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# Evidence that High Affinity IgE Can Develop in the Germinal Center in the Absence of an IgG1-switched Intermediate

**Qiang Chen**,

**Hong Liu**,

**Noelle Luling**,

**Julia Reinke**,

**Alexander L. Dent**

Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, Indiana, USA.

## Abstract

High affinity allergen-specific IgE is essential for the severe allergic anaphylaxis response. High affinity antibodies (Abs) are formed by successive rounds of selection of Ag-specific B cells in the germinal center (GC), however several studies have shown that IgE+ GC B cells are impaired in their ability to undergo selection in the GC. A pathway, known as the "indirect switching pathway" for IgE, has been described whereby Ag-specific B cells initially switch to the IgG1 isotype and undergo affinity selection in the GC, with a secondary switch to the IgE isotype after affinity selection. In previous work, using a food allergy model in mice, we investigated how high affinity IgE develops in the GC but we did not test the indirect switching model. Here we analyzed the importance of the indirect switching pathway by constructing IgG1-cre Bcl6-fl/fl mice. In these mice, once B cells switch to IgG1, they delete Bcl6 and thus cannot enter or persist in the GC. When we tested IgG1-cre Bcl6-fl/fl mice with our food allergy model, we found that as expected, IgG1 Abs had decreased affinity, but unexpectedly the affinity of IgE for allergen was unchanged. IgG1-cre Bcl6-fl/fl mice underwent anaphylaxis in response to allergen, consistent with the formation of high affinity IgE. Thus, in a food allergy response, high affinity IgE can be efficiently formed in the absence of indirect switching to IgG1, either by direct selection of IgE+ GC B cells or indirect selection of IgM+ GC B cells that later switch to IgE.

### Keywords

IgE; germinal center; affinity maturation; Ig switch; allergy; anaphylaxis

Correspondence should be addressed to: Alexander Dent, Ph.D., Department of Microbiology and Immunology, Indiana University School of Medicine, 950 W. Walnut St., R2 302, Indianapolis, IN 46202, 317-274-7524 (phone), 317-274-7592 (FAX), adent2@iupui.edu.

Disclosures

The authors declare no conflict of interest with this work.

## Introduction

Atopic and allergic diseases have increased greatly over the last several decades, and currently allergic diseases are extremely prevalent in the United States (1, 2). Food allergy is a particularly common allergic disease that has appeared as a “second wave” of allergic disease in the past 30 years (3). Up to 8% of children and 2% of adults are affected by food allergies (3). Moreover, other common diseases such as gastroenteritis and inflammatory bowel syndrome (IBS) may have an underlying component of food allergy driving the pathology (4). There are few effective treatments for food allergy and thus new treatments are in high demand. IgE antibodies specific for food allergens is the primary mediator of the food allergy response and cross-linking of mast cell-bound IgE by allergens induces a powerful inflammation and even anaphylaxis (5, 6). The molecular and cellular pathways that regulate the initial development of IgE specific for food antigens are still poorly understood.

Despite the importance of IgE in allergic disease, we are only beginning to understand the details of how IgE responses are initiated by interactions between T cells and B cells *in vivo*. Previous research on the regulation of IgE responses has focused on signals that regulate the development of IgE-expressing B cells and the stages of differentiation through which these cells transit. IL-4, provided by T cells, is critical in promoting IgE class switching (7, 8) and Stat6, a major IL-4 signaling transcription factor, plays an intrinsic role in the induction of the epsilon (ε) sterile transcript that is induced prior to IgE switching (9–12). Along with IL-4, CD40 signaling can help promote IgE responses, in part by overcoming negative effects of IL-21 on IgE switching (13). IgE-switched B cells can develop from either the germinal center (GC) reaction (14–20), or by GC-independent and T cell-independent pathways (21, 22). The GC micro-environment is required for Ig somatic hyper-mutation and affinity selection (23–25).

