days); IgE⁺ and IgG1⁺ PCs (60 days and 234.4 days respectively) and clinically-relevant cell-bound IgE (67.3 days).

Conclusions: These findings can explain lifelong food allergies observed in humans as the consequence of allergen exposures that recurrently activate memory B cells and identify these as a therapeutic target with disease-transforming potential.

GRAPHICAL ABSTRACT



KEY MESSAGES

- Clinically relevant lifelong IgE titres in food allergy are not sustained by long-lived IgE⁺ PCs.
- Lifelong food allergy is conferred by allergen-specific long-lived memory B cells that replenish the IgE⁺ PC compartment upon allergen re-exposure in an IL-4 and CD4 T cell-dependent manner.
- Transformative therapies for IgE-mediated food allergies should focus on disabling the machinery that maintains and activates memory responses upon re-exposure to allergen.

CAPSULE SUMMARY: R. Jimenez-Saiz and D.K. Chu *et al.* examine lifelong IgE responses in food allergy and identify that allergen-specific long-lived memory B-cells and IL-4 producing CD4 T cells are critical to this process by re-generating reservoirs of IgE-secreting plasma cells.

KEY WORDS: IgE, Mast cells, Memory B cells, Plasma cells, Th2, Food allergy, Anaphylaxis, IgG1, Peanut allergy, Germinal centers.

ABBREVIATIONS: Ag, antigen; MC, mast cell; PC, plasma cell; Ig, immunoglobulin; Bcl-6^{B-cell} KO, B cell specific Bcl-6 knockout (Mb1-cre x BCL6^{fl/fl}); OVA, ovalbumin; Th2, T helper type 2; CYC, cyclophosphamide; BZ, bortezomib; CFSE, Carboxyfluorescein succinimidyl ester; mLN, mesenteric lymph nodes; BM, bone marrow; LP, lamina propria; GC, germinal center; WT, wild-type.

INTRODUCTION

Food allergy is typified by pathogenic Th2 responses and is of growing clinical and public concern¹⁻³. The clinical manifestations of type 1 hypersensitivity reactions to foods are mediated by immunoglobulin (Ig) E cross-linking of antigen (Ag) on mast cells (MCs) via FcεRI^{4, 5}. Clinical symptoms and signs range in severity from mild urticaria, wheezing, vomiting and diarrhea, to anaphylaxis, a systemic reaction that is rapid in onset and life-threatening⁶. An issue of grave concern that remains poorly understood is that a number of food allergies (e.g. fish, shellfish, tree nuts, peanuts)^{7, 8} are lifelong while no disease-transforming therapies exist. In this study, we investigated cellular processes underlying the persistence of IgE-mediated clinical reactivity in an established model of peanut allergy and anaphylaxis⁹⁻¹¹.

Current understanding on the maintenance of food allergy is predominantly extrapolated from immunity to viruses and vaccines¹², and proposes that after Ag-dependent T cell interactions, germinal center (GC) B cells can generate high affinity IgE-producing plasmablasts^{13, 14}. Plasmablasts exit GCs and relocate to the bone marrow (BM) where they terminally differentiate into mitotically quiescent plasma cells (PCs)¹⁵ and secrete IgE for the lifetime of the individual¹⁶⁻¹⁹. Although the establishment of PCs in the BM upon systemic and mucosal Th2 immunization has been documented²⁰⁻²², the lifespan of IgE-producing PCs remains poorly characterized, particularly as it refers to food allergens. This is in part due to the paucity of studies extending beyond 3 months post-sensitization as well as the historical methodological challenges of identifying rare populations of Ag-specific IgE⁺ PCs^{19, 23}. Importantly, the spatiotemporal distribution of memory B cells upon Th2 mucosal immunization and, particularly, their clinical relevance, remain largely unexplored²⁴.

In this study, we identified immunological mechanisms underlying lifelong IgE responses with direct relevance to food allergy. Our data show that IgE-mediated clinical reactivity to peanut, in allergic mice that remained unexposed to the allergen, is not lifelong. However, the potential to develop

anaphylaxis upon peanut re-exposure is maintained for virtually the lifetime of the mouse due to long-lasting B cell memory responses dependent on IL-4-producing CD4 T cells. Re-activated memory B cells regenerate the IgE⁺ PC compartment with ensuing IgE production. We define the half-lives of antigen-specific: GCs; IgE⁺ and IgG1⁺ PCs and clinically-relevant cell-bound IgE. These data demonstrate that long-lived, allergen-specific memory cells are critical in the maintenance of food allergy, and, consequently, identify this cell population as a key therapeutic target in IgE-mediated reactions.

METHODS

Supplemental information can be found in the Methods section in this article's Online Repository at www.jacionline.org.

Mice. Age-, sex-, vendor-, and strain-matched controls were used in all the experiments. IgE-deficient mice (Igh-7^{tm1Led})²⁵ were kindly provided by Dr. H. Oettgen (Harvard Medical School, Boston, MA) and bred in house. BCL6fl/fl mice and Mb1-cre mice were generously provided by Dr. T. Takemori (Riken Institute, Yokohama, Japan) and Dr. M. Reth (Max Planck Institute, Freiburg, Germany) respectively. C57BL/6 mice were purchased from Charles River and immunodeficient Rag2^{-/-} γc^{-/-} mice (B6-Rag2^{tm1Fwa} Il2rg^{tm1Wjl}) were obtained from Taconic. All procedures were approved by the McMaster University Research Ethics Board.

Statistics. Half-life was calculated using the following equation: (elapsed time × log2)/[log(beginning amount/ending amount)]²⁶. Data were analyzed and graphed with GraphPad Prism 6 software (GraphPad Software). Continuous data are expressed as means ± SEMs and were analyzed by using 1- or 2-way ANOVA with Bonferroni *post hoc* tests and unpaired Student's *t* test. Differences were considered statistically significant at a *P* value of less than .05.

RESULTS

IgE-mediated clinical reactivity to food allergens wanes with time despite storage of IgE on MCs

We used a well-established model of food allergy that mimics multiple features of the human disease^{4, 9-11, 27-30} to evaluate the kinetics of clinical reactivity and circulating specific IgE and IgG1 over time. Mice were sensitized to peanut, then separate groups were challenged with the allergen at different times post-sensitization for up to 15 months (approximately 75% of the lifespan of a mouse)³¹. Each group was challenged only once. Anaphylaxis was assessed by measuring core temperature over 40 minutes (**Fig. S1 A**), represented as area under 39°C for clarity (**Fig. 1 A and Fig. S1 B**), and hematocrit (**Fig. 1 B**), with hemoconcentration indicating vascular leakage. On average, allergic mice experienced $\geq 5^{\circ}\text{C}$ drop in core temperature, and $\geq 150\%$ increase in hematocrit. Clinical reactivity remained significant for up to 6 months post-sensitization. Since anaphylaxis is mainly mediated by IgE and, to a lesser extent, IgG1⁴, we measured serum levels of peanut-specific IgE and IgG1 by ELISA. Serum levels of peanut-specific IgE peaked at 2 weeks post-sensitization and gradually decreased over time, until being undetectable at 6 months post-sensitization (**Fig. 1 C**). This correlated in a strong positive manner with clinical reactivity ($R^2=0.923$) (**Fig. 1 E**). In contrast, sustained serum levels of peanut-specific IgG1 were detected for up to 15 months post-sensitization (**Fig. 1 D**) and showed a weak positive correlation with clinical reactivity ($R^2=0.159$) (**Fig. 1 E**).

Paradoxically, when peanut-specific IgE was no longer significant in serum at 6 months post-sensitization (**Fig. 1 C**), the mice still underwent severe anaphylaxis upon challenge (**Fig. 1 A and B**). We hypothesized that this could be due to allergen cross-linking of cell-bound IgE. To test this, we generated a system to track the spatial distribution of IgE by serum transfer from wild-type (WT)

allergic mice to IgE-deficient mice (**Fig. 2 A**). As shown in **Fig. 2 B**, serum levels of peanut-specific IgE were elevated at 6 h post-transfer, declined by 24 and 48 h, and were no longer detected after 168 h (7 days). To determine whether the decrease in circulating peanut-specific IgE was due to the uptake of IgE by MCs, we measured MC bound-IgE in recipient mice by flow cytometry (**Fig. 2 C**). As early as 6 h post-transfer MCs in IgE-deficient animals were loaded with IgE, reaching almost 100% saturation by 24 h. Over 70% of MCs were still coated with IgE at 7 days post-transfer (**Fig. 2 D**). To determine the duration of clinical reactivity due to MC-bound IgE, separate groups of IgE-deficient mice were passively immunized to peanut and challenged once at different times post-transfer for up to 120 days (**Fig. 2 E and F**). Clinical reactivity peaked 2 days post-transfer and was comparable to that observed in actively immunized peanut-allergic mice (**Fig 1 A and B**). From days 2 to 15 clinical reactivity decreased and reached a plateau that was maintained for up to 75 days; anaphylaxis was no longer evident after 100 days post-transfer. These data provided a calculated half-life for MC-bound IgE-mediated reactivity of 67.3 days (95% CI, 46.5-88.1) and demonstrate that undetectable serum allergen-specific IgE does not preclude clinical reactivity upon allergen exposure.

Persistent IgE production requires GC formation by Bcl-6 competent B cells

We sought to identify whether the transient IgE titres were due to follicular or extrafollicular generation of antibody producing cells. Affinity maturation resulting in the sustained production of high affinity antibodies occurs in GCs and the binding affinity of IgE to allergen contributes to the severity of anaphylaxis¹³. To examine Ag-specific GC reactions during allergen sensitization we used ovalbumin (OVA), a model Ag that can be easily tracked. We first verified that Th2 humoral responses and clinical reactivity to OVA for up to 6 months post-sensitization were comparable to those observed with peanut (**Fig. S1 C-E**). To quantify the duration of GC activity, we identified Ag-specific GC B cells by flow cytometry based on the co-expression of CD95 and GL7, and binding to OVA (**Fig. 3 A**). OVA-specific GC B cells were present at 6 days post-sensitization both in the

spleen (**Fig. 3 B**) and the mesenteric lymph nodes (mLN) (**Fig. 3 C**), becoming undetectable by 34 days in the spleen (**Fig. 3 B**) and 60 days in the mLN (**Fig. 3 C**). The calculated half-life of GC reactions in this system is 23.3 days (95% CI, 12.6-34.0).

The development of GCs relies on the transcriptional repressor Bcl-6^{32, 33}. To evaluate the role of GCs in allergen-specific antibody production and anaphylaxis, we investigated mice with a deficiency in Bcl-6 restricted to the B cell lineage (Mb1-cre x BCL6^{fl/fl}; hereafter denoted as Bcl-6^{B-cell} KO). Peanut-allergic Bcl-6^{B-cell} KO mice reacted clinically to allergen challenge in a manner comparable to WT mice (**Fig. 3 D and E**). Serum levels of peanut-specific IgE (**Fig. 3 F**) and IgG1 (**Fig. 3 G**) decreased significantly faster over time in Bcl-6^{B-cell} KO mice compared to WT mice. These data suggest that extrafollicular antibody production can facilitate hypersensitivity reactions early after sensitization, but that sustained humoral IgE/IgG1 responses are dependent on GC-derived PCs.

Poor survival of cyclophosphamide-resistant Ag-specific IgE⁺ PCs generated upon sensitization to food allergens

Systemic immunization with Th2 adjuvants can lead to the production of IgE⁺ long-lived PCs that home to the BM, which have been examined up to 80 days post-sensitization²². To directly identify Ag-specific IgE⁺ PCs elicited by intestinal allergic sensitization, we took advantage of a recently validated flow cytometric method¹⁴. Our gating strategy and in-house re-validation are shown in **Fig. 4A** and **Fig. S2A**. We evaluated Ag-specific IgE⁺ or IgG1⁺ PCs in the mLN, lamina propria (LP) of the small intestine, spleen and BM for up to 9 months post-immunization. At 1 month post-sensitization, we detected significant percentages of Ag-specific IgG1⁺ PCs in the mLN (**Fig. S2 B**) and Ag-specific IgE⁺ or IgG1⁺ PCs in the BM (**Fig. 4 B**). Neither population was detected in the spleen or LP of the small intestine (data not shown). In the mLN, Ag-specific IgG1⁺ PCs decreased over time until becoming undetectable by 3 months post-sensitization (**Fig. S2 B**). In the BM, Ag-

specific IgE⁺ PCs were detected for up to 3 months post-sensitization (**Fig. 4 B**), in agreement with the kinetics of peanut-specific IgE in serum (**Fig. 1 C**); the calculated half-life for Ag-specific IgE⁺ BM PCs is 60 days (95% CI, 27-93). In contrast, Ag-specific IgG1⁺ BM PCs were detected for at least 9 months post-sensitization (**Fig. 4 C**), also consistent with the Ag-specific IgG1 titer in serum (**Fig. 1 D**); the calculated half-life of Ag-specific IgG1⁺ BM PCs is 234.4 days (95% CI, 45.7-426.1).

Long-lived PCs are mitotically quiescent cells, classically defined by their resistance to cytotoxic agents such as cyclophosphamide (CYC), a chemotherapeutic drug that kills proliferating cells³⁴. Bortezomib (BZ) is a 20S proteasome inhibitor that eliminates cells that produce high amounts of proteins, notably PCs³⁵. Therefore, to determine if the BM-resident PC population generated upon mucosal Th2 immunization was proliferating (short-lived) or non-dividing (long-lived)¹⁵, mice were treated with either CYC or with BZ once GC activity had ended (2.5 months post-sensitization). In agreement with previous studies, BZ treatment depleted PCs in the BM (**Fig S3 A and B**) and the spleen (**Fig. S3 C and D**), whereas CYC treatment dramatically reduced both the B and T cell compartments (**Fig S3 E-G**). Mice were sensitized to peanut and then treated with either CYC or BZ when GC activity had ended (2.5 months post-sensitization). After these treatments, mice were left un-manipulated for 100 days, allowing for clinical reactivity due to cell-bound IgE to wane, and then challenged with allergen (**Fig. 4 D**). Compared to vehicle-treated mice, serum levels of peanut-specific IgE (**Fig. 4 E**) and IgG1 (**Fig. 4 F**) after CYC-treatment (1-2 weeks post-treatment) were not affected and CYC-treated mice exhibited full anaphylaxis upon challenge (**Fig. 4 G and H**). In contrast, BZ-treatment exhibited greatly reduced serum levels of peanut-specific IgE (**Fig. 4 I**) and IgG1 (**Fig. 4 J**). Accordingly, BZ-treated mice did not develop anaphylaxis upon allergen challenge (**Fig. 4 K and L**). Treatment with BZ immediately after sensitization was ineffective due to ongoing GC activity (**Fig. S3 H-J**), and challenge immediately after BZ-treatment did not impair clinical reactivity due to residual cell-bound IgE (**Fig. S3 K-M and Fig 2 E and F**). These data demonstrate

the existence of cytotoxic-resistant, Ag-specific IgE⁺ long-lived PCs in the BM, which have a much shorter half-life compared to IgG1⁺ PCs of the same Ag specificity.

Allergen re-exposure activates long-lived memory B cells to reinvigorate IgE-humoral immunity

We questioned if the abrogation of clinical reactivity upon PC depletion was due a transient or permanent elimination of IgE-humoral immunity. To test this, peanut-allergic mice were treated with BZ at 2.5 months post-sensitization to deplete PCs, rested for 100 days to allow for reactivity due to mast cell-bound IgE to wane, and then challenged. Some mice received allergen orally, which in the absence of adjuvant will not drive a primary immune response (**Fig. 5 A**). Re-exposure to allergen at this time did not induce any anaphylactic reactions (data not shown). Serum levels of peanut-specific IgE (**Fig. 5 B**) and IgG1 (**Fig. 5 C**) were assessed two weeks after the allergen re-exposures. Peanut-specific IgE/IgG1 were undetectable in BZ-treated mice that had not been re-exposed to allergen. In contrast, serum levels of peanut-specific IgE/IgG1 were elevated in BZ-treated mice that had been re-exposed to peanut either systemically or mucosally. Notably, when these mice were later challenged with peanut, the mice now underwent severe anaphylaxis (**Fig. 5 D and E**). Thus, clinically silent allergen re-exposure of previously allergic mice fully regenerates disease expression.

The rapid appearance of circulating peanut-specific IgE/IgG1 upon allergen re-exposure in BZ-treated mice suggested that a memory response was triggered. To assess this, we evaluated Ag-specific IgE⁺ and IgG1⁺ memory B cells (**Fig. 5 F**) in the LP of the small intestine, mLN and spleen at 9 months post-sensitization (**Fig. 5 G-I**). While Ag-specific IgE⁺ memory B cells were undetectable (data not shown), Ag-specific IgG1⁺ memory B cells were abundant in the LP of the small intestine (**Fig. 5 G**), and in lower numbers in the mLN (**Fig. 5 H**) and the spleen (**Fig. 5 I**).

To test the impact of allergen re-exposure on the memory B cell compartment, allergic mice were given Ag orally as it leads to tolerance (in the absence of adjuvant)³⁶ and, therefore, does not

stimulate *de novo* sensitization. Oral allergen exposure induced Ag-specific IgG1⁺ memory B cell expansion in the LP of the small intestine (**Fig. 5 G**), mLN (**Fig. 5 H**) and spleen (**Fig. 5 I**); in addition, a number of Ag-specific, IgE-expressing B cells became apparent (**Fig. 5 J-L**). Likewise, Ag-specific IgE⁺ PCs, which were not present in the BM at 9 months post-sensitization, re-populated the BM following allergen exposure (**Fig. 5 M**). In contrast, Ag-specific IgG1⁺ PCs were still present in the BM at 9 months and remained unchanged after allergen exposure (**Fig. 5 N**). The re-population of the BM with Ag-specific IgE⁺ PCs resulted in the emergence of high levels of Ag-specific IgE in the systemic circulation (**Fig. 5 O**) while Ag-specific IgG1 remained at a stable and elevated level (**Fig. 5 P**). These data show that Th2 mucosal immunization results in the establishment of both systemic and local long-lived allergen-specific memory B cells.

IgE production upon recall relies on memory B cell responses and IL-4-producing CD4 T cells

To further investigate the persistence of the memory response to allergen re-exposure, we extended our observation period until the mice were ≥ 18 months of age (over 75% of the lifespan of a mouse). Peanut sensitized mice were re-exposed to peanut allergen either systemically or mucosally at 15 months post-sensitization. Peanut-specific IgE was undetected at 15 months post-sensitization but increased rapidly two weeks following oral allergen re-exposure (**Fig. 6 A**); while serum levels of peanut-specific IgG1 remained elevated for the lifespan of the mice (**Fig. 6 B**). Soy exposure at regular intervals in these experiments did not appear to cause cross-reactivity or perpetuate allergy. Sensitized mice not re-exposed to peanut for over 15 months did not clinically react to challenge (**Fig. 6 C and D**). In contrast, mice that had been re-exposed to peanut at 15 months post-sensitization exhibited a full anaphylactic reaction when challenged (**Fig. 6 C and D**).

To gain further insights into memory lymphocyte responses at this time, splenocytes from peanut-allergic mice were CFSE-labeled and cultured with peanut to evaluate proliferation of memory B and CD4 T cells, and plasmablasts by flow cytometry (**Fig. 6 E-G**). We observed significant increases in

proliferation of memory B and CD4 T cells (**Fig. 6 E and F**), as well as plasmablasts (**Fig. 6 G**), in response to peanut exposure at 15 months post-sensitization. In addition, stimulation of these cells caused the production of hallmark Th2 cytokines, IL-4 and IL-5 (**Fig. 6 H and I**). Therefore, allergen-specific memory B and CD4 T cells established during mucosal immunization can last for a lifetime.

Whether or not memory humoral responses require T cell help is contentious^{37, 38}, including the requirement for IL-4 in secondary IgE responses^{39, 40}. To evaluate the contribution of memory CD4 T cells to secondary B cell responses to food allergens, we developed an *in vivo* model to adoptively transfer immune memory. WT allergic mice were treated with depleting anti-CD4 antibody or vehicle between 1 and 3 months post-sensitization; splenocytes from either of these donors were then transferred into immunodeficient Rag2^{-/-} γ c^{-/-} recipients (**Fig. 7A**), which lack T, B and NK cells. The extent of CD4 T cell depletion achieved with anti-CD4 reached over 99% in both the spleen (**Fig. S4 A**) and LP of the small intestine (**Fig. S4 B**) of donor mice. Treatment with anti-CD4 was continued in the recipient mice after adoptive transfer, which were orally exposed to Ag 3 times a week for 3 weeks followed by allergen challenge (**Fig. 7A**). As expected, following allergen exposure, serum levels of Ag-specific IgE and IgG1 (**Fig. 7 B and C**) were increased in mice that received splenocytes from allergic mice. Importantly, mice that received sensitized splenocytes depleted of CD4 T cells did not generate Ag-specific IgE and IgG1 following allergen exposure (**Fig. 7 B and C**). Consistent with these observations, anaphylaxis was observed in mice that received splenocytes from sensitized mice but not in those depleted of CD4 T cells (**Fig. 7 D and E**). These data show that CD4 T cell-help is required to replenish IgE/IgG1 humoral immunity during re-call responses following allergen exposure.

We next investigated mechanisms of CD4 T cell help during secondary B cell responses to food allergens. To address this, peanut-allergic mice were treated with depleting anti-CD4 antibody or vehicle *in vivo* before their splenocytes were labeled with CFSE and stimulated *ex vivo* with peanut

in the presence of blocking antibodies to IL-4, CD40L and/or an isotype control. CD4 T cells facilitated proliferation of memory B cells upon allergen stimulation in an IL-4- and CD40L-independent manner (**Fig. 7 D, Fig. S4 C-D**). However, the lack of CD4 T cells, IL-4, or both remarkably reduced the frequency of proliferating plasmablasts (**Fig 7 G, Fig. S4 E-F**). These data indicate that CD4 Th2 cells provide critical help for the differentiation of proliferating memory B cells into plasmablasts via IL-4 secretion and independently of CD40L interactions.

DISCUSSION

Deciphering the generation and maintenance of food allergy in humans is challenging given that the critical events underlying these processes are clinically silent and occur in inaccessible tissues. Furthermore, inadvertent, subclinical allergen exposure cannot be controlled. In this study, we used an established model of peanut allergy and anaphylaxis to delineate the determinants of persistent IgE responses.