IgE-switched B cells can develop in the GC via two major pathways: a direct switching pathway from IgM to IgE that typically involves rapid differentiation into plasma cells (15, 18), or a sequential switching pathway where the cells start out initially switching to IgG1, undergo successive rounds of mutation and selection as IgG1+ GC B cells, then switch to IgE at a late GC step, before final differentiation into plasma cells (14, 16, 17, 19). Since IgE+ switched GC B cells have altered antigen receptor signaling and undergo increased differentiation and apoptosis, selection of high affinity IgE+ GC B cells is considered to be less efficient than for B cells expressing other Ig isotypes (18, 26, 27). Thus, GC B cells that undergo direct switching to IgE typically have low affinity for antigen (Ag), whereas IgE-switched cells that go through an initial IgG1+ phase can more easily undergo affinity selection to express high affinity IgE (16, 28). High-affinity IgE is pathogenic and is required for anaphylaxis (16, 29). Despite this previous work, much is still unknown about what factors control the initial switch to IgE *in vivo* and what controls the development of IgE+ B cells in the GC.

Several studies, including our own work and research with collaborators, have shown that IL-4-producing T follicular helper (Tfh) cells are essential for the development of Ag-specific IgE (30–33). Recently, a special subset of Tfh cells, Tfh13 cells, were found to

be required for the production of high affinity IgE (29, 34). However, it is not yet clear how Tfh13 cells exactly promote high affinity IgE selection. Tfh13-mediated selection may act directly on IgE+ GC B cells and aid in their selection or Tfh13 cells may select high affinity IgG1+ GC B switched cells and then induce switching to IgE. Overall, exactly how high affinity IgE responses develop is an unsettled question.

We recently explored how high affinity Ag-specific IgE responses develop in the GC using a food allergy model with peanut allergen in mice (35). Our findings on IgE development in this study did not clearly fit into a pathway where high affinity IgE develops from high affinity IgG1+ memory B cells induced to switched to IgE (28, 36). First, we observed that Ag-specific IgE Abs were detected in sera only two days after Ag-specific IgG1 Abs appeared, and there was no clearly defined signal to induce the switch from IgG1 to IgE (35). Second, we found that a longer interval (two weeks) between allergen priming signals led to loss of the IgE response, which is inconsistent with a role for memory B cells in the production of IgE (35). We therefore wondered if sequential switching from IgG1 to IgE is absolutely required for the production of high affinity antigen-specific IgE in our food allergy model. We decided to test this by analyzing IgE responses in mice where IgG1+ GCB cells are blocked by the use of a cre recombinase driven by expression of the IgG1 constant region (37) that deletes Bcl6, a key gene that is required for the GC B cell stage of B cell differentiation (38–41). We show that while this mouse genetic system blocks the development of high affinity IgG1 after food allergy priming as expected, we still observe the development of high affinity IgE that can promote anaphylaxis. We conclude that, at least for IgE that develops in the gut, high affinity IgE does not necessarily need to develop from an IgG1+ GC B cells stage.

## Materials and Methods

### Mice

All mutant mice were on a C57BL/6 background. B6.129P2(Cg)-*Ighg1*$^{tm1(cre)Cgn}$/J mice (37) were obtained from The Jackson Laboratory. Bcl6-flox mice were described previously (42). Cγ1-Cre Bcl6-fl/fl mice were achieved by crossing Cγ1-Cre mice with Bcl6-flox mice. Six-to ten-week-old male and female mice were used for most experiments. Mouse littermate comparisons were used whenever possible. Control and experimental mouse cohorts were age and sex matched. Mice were bred under specific pathogen–free conditions at the laboratory animal facility of the Indiana University School of Medicine. All experiments and handling of animals were conducted according to protocols approved by the IACUC of the Indiana University School of Medicine.

### Mice sensitization and immunizations

For gut sensitizations, mice were deprived of food for 2 h, and then each mouse was fed 300 uL 1.5% $NaHCO_3$ water (i.g.). Thirty minutes later, each mouse was given 1 mg peanut protein extract (Greer Laboratories) or 16x (4-hydroxy-3-nitro- pheny1)acetyl (NP)-conjugated chicken Ovalbumin ($NP_{16}$-OVA) (Biosearch) together with 10 μg cholera toxin (Sigma) according to the setting of experiments (43, 44). For $NP_{16}$-OVA + Alum immunizations, 100 μg of $NP_{16}$-OVA were mixed with Alum (Sigma) and then injected into

mice (i.p.). Mice were sacrificed on the indicated days and the mesenteric lymph nodes (mLN) and spleen were harvested. Serum was also collected.