We found that clinical reactivity to food allergens is not lifelong as serum levels of allergen-specific IgE, the key effector molecule in anaphylaxis, disappeared over time. However, anaphylactic reactions persisted in the absence of detectable IgE. Kubo and colleagues reported severe anaphylaxis in B cell deficient mice challenged with TNP-BSA 56 days post-transfer of TNP-IgE, but did not precisely define the duration of reactivity due to cell-bound IgE⁴¹. Using a serum transfer approach, we determined that MCs remained loaded with clinically-relevant levels of IgE even in the absence of detectable serum IgE. Anaphylactic reactions to allergen, in the absence of circulating IgE, lasted 100 days (half-life 67.3 days). These findings explain why a proportion of patients with clinical allergy paradoxically have undetectable levels of serum allergen-specific IgE

We showed that Th2 mucosal sensitization entailed GC activity which, in turn, has been implicated in the generation of high affinity antibodies⁴². Serum levels of peanut-specific IgE peaked earlier

than IgG1, likely due to the predisposition of IgE⁺ B cells to commit to a PC fate¹⁴. The genetic deletion of GC B cells did not affect early humoral responses or clinical reactivity. However, serum titers of specific IgE/IgG1 decreased rapidly over time as a result of this GC-independent humoral response, which is consistent with the short-lived nature of extrafollicular PCs^{43, 44}. These data suggest that the extrafollicular pathway can produce IgE of sufficient affinity to cause anaphylaxis plausibly via random generation of B cell clones that, without further B cell receptor editing, have high enough affinity towards food allergens to elicit anaphylaxis. In addition, since peanut is a complex matrix comprising multivalent allergens⁴⁵ it is possible that IgE avidity contributes to the ability of the lower affinity of antibodies to cause pathology.

Th2 mucosal sensitization produced Ag-specific IgE⁺ PCs that homed to the BM. While the majority of IgE⁺ PCs are thought to be short-lived¹⁴, we demonstrated that a population of cytotoxic-resistant and, consequently, long-lived Ag-specific IgE⁺ PCs were generated by allergic sensitization. This population is thought to be responsible for persistent IgE responses¹⁶⁻¹⁸. Using a comprehensive approach that integrated direct identification of Ag-specific IgE⁺ PCs, measurement of Ag-specific IgE in serum and clinical readouts, we determined that the half-life of these cells was substantially shorter (60 days) than previously proposed^{16, 18}. This is in contrast to Ag-specific IgG1⁺ PCs whose half-life we calculated to be 234.4 days, or approximately 4-fold longer than IgE⁺ PCs of the same specificity. Given the brief and more apoptotic GC phase of IgE⁺ B cells^{14, 46}, as well as the relatively inefficient migration of IgE⁺ plasmablasts to the BM⁴⁷, the initial number of Ag-specific IgE⁺ PC was expectedly lower compared to Ag-specific IgG1⁺ PCs. However, their rate of decay was nearly 3 times higher (slope value: -17.2 for IgE⁺ vs -5.2 for IgG1⁺). These data suggest that, besides differences in competition for survival signals⁴⁴, isotype-related intrinsic mechanisms and/or a GC-imprinted fate regulate the lifespan of IgE⁺ PCs. Thus, the limited half-life of BM-resident Ag-specific IgE⁺ PCs elicited upon Th2 mucosal sensitization cannot fully account for persistent food allergy.

We showed that mice became clinically non-reactive to allergen challenge after either natural or drug-induced extinction of allergen-specific IgE⁺ PCs. However, mice underwent severe anaphylaxis after subsequent allergen exposures. This argues for the lifelong persistence of allergen-specific memory B cells that can be activated by allergen, or potentially also by inflammatory environmental stimulus⁴⁸, to reconstitute the IgE⁺ PC compartment. These findings provide an explanation for why certain food allergies can last for a lifetime. They also raise the possibility that unintended or intended, such might be the case of oral immunotherapy, subclinical exposures to allergen may be sufficient to fully regenerate and/or perpetuate disease. In this regard, allergen-specific memory lymphocytes have been detected in peanut-allergic patients after 12-24 months of oral immunotherapy⁴⁹.

The dynamics that control the fate of memory B cells during secondary responses to foods remain largely unexplored²⁴. We showed that Th2 mucosal sensitization generated local and systemic, long-lasting Ag-specific memory B cells that expanded upon mucosal or systemic allergen exposure. The expansion of IgG1⁺ memory B cells was accompanied by BM reconstitution with Ag-specific IgE⁺ PCs while the population of IgG1⁺ PCs of the same specificity remained unchanged; this implies that the recall response was driven by IgG1⁺ memory B cells that class-switched and differentiated into IgE⁺ PCs. Although sequential class switching from an intermediate IgG-expressing B cell to IgE has been reported in patients with atopic dermatitis⁵⁰ and *ex vivo* tonsil B cell cultures⁵¹, the relevance of both direct and sequential class switching to IgE in food allergic patients remains to be elucidated^{52, 53}.

Even though GC activity was induced during the primary immune response (sensitization), it was absent during recall responses. These data reinforce the notion that class-switched memory B cells, rather than IgM⁺ B cells, lead the secondary response^{54, 55}, and suggest that the strength of B cell receptor-allergen binding is a major fate decision checkpoint for memory B cells⁵⁶. Our data on the activation of recall responses are in agreement with a recent publication that showed that in a system

involving transfer of splenic B cells from systemically sensitized mice along with CD4 T cells, followed by two systemic challenges, food allergy was elicited in naïve recipient in a CD4 T cell-dependent manner¹⁷. Mechanistically, we found that CD4 T cells facilitated B cell proliferation independently of CD40L interactions, which is in agreement with the low degree of co-stimulation required by memory CD4 T cells⁵⁷ and the absence of GC activity observed during re-call responses to *N. brasiliensis*⁵⁸. Furthermore, we show that CD4 T cell-secreted IL-4 was central for PC proliferation and orchestrated class switching of IgG1⁺ memory B cells. These data explain observations in models of helminth infection in which serum increases of total IgE upon recall were dependent on IL-4⁵⁹. Hence, we propose a model of secondary Th2 responses to food allergens in which class-switched memory B cells are instructed by IL-4-producing CD4 T cells to proliferate and directly become IgE⁺ PCs without additional rounds of affinity maturation.

There is a dearth of knowledge regarding the essential events for the maintenance of Th2 humoral immunity to allergens. While targeting IgE⁺ PCs has been proposed as a potential cure of food allergy^{18,60}, our data show that IgE-mediated clinical reactivity to food allergens is not sustained by BM resident Ag-specific IgE⁺ PCs. Instead, long-lived mucosal and systemic allergen-specific memory B cells, critically assisted by IL-4-producing CD4 T cells, replenish the allergen-specific IgE⁺ PC compartment upon allergen exposure. These findings intimate that lifelong food allergies observed in humans may be the consequence of allergen exposures that recurrently activate memory B and CD4 T cells and provide a comprehensive and invaluable roadmap for the precise design of therapeutic interventions. Our findings indicate that efforts to disable the machinery that maintains and activates memory responses may lead to the development of transformative therapies for IgE-mediated food allergies.

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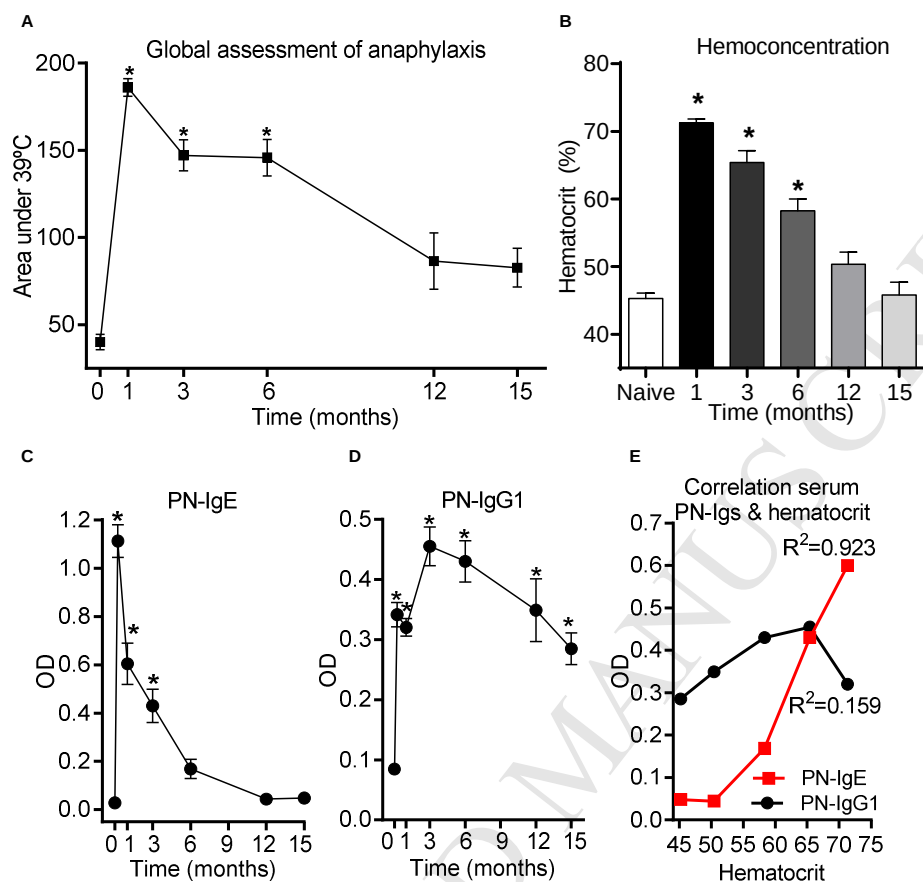


Figure 1. Duration of IgE-mediated clinical reactivity to food allergens. Separate groups of mice were orally sensitized to peanut (PN) and left un-manipulated until challenge 1, 3, 6, 12 or 15 months later. Anaphylaxis was assessed by hypothermia (A) and hemoconcentration (B). Serum PN-IgE (C) and IgG1 (D) before challenge. Correlation of serum PN-Igs and clinical reactivity (hematocrit) (E). Pooled data from 2-3 independent experiments and represented as mean $\pm$ SEM, $n=10-20$. * $P<0.05$ vs naïve.