## Switching region sequencing

Amplification of region from Sμ to Sε were done using nested PCR with DNA Taq Polymerase (Roche). Primers were listed in Table 1. Genomic DNA extracted from target cells were used as template. PCR-amplified junctions were cloned into T-A vectors using TOPO TA Cloning Kit (Invitrogen). After miniprep using QIAprep Spin Miniprep Kit (QIAGEN), these clones were sequence in ACGT Inc. The sequences were deposited in NCBI GenBank database (OP925265–OP925329) under https://www.ncbi.nlm.nih.gov/genbank/

## Somatic hypermutation analysis of V186.2 gene

Somatic hypermutation V186.2 in IgE switched GC B cells was analyzed using a similar approach as Heise et al. (45). Briefly, on day 15, after two rounds of immunization with $NP_{16}$-OVA + Alum (days 1 and 8) or two rounds of sensitization with $NP_{16}$-OVA + cholera toxin (days 1 and 8), GC B cells were isolated by FACS, RNA was prepared and first-strand cDNA was synthesized using Transcriptor First Strand cDNA Synthesis Kit (Roche) with the 3' primer Cε-cDNA (Table 1). Amplification of the V-D-J gene segment 5' of Cε from the cDNA was done with the primers V186.2-leader and Cε-cDNA (Table 1) using Taq DNA Polymerase (Roche). A second round of PCR was then performed using nested primers V186.2-nested and Cε-PCR (Table 1). PCR-amplified products were cloned into T-A plasmid vectors using TOPO TA Cloning Kit (Invitrogen). After miniprep using QIAprep Spin Miniprep Kit (QIAGEN), clones were sequenced commercially (ACGT Inc.). V186.2 sequences were analyzed for Vh186.2 identity and mutations using the immunoglobulin Vbase2 online database (http://www.vbase2.org/vbase2.php). Unique clones were determined by mutation patterns, use of specific D and J gene segments and unique sequences at the V-D-J joining site. The sequences were deposited in NCBI GenBank database (OP925330–OP925362)

## Flow cytometry

Cell suspensions from mLNs were prepared and filtered through a 40-mm cell strainer (Fisherbrand). Cells were washed and diluted in PBS with 1% FBS and were stained with Fc block (Biolegend) for 5 min, followed by surface staining for the indicated markers. Following labeled Abs were used: anti-CD38 (90), anti-B220 (RA3–6B2), anti-GL7 (GL7), anti-IgG1 (RMG1–1) were obtained from BioLegend. Samples were acquired on an LSR2 flow cytometer (Becton Dickinson) and analyzed with FlowJo V10.6 (TreeStar).

## ELISA

For the measurement of antigen-specific IgE, 96 well Nunc-Immuno plates (Sigma) were coated with 5 μg/mL IgE Ab (Clone: LO-ME-3, BIO-RAD) in 0.1 M Carbonate buffer (pH 9.5) overnight at 4 °C. Wells were blocked with 1% BSA for at least 1 h at room temperature and diluted serum was added and incubated at room temperature for 2 h. For peanut (PN)-specific IgE, peanut protein extract was labeled with biotin (Sigma) and added into wells for

one hour. For NP-specific IgE, $NP_4$-BSA and $BP_{27}$-BSA were labeled with biotin and added for incubation. Poly-HRP streptavidin (Pierce Endogen) was then added and incubated for 0.5 h (1:5000). For the measurement of PN-specific IgG1, 96 well Nunc-Immuno plates were coated with 5 μg/mL peanut protein extract in 0.1 M Carbonate buffer (pH 9.5) overnight at 4 °C. For NP-specific IgG1, plates were coated with 5 μg/mL $NP_4$-BSA and $NP_{27}$-BSA. Wells were then blocked with 1% BSA for at least 1 h at room temperature and diluted serum was added and incubated at room temperature for 2 h. An anti-mouse IgG1 (Clone: A85–1, BD Pharmingen) was used as secondary Ab (2 μg/mL) followed by adding avidin-HRP (Invitrogen) for 0.5 h (1:2000). For antigen-specific IgG2a and antigen-specific IgG2b, anti-mouse IgG2a (Clone: R19–15, BD Pharmingen) and anti-mouse IgG2b (Clone: R12–3, BD Pharmingen) were added as the secondary Ab. For the measurement of total IgE, 96 well Nunc-Immuno plates were coated with 2 ug/mL anti-mouse IgE (Clone: R35–72, BD Pharmingen) overnight at 4 °C. Wells were blocked with 1% BSA for at least 1 h at room temperature and diluted serum was added and incubated at room temperature for 2 h. An anti-mouse IgE (Clone: R35–118, BD Pharmingen) was used as secondary Ab (2 μg/mL) followed by adding avidin-HRP (Invitrogen) for 0.5 h (1:2000). After the incubation with HRP, TMB Substrate Reagent Set (BD Pharmingen) was added for the reaction development.