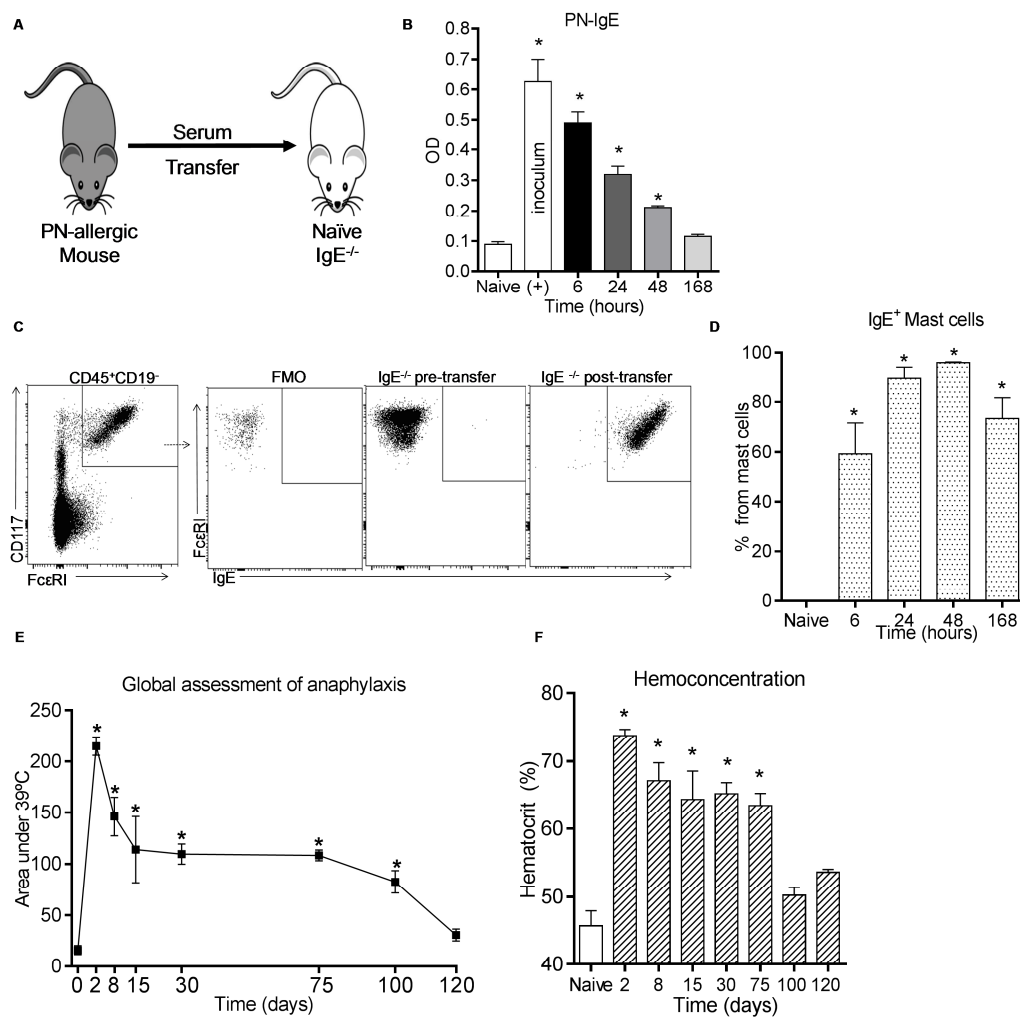


Figure 2. Duration of reactivity due to cell-bound IgE. Separate groups of IgE deficient mice were passively sensitized to peanut (PN) (A). Serum PN-IgE after transfer (B). Recipient peritoneal IgE⁺ MCs (C, D). Recipient mice were challenged to PN at different times post-transfer and clinical reactivity evaluated by hypothermia (E) and hemoconcentration (F). Pooled data from 2-3 independent experiments and represented as mean ± SEM, n= 3-5 per experiment. *P<0.05 vs naïve.

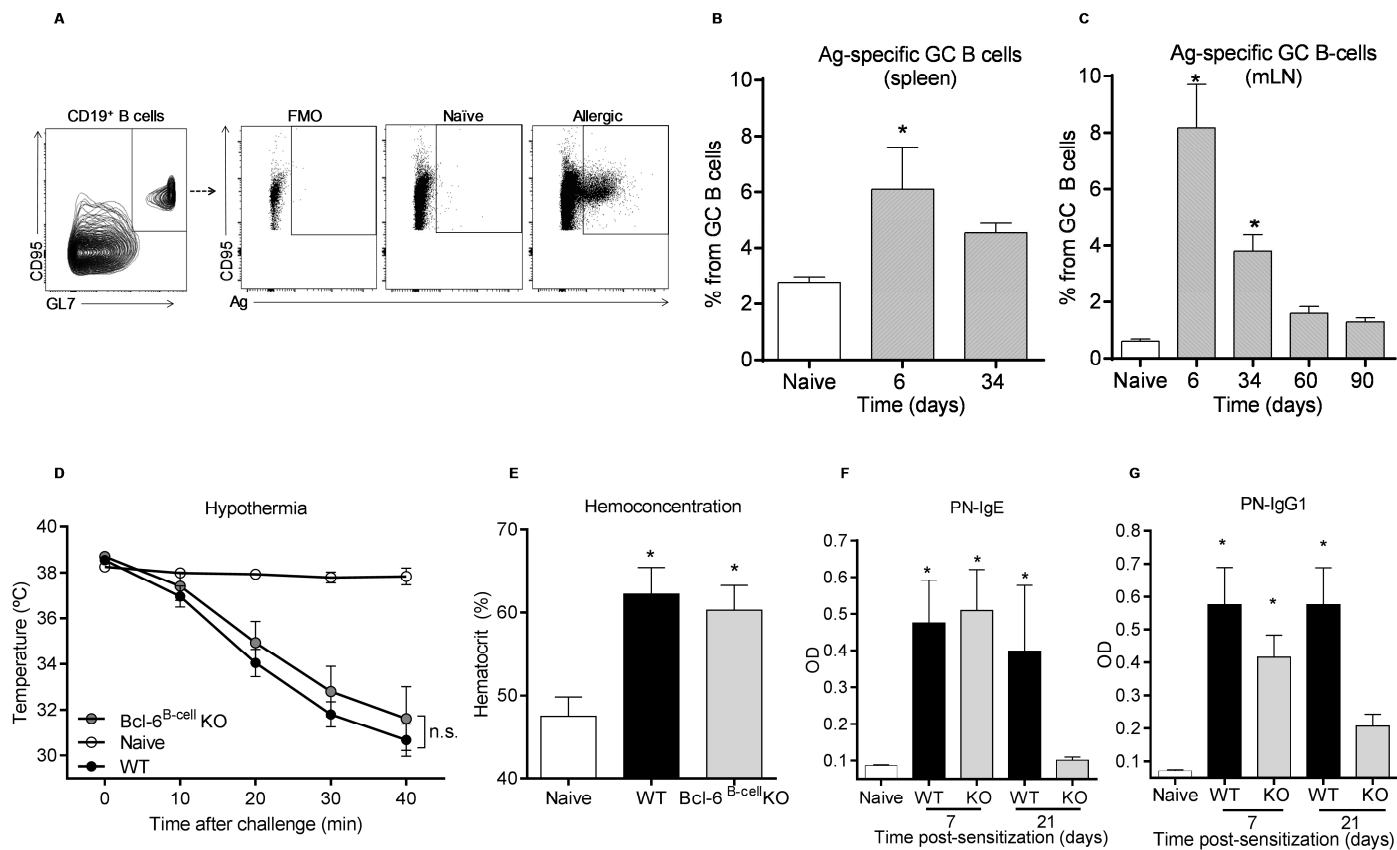


Figure 3. Persistent IgE production requires GC formation by Bcl-6 competent B cells. Ag-specific GC B cells (A). GC activity in spleen (B) and mLN (C) at different times post-sensitization. Bcl-6^{B-cell KO} and WT mice were sensitized to peanut (PN) and anaphylaxis evaluated by hypothermia (D) and hemoconcentration (E). Serum PN-IgE (F) and IgG1 (G) at different times post-sensitization. Representative data from 2-3 independent experiments and represented as mean $\pm$ SEM, n=4-6 per experiment. *P<0.05 vs naïve; n.s., nonsignificant.

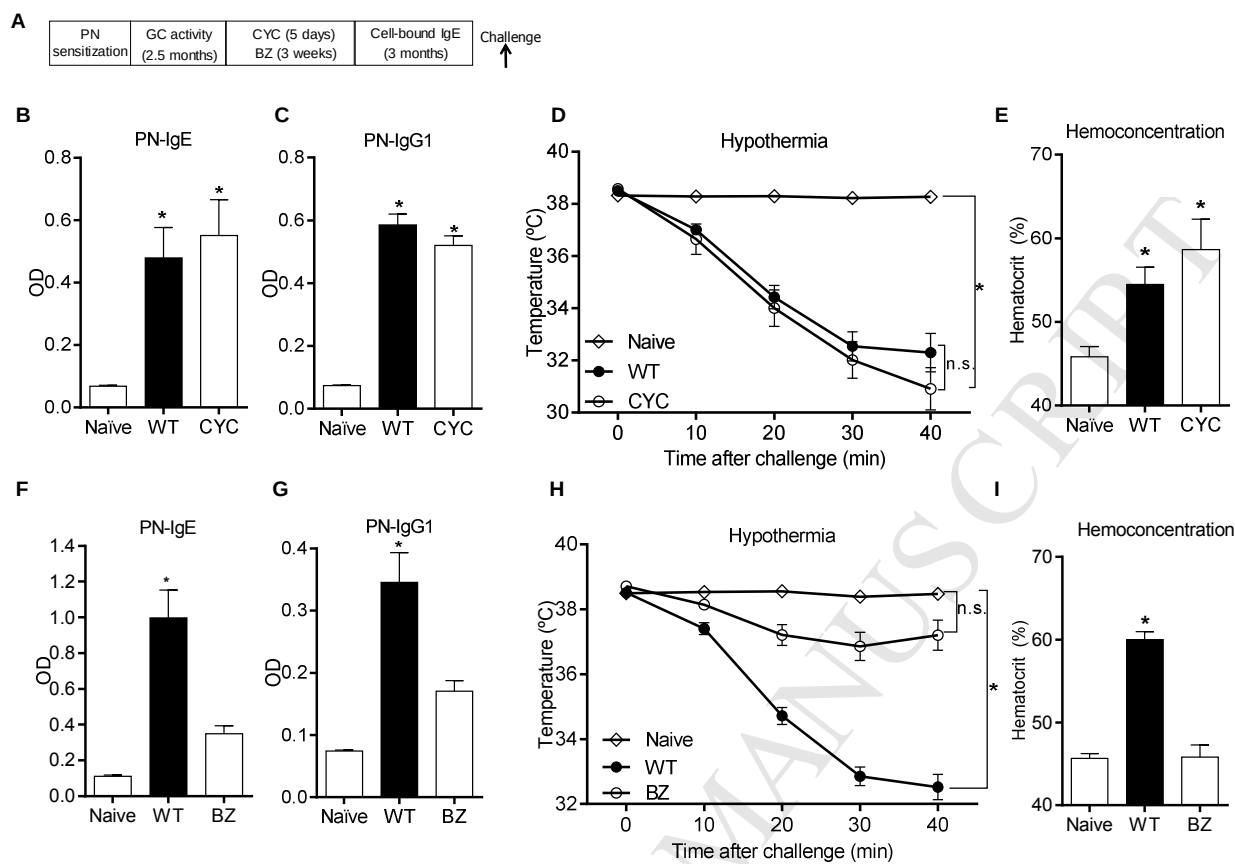


Figure 4. Poor survival of CYC-resistant Ag-specific IgE⁺ PCs generated upon sensitization to food allergens. Ag-specific IgE⁺/IgG1⁺ PCs were identified in the BM by flow cytometry (A) and assessed at different times post-sensitization (B, C). Separate groups of mice were sensitized to peanut (PN), treated with CYC, BZ or a vehicle control (VEH), and later challenged (D). PN-IgE/IgG1 with and without CYC (E, F) or BZ (I, J). Anaphylaxis-induced hypothermia (G, K) and hemoconcentration (H, L) after challenge. Mean ± SEM, n=6-16 pooled from 5-8 (A-C) and 2-3 (E-L) experiments. *P<0.05 vs naïve. n.s., nonsignificant.

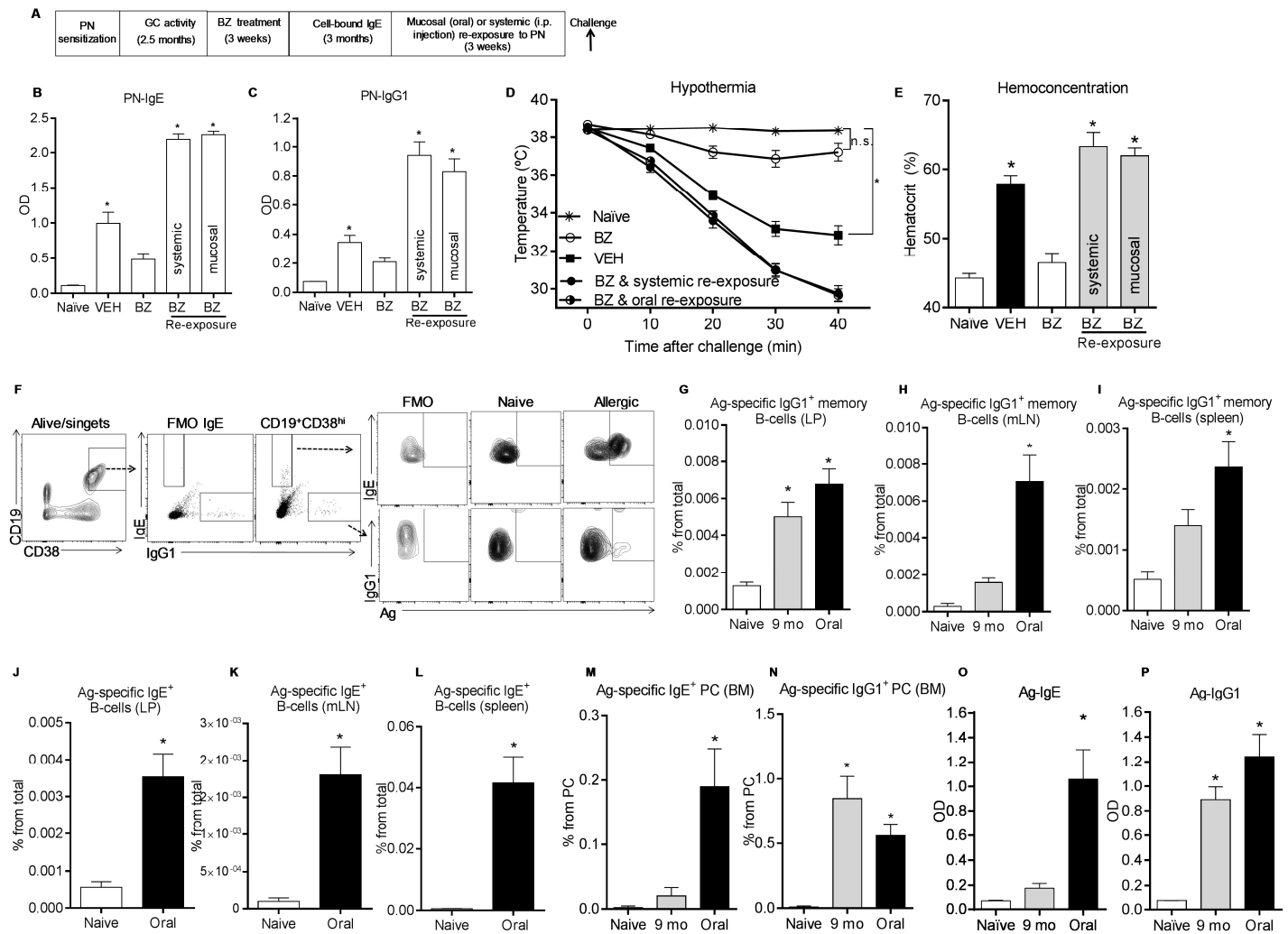


Figure 5. Allergen re-exposure reinvigorates IgE-humoral immunity. Peanut (PN)-allergic mice treated with BZ were exposed to PN, either mucosally or systemically, and challenged 2 weeks later (A). Serum PN-IgE (B) and IgG1 (C). Anaphylaxis-induced hypothermia (D) and hemoconcentration (E) after challenge. Mice were sensitized and 8 months later, either left unmanipulated or re-exposed to oral allergen before analysis at 9 months. Ag-specific IgE⁺/IgG1⁺ memory B cells in LP of the small intestine, mLN and spleen (G-L). Ag-specific PCs in BM (M, N) and serum Ag-IgE (O) and IgG1 (P). Mean $\pm$ SEM, n=9-15 pooled from 2-3 experiments. *P<0.05 vs naïve; n.s., nonsignificant.

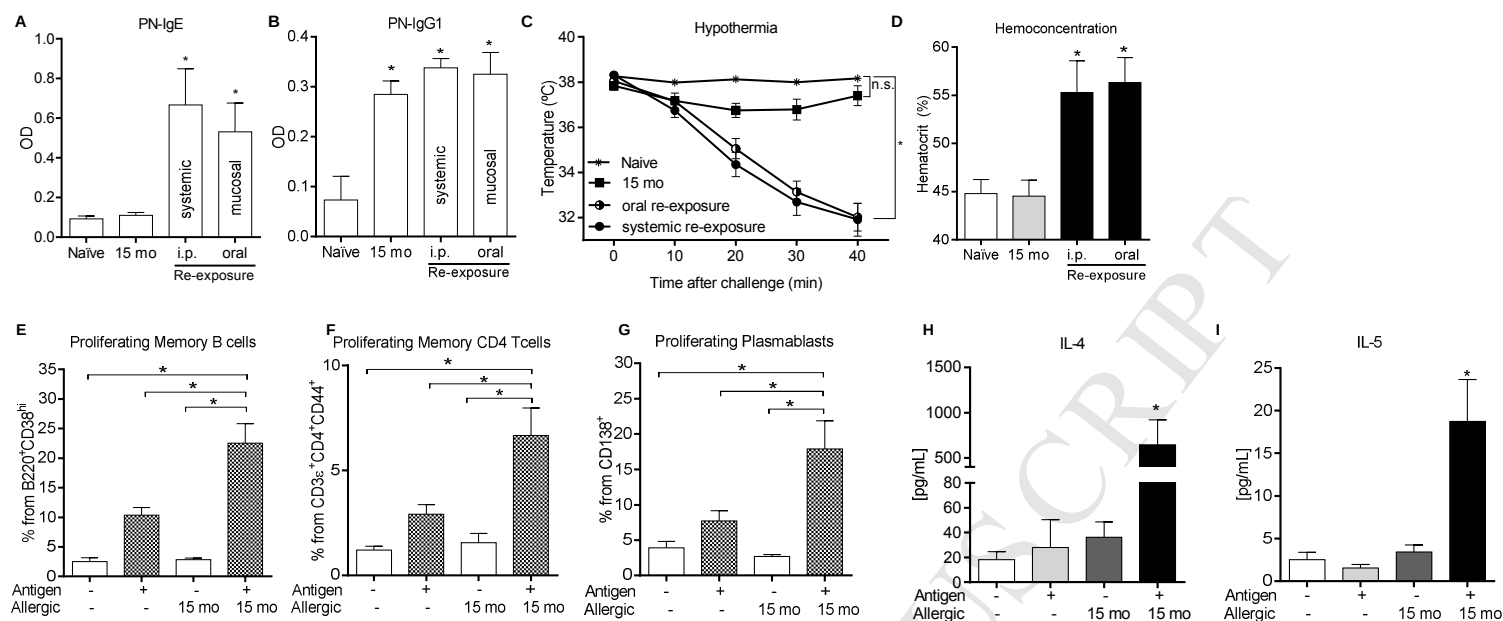


Figure 6. Memory responses to peanut at 15 months post-sensitization. Mice were sensitized to peanut (PN), rested for 15 months and then re-exposed to PN, either mucosally or systemically, before challenge to PN 2 weeks later. Serum PN-IgE (A) and IgG1 (B) before challenge. Anaphylaxis-induced hypothermia (C) and hemoconcentration (D). Proliferation of memory B (E) and CD4 T cells (F), and plasmablasts (G) from spleens at 15 months. Th2 cytokines from culture supernatants (H, I). Mean $\pm$ SEM, $n=8-15$ pooled from 2-3 experiments. * $P<0.05$ vs naïve; n.s., nonsignificant.

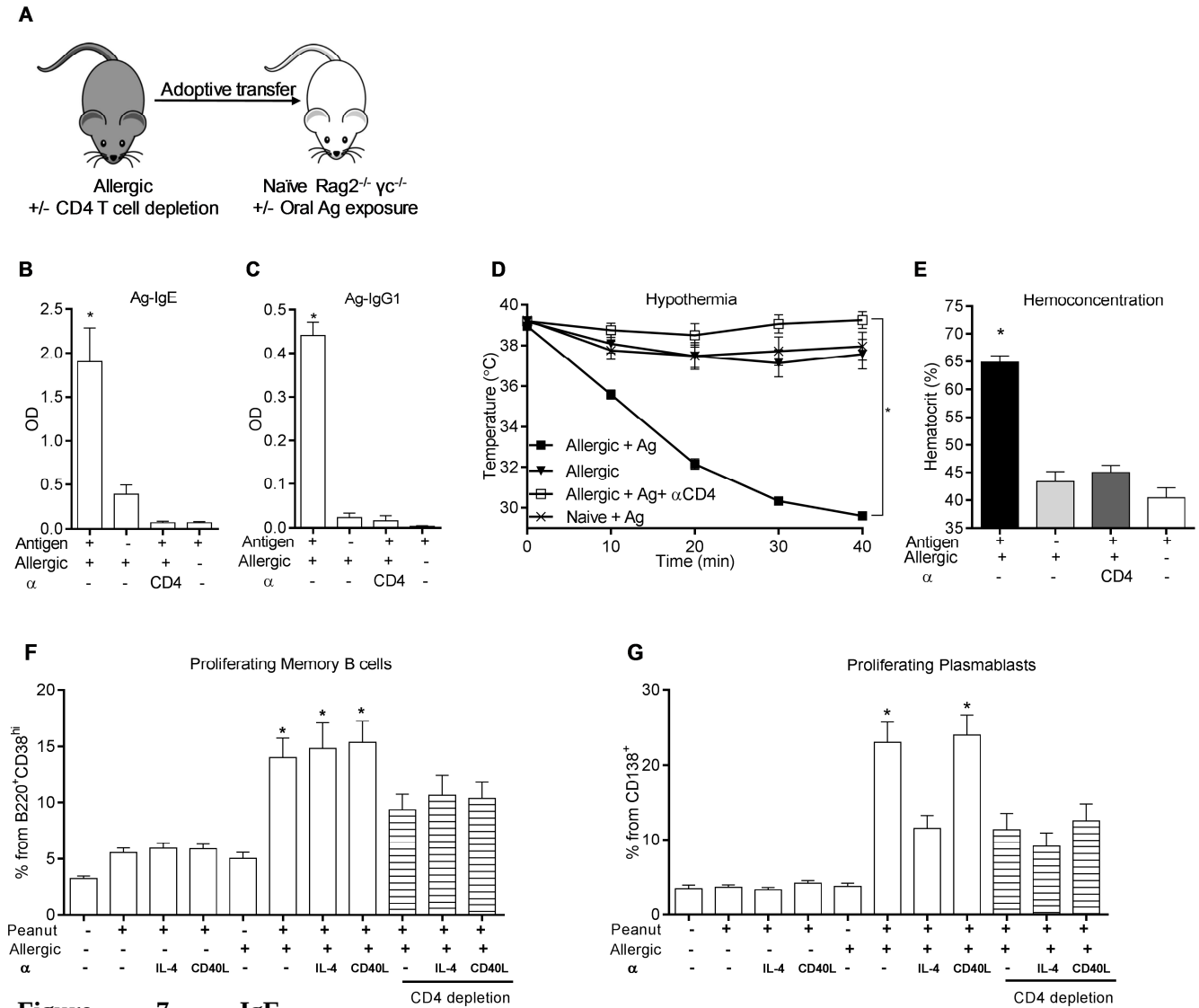


Figure 7. IgE

production upon recall relies on memory B cell responses and IL-4-producing CD4 T cells. Spleen cells from allergic mice were adoptively transferred into immunodeficient mice and then recipients were exposed to oral allergen for 3 weeks (A). Serum Ag-IgE (B) and IgG1 (C). Anaphylaxis-induced hypothermia (D) and hemoconcentration (E). Proliferation of memory B cells (F) and plasmablasts (G) in the presence or absence of CD4 depletion, IL-4 neutralization, and/or CD40L blockade from spleens. Mean $\pm$ SEM, n=8-15 pooled from 2-3 experiments for A-D and n=15-20 pooled from 4 experiments for E and F. *P<0.05 vs naïve for A-E; *p<0.001 vs naïve for F and G.