### Assessment of anaphylaxis

To induce anaphylaxis, 2 mg peanut without cholera toxin (CT) was injected (i.p.) per mouse at the indicated times after gut sensitization. Mice were monitored every 10 min after challenge for the rectal (core) body temperature (Braintree Scientific). For Hematocrit measure, blood was collected in Heparinized capillary tubes (Fisherbrand). After centrifugation, percent hematocrit was calculated ((mm of packed RBC/mm of total volume) × 100).

### QPCR

Expression of Cre mRNA were performed using FastStart Universal SYBR Green Master (Roche) according to manufacturer's instruction. Primer of Cre-F and Cre-R were used (Table 1). Beta-2-microtubulin (B2M) were used as internal control.

### Statistical analysis

All data analysis was performed using GraphPad Prism software (GraphPad Software). Graphs show the mean ± SEM. Unless otherwise stated, Two-tailed Student's t test or One-way ANOVA with Tukey's post hoc analysis was used. All ELISAs were analyzed using Two-way ANOVA. Significant differences ($P < 0.05$) and some non-significant differences are indicated in the figures. All the statistical details of experiments can be found in figure legends. The investigators were not blinded for the analyses.

## Results

### Analysis of switch regions for evidence of indirect switching to IgE.

After our initial characterization of how high affinity Ag-specific IgE responses develop in a mouse food allergy model (35), we wondered if the IgE+ B cells developed through a direct

or indirect IgE switching pathway (Fig. 1A). To test this, we initially set out to determine if we could detect remnants of the γ1 switch (Sγ1) region in the Sμ-Sε switch junction that brings the VDJ exon upstream of the Cε region, which would be indicative of an indirect IgE switch pathway (Fig. 1A) (16). We PCR amplified Sμ-Sε switch junctions from purified mouse B cells, then cloned, sequenced the PCR products and aligned sequences to the mouse genome (Fig. 1B, Suppl. Table). We initially analyzed Sμ-Sε switch junctions after OVA-Alum immunization, as Sγ1 remnants were observed in this response previously (16). As shown in Table 2, if we count only unique switch region joins, we found 3/28 clones with Sγ1 remnants, a rate of ~11%. We then analyzed Sμ-Sε switch junctions amplified and cloned from B cells after food allergy priming with peanut protein plus cholera toxin (PCT). Here, analyzing only unique switch region joins, we see 3/18 clones with Sγ1 remnants, a rate of ~17% (Table 2). Thus, in the food allergy response, there was a trend towards a higher rate of Sγ1 remnants.

The two types of Sγ1 remnants we found mapped to the approximate center of the Sγ1 region and outside of the tandem repeat region within the larger Sγ1 region (Fig. 1C). Xiong et al designed PCR primers specific for the tandem repeat region of Sγ1 that were used to monitor Sγ1 remnants in Sμ-Sε switch junctions following immunization with OVA-Alum (16). We were not able to obtain clear PCR products using the Sγ1 PCR primers used by Xiong et al (16) (data not shown), consistent with the genomic location of the Sγ1 remnants we identified being outside of this repeat region (Table 2 and Fig. 1C). Overall our results indicate that there is some degree of indirect IgE switching in our mouse food allergy model. At the same time, we are unable to conclude whether IgE+ B cell clones containing Sγ1 remnants are critical for the high affinity anaphylaxis-inducing IgE produced in the food allergy response (35).

We next analyzed differences in mutation rates between the clones with Sγ1 remnants and without Sγ1 remnants, in order to determine if the degree of switch DNA mutation induced by AID could indirectly indicate the degree of selection that occurred in the GC for the two types of clones. As shown in Figure 1D, indirectly switch clones had the same level of mutations as directly switched clones. Thus, indirectly switched clones do not show signs of longer GC transit than directly switched clones. A complication to this switch region analysis is that Sε switch junctions resulting from indirect switching with an Sγ1 intermediate can be formed where remnants of Sγ1 are lost in the final switch (Fig. 1A) (16).