FIGURE S1

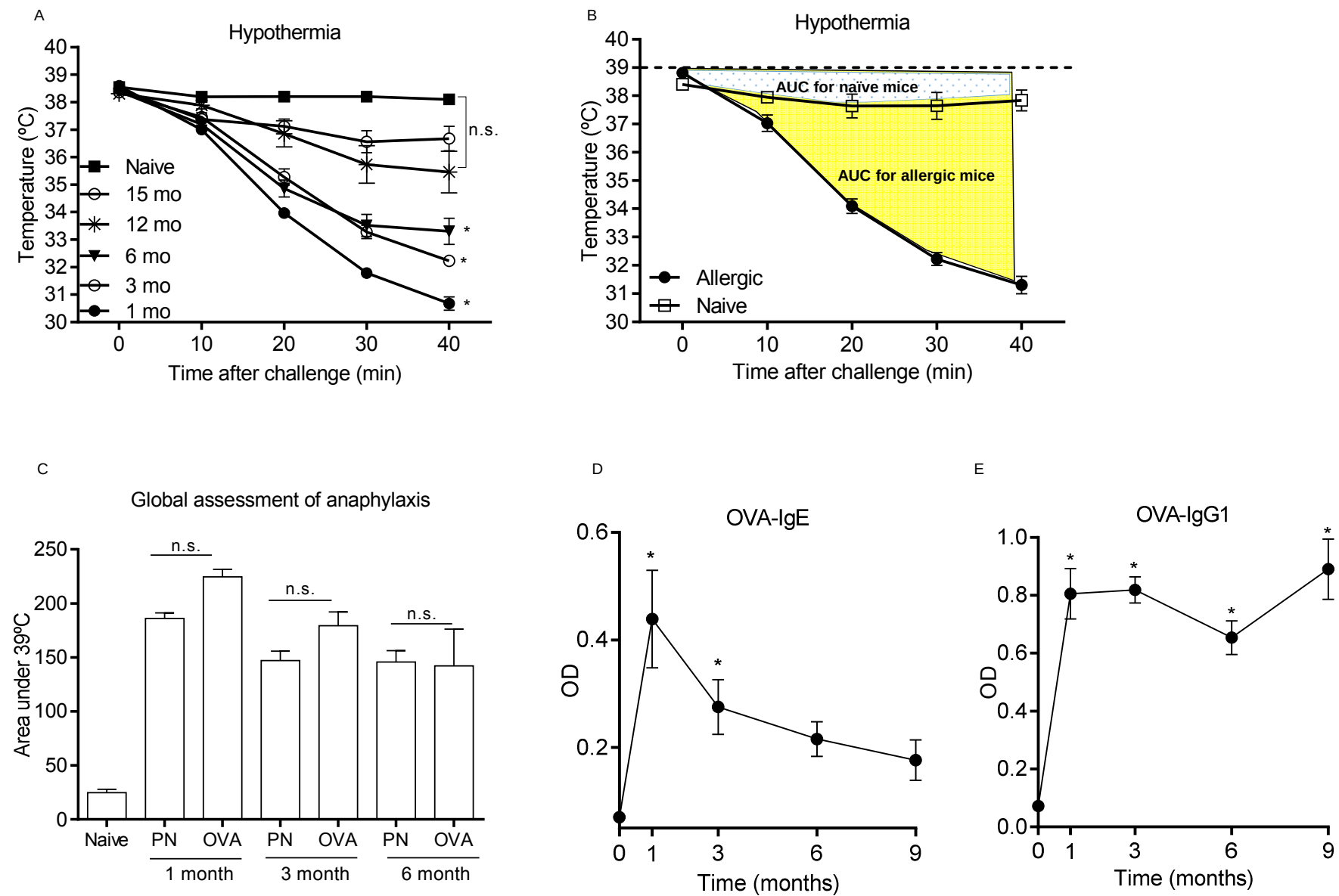


FIGURE S2

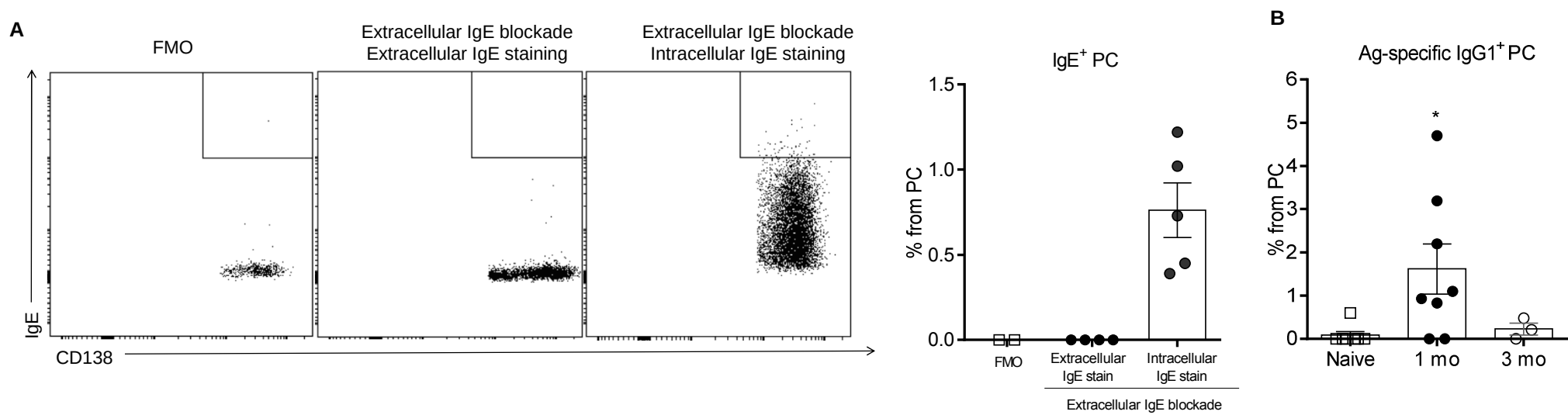


FIGURE S3

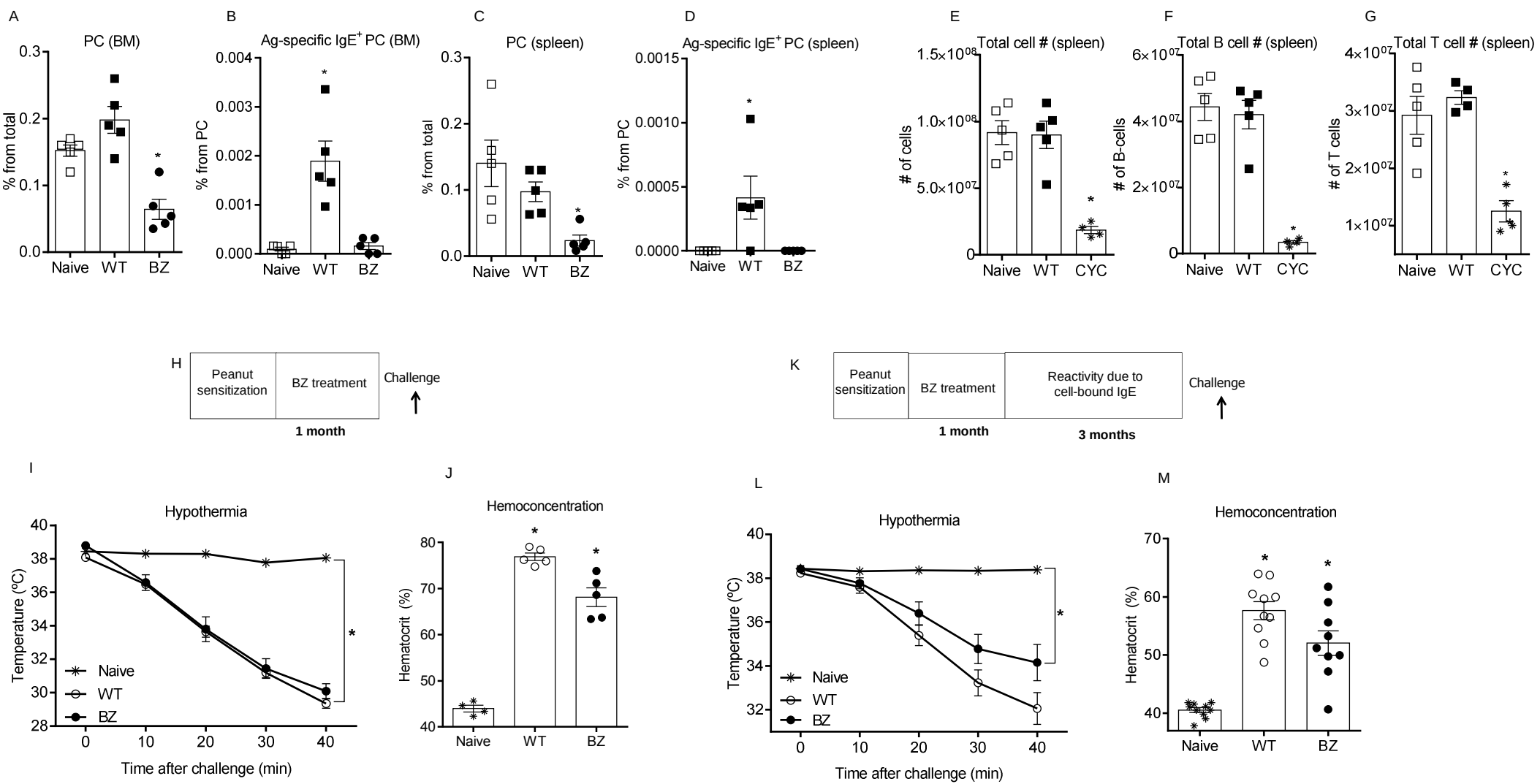
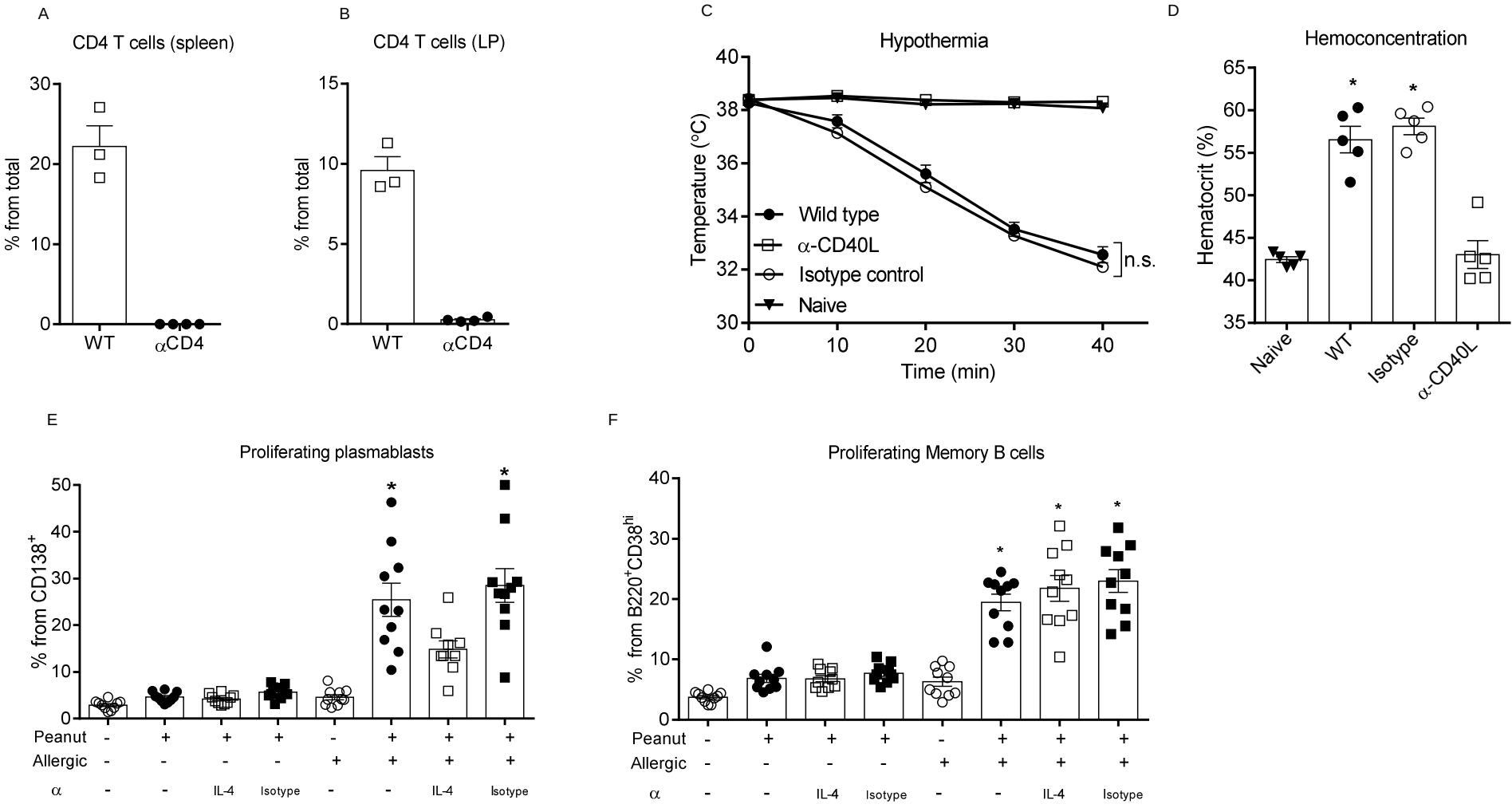


FIGURE S4



SUPPLEMENTARY METHODS

Food allergy model. 3.75 mg of peanut butter (~1 mg of protein; Kraft), or 1 mg of OVA (A5503, Sigma-Aldrich), with 5 µg of cholera toxin (List Biologicals) in 0.5 mL of PBS was administered intragastrically (Delvo), weekly for 4 weeks, with systemic challenge to 5 mg of crude peanut extract (Greer) or OVA at different times post-sensitization. Serum was collected by retro-orbital bleeding 24 h before challenge and analyzed for peanut-specific Igs¹ or OVA-IgE (500840, Cayman) and OVA-IgG². Core temperature was assessed rectally with a dual thermometer (23226-656, VWR), and hematocrit by centrifuging (HemataSTAT II, Fisher Scientific) anticoagulated blood at 40 min after challenge.

Intestinal cell isolation. After flushing intestinal contents with cold PBS, fat was removed manually and intestines were opened longitudinally and cut into approximately 3-5 mm pieces. Mucus was eliminated by means of a 15 minute-wash at 37°C, under shaking, in PBS containing 10 mM HEPES and 4 µM DTT (Sigma). Epithelial cells were removed by 3 rounds of 10 min-washes at 37°C, under shaking, in PBS containing 10% FBS, 10 mM HEPES and 5 mM EDTA. Then, the tissues were digested in 0.125 U/mL Collagenase A (Roche) with 130 U/mL DNase I (Roche) in 10% FBS containing RPMI for 50–60 min in a shaker at 37°C. Lastly, the digested tissues were pressed through a 40 µm nylon strainer (Falcon) and immune cells purified via 40/70% Percoll (GE Healthcare) gradient and centrifugation³.

MLNs and BM processing. MLNs were triturated between frosted slides in HBSS, washed and filtered (40µm). Legs were collected and BM processed as described³.

Flow cytometry. Antibodies were obtained from eBioscience, BD, or BioLegend: CD45-Alexa Fluor 700 or Pacific Blue; CD3-Brilliant Violet 785 or Qdot; CD4-Brilliant violet 421; CD44-APC; CD19-PerCP.Cy5.5 or PE-Cy5; B220-Alexa Fluor 700; CD38-PE or PE-Cy7; CD95-PE-Cy7; GL7-Brilliant Violet 421; CD117-PE-Cy7; FcεRI-APC; IgD-Pacific Blue; IgG1-Brilliant Violet-421; CD138-APC; IgE-PE (clone RME-1). OVA-FITC was obtained from Molecular Probes (023020, ThermoFisher). In all assays, cells were incubated with anti-FcγRII/III before incubation with fluorochrome-conjugated

antibodies. Dead cells were excluded by propidium iodide uptake (Sigma) or fixable viability dye eFluor780 (eBioscience), and gated on singlets. On average, a minimum of 10^6 and 200,000 alive and singlet cells were analyzed for experiments evaluating PCs, and GCs, memory B cells and MCs respectively. Experiments that involved detection of IgE⁺ B cells included un-conjugated anti-IgE (clone RME-1) together with anti-FcγRII/III and exclusive detection of intracellular IgE via IgE-PE (clone RME-1) upon fixation/permeabilization of cells (BD Biosciences). Fluorescence minus one (FMO) and isotype controls were used for gating. Data were acquired on a LSR II or Fortessa (BD) and analyzed using FlowJo (Treestar).

Passive sensitization model. Sera from peanut-allergic mice was administered to IgE-deficient mice. The sera were serially transferred via i.p. injection as follows: 200 μL; 300 μL; and 500 μL with an interval of 48 h between each administration. Peritoneal lavage was performed with ice-cold 10 mM EDTA in PBS. IgE-bearing peritoneal MCs were quantified using flow cytometry as CD45⁺CD117⁺FcεRI⁺IgE⁺ cells.

Antibody and drug administration. CD4 T cell depletion was accomplished via 2-3 i.p. administrations of 200 μg of anti-CD4 (clone GK1.5; made-in house) over a one-week period. PCs were depleted with BZ (PS-341, Selleckchem) via i.p. injections of 40 μg, two times per week, for 3 weeks. 10-15% mortality was associated with BZ treatment. Proliferating cells were eliminated with CYC (C7397, Sigma-Aldrich) via daily i.p. injection of 1 mg for 5 consecutive days⁴.

Evaluation of proliferation *ex vivo* and cytokine production. Spleens were pressed through 40 μm nylon strainers and red blood cells lysed with ACK before being re-suspended in RPMI supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, 55 μM 2-mercaptoethanol (21985-023, Gibco), 1 mM sodium pyruvate, 10 mM HEPES and 0.1 mM MEM NEAA (11140, Gibco). Viable cells were counted via Trypan Blue (Gibco) exclusion and re-suspended at 8×10^6 cells/mL for further CFSE (Sigma) labelling⁵. 800,000 alive cells were cultured in sextuplicates in medium alone or with 250 μg/mL

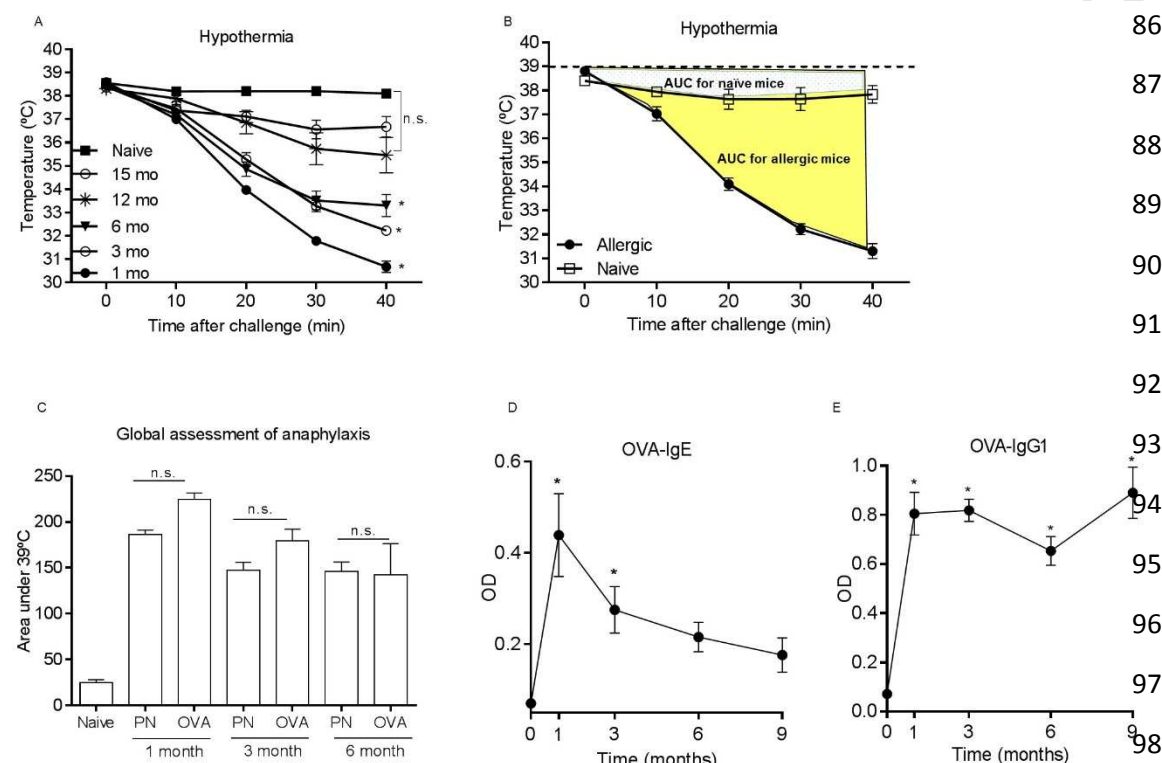
of crude peanut extract; 20 µg/mL of anti-IL-4 (BE0045, Bio X cell); 20 µg/mL of anti-CD40L (MedImmune); 20µg/mL of rat IgG1 isotype control (BE0088, Bio X cell), in flat-bottom 96-well plates (BD). After 4-5 days of culture at 37°C and 5% CO₂, the sextuplicates were pooled, cells were collected for flow cytometry analysis and cell-free supernatants were stored at -80°C until further analysis. Cytokines in cell-free supernatants were quantified using Luminex (Millipore).

Adoptive transfer model. Spleens from OVA-allergic and naïve mice were harvested and processed under sterile conditions as described above. Some groups were subjected to *in vivo* CD4 T cell depletion as described above. Once processed, 20 x 10⁶ alive splenocytes were injected i.v. in a total volume of 400 µL µL into recipient immunodeficient Rag2^{-/-} γc^{-/-}. Recipient mice were gavaged with 1 mg of OVA, 3 times a week for 3 weeks. After the last gavage the mice rested 8-10 days before blood was collected and clinical reactivity evaluated.