Notably, in both the OVA-Alum and PCT responses, we saw a similar type of Sγ1 remnant-containing switch clone, with a nearly perfectly conserved 122 bp long Sγ1 sequence and conserved Sε flanking sequences (Suppl. Table). This 122 bp Sγ1 remnant was the major type of Sγ1 remnant in our analysis and may represent an unusually frequent breakpoint for indirect IgE switching. On the other hand, we cannot completely rule out that this repeated switch region is a contaminant in our PCR and cloning system, if we assume that the specific joins in a given switching event are random, making it highly unlikely that a very specific Sμ- Sγ1-Sε switching event occurs more than once. While the vast majority of the switch junctions between experiments were unique, we did observe two other switch junctions appear in more than one experiment (clones 29 and 31, Suppl. Table). This again

suggests either some switch regions are frequently used or that we had some low level of PCR contamination in our analysis. Since we first detected the Sγ1 remnant in B cells from the food allergy model and not after OVA-Alum immunization, the food allergy switch clones would be the initial source of contamination if in fact there was contamination. Thus, our basic conclusion that Sμ-Sγ1-Sε switching can occur in the food allergy response remains intact. However, our data also show that the extent of indirect switching pathway usage in the food allergy IgE response is unlikely to be definitively revealed by Sε switch region sequencing. We therefore decided to use another approach to address this question.

### Rationale for Cγ1-cre Bcl6-fl/fl mice.

In previous work showing a role for the indirect pathway in the generation of high affinity IgE, "hMT" mice were used in which the sterile γ1 promoter is replaced by an irrelevant promoter (16). In this mouse model, switching to Cγ1 is blocked, IgG1 cannot be produced and the indirect IgE switching pathway is blocked, leading to lower affinity IgE (16). We worried that completely blocking the formation of IgG1 might lead to unexpected effects on the Ab response. Thus we devised an alternate mouse model more specifically focused on IgG1+ B cell selection in the GC. In this model, a Cγ1-cre knock-in allele (37) deletes a floxed Bcl6 gene. In these Cγ1-cre Bcl6-fl/fl mice, B cells that turn on expression of the IgG1 constant region at any stage of differentiation will delete Bcl6, disabling the ability of the B cell to enter the GC or remain in the GC stage (38–41). Figure 2 shows the predicted result of knocking out Bcl6 by Cγ1-cre on the generation of high-affinity IgG1 and high-affinity IgE according to the indirect IgE switching model. Loss of GC differentiation by IgG1+ B cells is expected to cause loss of affinity maturation and thus lower affinity IgG1 responses in Cγ1-cre Bcl6-fl/fl mice. If indirect switching is crucial for high affinity IgE responses, we should also see lower affinity IgE responses in Cγ1-cre Bcl6-fl/fl mice. However, if high affinity IgE responses can be generated by an alternative pathway other than by indirect switching through IgG1, we should still observe high affinity IgE responses in Cγ1-cre Bcl6-fl/fl mice.

### Generation and preliminary analysis of Cγ1-cre Bcl6-fl/fl mice.

We initially wanted to verify that the Cγ1-cre allele was not induced non-specifically in IgE+ B cells, as that would complicate our analysis. We therefore mated Cγ1-cre mice to Verigem IgE reporter mice so that we could readily analyze IgE+ B cells (17). Cγ1-cre Verigem+ mice were then primed i.g. with PCT four times to induce a strong peanut-specific IgE response (33, 43). B cells were then isolated, stained for IgM and IgG1, then sorted by FACS, obtaining purified IgM+, IgG1+ and IgE+ B cells. RNA and cDNA were prepared from these cells and expression of Cre was analyzed by QPCR. As shown in Figure 3A, only IgG1+ B cells expressed significant amounts of cre mRNA, providing evidence that the Cγ1-cre allele wasn't induced in IgE-expressing B cells.