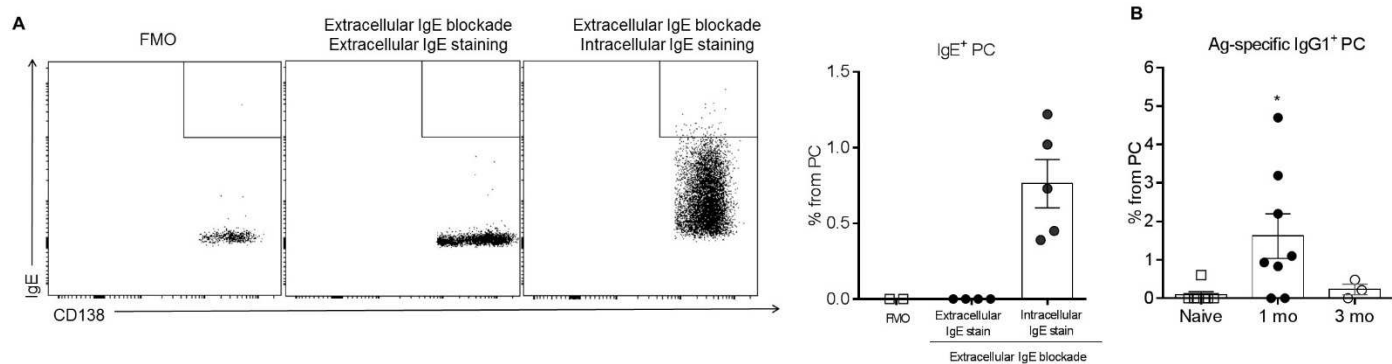
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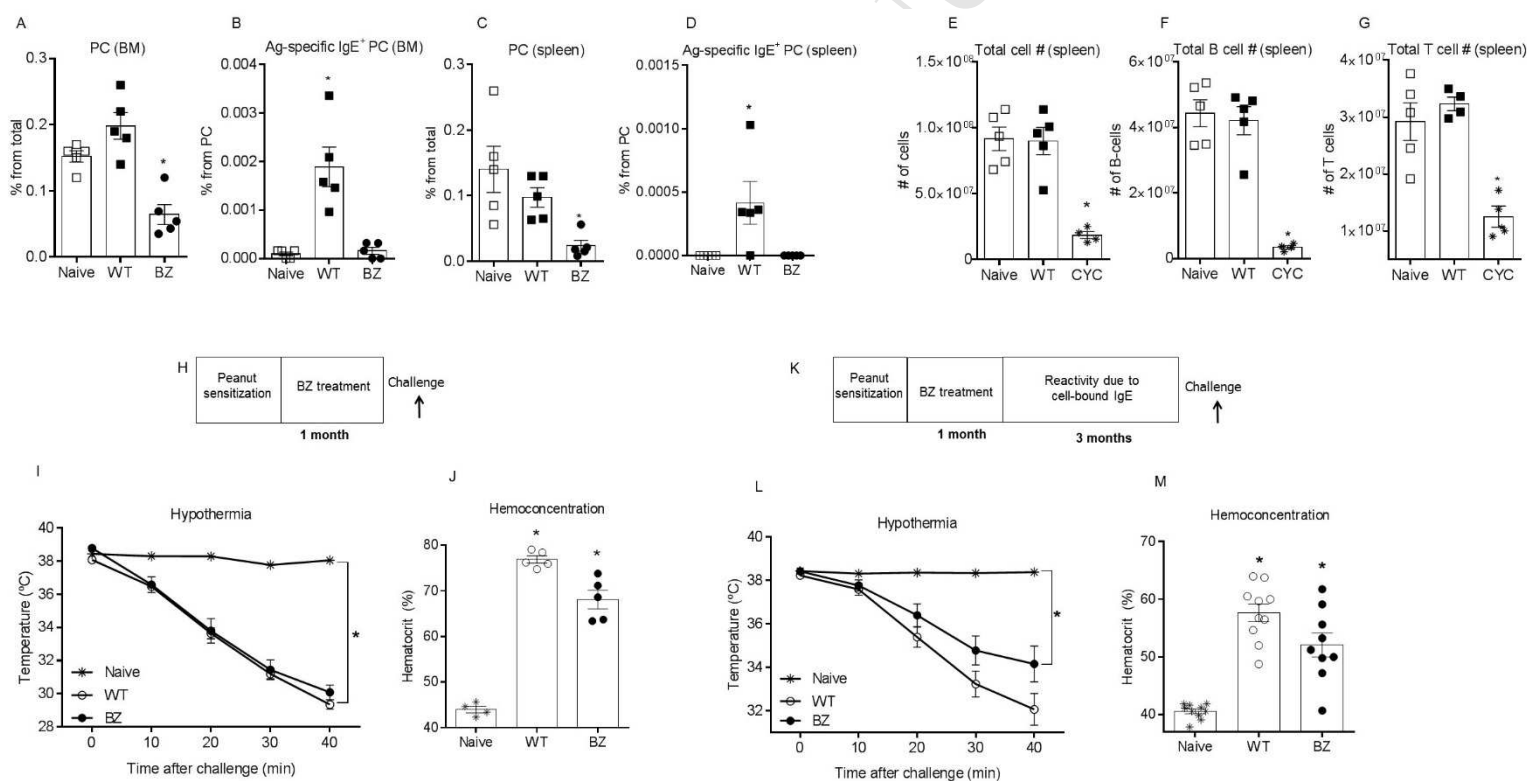
SUPPLEMENTAL FIGURES



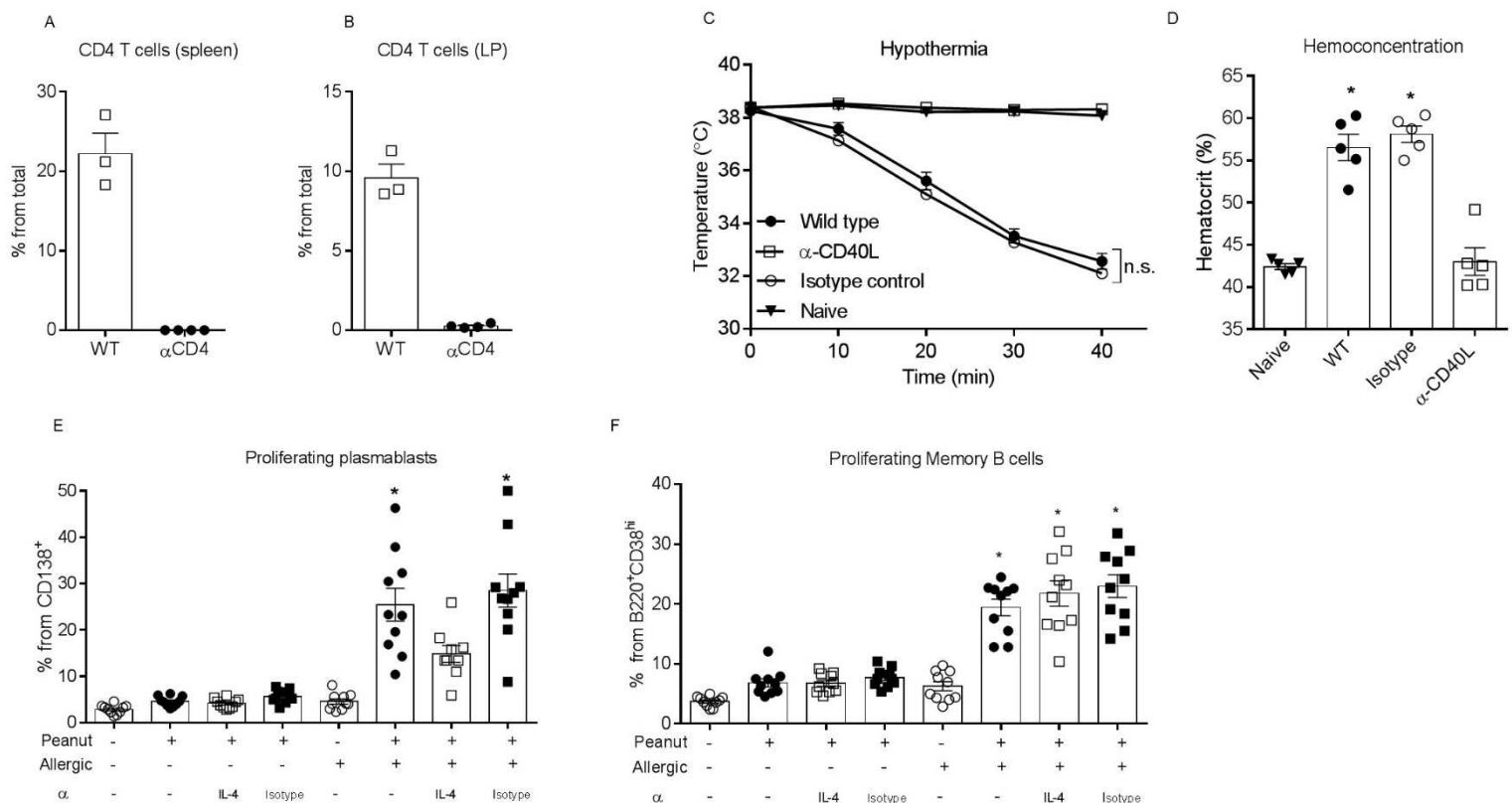
Supplemental Figure 1. Separate groups of mice were orally sensitized to peanut (PN) and left un-manipulated until challenge 1, 3, 6, 12 and 15 months later. Anaphylaxis was evaluated by hypothermia (A); for clarity, the area under 39°C (AUC) over 40 minutes was calculated as shown in panel B (See also Fig 1). Separate groups of mice were orally sensitized to PN and OVA, left un-manipulated until challenge 1, 3 and 6 months later. Anaphylaxis was evaluated by hypothermia (C). Sera was collected at different times post-sensitization for OVA-IgE (D) and OVA-IgG1 (E) detection (See also Fig. 2). Mean $\pm$ SEM, $n=10-20$ pooled from 2-3 experiments. * $P<0.05$ vs naïve, or as indicated; n.s., nonsignificant.



Supplemental Figure 2. Intracellular detection of IgE⁺ PCs (A) and assessment of Ag-specific IgG1⁺ PCs in the mLN at 1 and 3 months post-sensitization (B) (See also Fig. 3). Mean $\pm$ SEM, $n=6-10$ representative data from 2 independent experiments. *P<0.05 vs naïve.



Supplemental Figure 3. Impact of BZ treatment on PCs and Ag-specific IgE⁺ PCs in BM (A, B) and spleen (C, D). Impact of CYC treatment on total cell count (E) and number of B (F) and T (G) cells. Challenge of peanut (PN)-allergic mice after BZ-treatment (H) did not impair clinical reactivity (I, J). BZ-treatment immediately after PN-sensitization (K) did not affect clinical reactivity (L, M) (See also Fig. 3). Mean $\pm$ SEM, $n=4-8$ representative data of 2-3 independent experiments. *P<0.05 vs naïve.



Supplemental Figure 4. The extent of antibody-mediated CD4 T-cell depletion in the spleen (A) and the LP (B) was evaluated by flow cytometry. Administration of anti-CD40L blocking antibody during systemic sensitization to peanut abrogated clinical reactivity upon challenge (C, D). The effect of anti-IL-4 and corresponding isotype control plasmablasts (E) or memory B cell (F) proliferation was evaluated by flow cytometry. Mean $\pm$ SEM, $n=3-6$ representative data of 2 independent experiments (See also Fig. 5). * $P<0.001$ vs naïve.