Cγ1-cre Bcl6-fl/fl mice were then generated; these mice were healthy and bred normally. We observed that naïve B cells developed at normal numbers in these mice (Fig. 3B). We next analyzed the GC B cell and plasma cell response in these mice and Cγ1-cre alone control mice following PCT priming (gating shown in Supplemental. Fig. 1). As shown in Figure 3C, the overall mLN GC B cell response in Cγ1-cre Bcl6-fl/fl mice was sharply

decreased compared to control mice, consistent with the original finding from Casola et al of Cγ1-cre activity in up to 75% of GC B cells (37). As expected, the IgG1+ GC B cell response was also significantly decreased in Cγ1-cre Bcl6-fl/fl mice compared to control mice (Fig. 3D). However, the strong decrease in overall GC B cells in Cγ1-cre Bcl6-fl/fl mice (~75%) contrasted with the relatively low percent of IgG1+ GC B cells in control mice (~6%). These data suggest that transcriptional activation of Cγ1 is induced in a high percentage of GC B cells without there being commitment to full IgG1 switching, and this cre expression is sufficient to delete Bcl6 and cause loss of the GC B cell phenotype. Despite the loss of GC B cells in Cγ1-cre Bcl6-fl/fl mice, these mice produced a normal number of IgG1+ plasma cells (Fig. 3E). Peyer's Patch responses showed a similar loss of GC B cells and IgG1+ GC B cells as in the mLN (Fig. 3F–G). Taken together, these data indicate that the loss in GC B cells in Cγ1-cre Bcl6-fl/fl mice leads to a compensatory increase in plasma cell differentiation so that the number of plasma cells is not significantly affected and possibly that a large number of IgG1+ plasma cells develop by a GC-independent pathway in this model.

### The food allergy IgE response in Cγ1-cre Bcl6-fl/fl mice.

We next wished to test how loss of GC B cells and IgG1+ GC B cells in Cγ1-cre Bcl6-fl/fl mice affected the Ab response. We first tested overall Ab levels in Cγ1-cre Bcl6-fl/fl mice after PCT priming. As shown in Figure 4A, we unexpectedly found higher titers of peanut-specific IgG1 Abs in the Cγ1-cre Bcl6-fl/fl mice compared to controls. These data indicate there is accelerated or increased development of Ag-specific IgG1+ plasma cells in the absence of Ag-specific IgG1+ GC B cell development in Cγ1-cre Bcl6-fl/fl mice. The levels of total and peanut-specific IgE were unchanged. Peanut-specific IgG2b was unchanged but peanut-specific IgG2a titers were decreased in Cγ1-cre Bcl6-fl/fl mice (Fig. 4B–E). The reason for the slight but significant loss of peanut-specific IgG2a Abs is unclear but may relate to the overall loss of GC B cells in Cγ1-cre Bcl6-fl/fl mice.

To address how Ab affinity was affected by the loss of GC B cells in Cγ1-cre Bcl6-fl/fl mice, we used the NP-protein conjugation system (45), where affinity to the NP hapten can be measured by comparing titers of Abs that reacts with sparsely protein-conjugated hapten versus titers of Abs that reacts with highly protein-conjugated hapten. We therefore primed control and Cγ1-cre Bcl6-fl/fl mice with $NP_{16}$-OVA plus CT i.g. instead of PCT for food allergy priming. As shown in Figure 5A–B, and as seen after peanut plus CT priming (Fig. 3C–D), Cγ1-cre Bcl6-fl/fl mice had fewer GC B cells and IgG1 GC B cells in the mLN compared to controls. Also, as seen with peanut plus CT priming (Fig. 4A), we saw higher titers of NP-specific IgG1 Abs in the Cγ1-cre Bcl6-fl/fl mice compared to controls (Fig.5C). At the same time, consistent with our prediction, the affinity of NP-specific IgG1 Abs in the Cγ1-cre Bcl6-fl/fl mice was strongly decreased compared to controls (Fig. 5C). Thus our Cγ1-cre Bcl6-fl/fl mouse model was effective at decreasing IgG1 affinity but not IgG1 titers to Ag in a food allergy model. However, the critical test of our model was whether or not loss of IgG1+ GC B cells affected the IgE response in this response. As shown in Figure 5D, neither the titer nor the affinity of NP-specific IgE was altered by loss of IgG1+ GC B cells in Cγ1-cre Bcl6-fl/fl mice.

We next analyzed somatic hypermutation of the IgE Vh genes in B cells responding to $NP_{16}$-OVA plus CT priming in control and Cγ1-cre Bcl6-fl/fl mice. We cloned and sequenced IgE heavy chain genes from purified GC B cells using PCR primers specific for the IgE (ε constant domain) gene and the Vh186.2 gene segment known to dominate anti-NP responses in C57Bl/6 mice (45). Consistent with the affinity analysis measured by ELISA, we found no significant differences in the levels of somatic hypermutation of the IgE heavy chain clones with Vh186.2-containing variable regions (Fig 5E). Furthermore significant numbers of mutations were observed in the variable region genes: 4–5 mutations per Vh186.2 segment on average (Fig 5E). These data indicate that high affinity IgE responses can be generated by somatic hypermutation in the GC, in the absence of indirect Ig switching through an IgG1+ intermediate B cell, at least in this food allergy model.

### The systemic IgE response in response in Cγ1-cre Bcl6-fl/fl mice.

As shown by Xiong et al (16), knocking out IgG1-intermediate switching strongly decreased IgE affinity in a systemic OVA-Alum immunization model. We therefore immunized control and Cγ1-cre Bcl6-fl/fl mice with $NP_{16}$-OVA plus Alum and tested IgG1 and IgE Ab responses. We analyzed GC B cells and IgG1+ GC B cells in these mice after $NP_{16}$-OVA-Alum immunization (Fig. 6A–B). These immune challenges produced the same pattern of decreased GC B cells and IgG1+ GC B cells as after PCT priming, although the loss of total GC B cells was not as severe as with PCT priming. As shown in Figure 6C, we again observed increased anti-NP IgG1 titers in Cγ1-cre Bcl6-fl/fl mice as well as loss of IgG1 affinity against NP, consistent with our findings in the food allergy model.

In contrast to the food allergy model however, we found elevated anti-NP IgE titers in Cγ1-cre Bcl6-fl/fl mice and loss of IgE affinity against NP (Fig. 6D). These data support previously published data (16), and also reveal that the importance of the indirect IgE switching pathway on IgE affinity varies depending on the type of IgE response. One explanation for these results is that there is a requirement for a greater amount of somatic hypermutation in the systemic response than in the food allergy model for the high affinity Ab response. By this argument, GC B cells in Cγ1-cre Bcl6-fl/fl mice could achieve the mutations necessary for high affinity IgE in the food allergy response but might not be able to accumulate the high level of mutations necessary for high affinity IgE responses after systemic immunization. To exclude this possibility, we again cloned and sequenced IgE heavy chain genes from GC B cells, but isolated after systemic $NP_{16}$-OVA plus Alum immunization, and compared the level of mutations to IgE heavy chain genes from GC B cells after $NP_{16}$-OVA plus CT oral priming. As shown in Figure 6E, we observe significantly more mutations in Vh186.2 genes in the oral priming model than after systemic immunization. These data indicate that the difference in IgE affinity between the two types of responses is not explained by a more demanding level of affinity selection in the systemic response.

### The anaphylaxis response in Cγ1-cre Bcl6-fl/fl mice.

To further confirm our results that loss of IgG1+ GC B cells did not affect IgE affinity in a food allergy priming system, we tested anaphylaxis, a functionally important measure of IgE affinity. Anaphylaxis is considered to be dependent on high-affinity IgE (16, 29).

Therefore we primed control and Cγ1-cre Bcl6-fl/fl mice with PCT and after one week, challenged the mice with peanut protein and measured subsequent changes in temperature and hematocrit. As shown in Figure 7A–C, we observed that both control and Cγ1-cre Bcl6-fl/fl mice underwent significant anaphylaxis as measured by a temperature drop and increased hematocrit. These data indicate that IgE affinity in Cγ1-cre Bcl6-fl/fl mice is sufficient to provoke an anaphylactic response. However, while there was not a significant difference in the anaphylaxis in control and Cγ1-cre Bcl6-fl/fl mice, there was less of a temperature drop in the Cγ1-cre Bcl6-fl/fl mice than the control mice. These data may indicate that there is somewhat lower IgE affinity in the Cγ1-cre Bcl6-fl/fl mice that led to less severe anaphylaxis.

When we analyzed the data from the challenged mice in more detail, we observed that at this challenge timepoint of the food allergy response, anaphylaxis was variable from mouse to mouse, with some mice responding strongly and some mice not responding at all (Supplemental Fig. 2A–C). The proportion of Cγ1-cre Bcl6-fl/fl mice that generated a full anaphylaxis response was almost two-fold lower than the proportion of control mice that generated full anaphylaxis responses, possibly indicating less IgE affinity in the non-responding mice. However, there was too much variability in these challenge experiments to draw firm conclusions about lower IgE affinity, and therefore we used a stronger PCT priming system to induce anaphylactic IgE (Fig. 7D). When we used 4 priming doses of PCT, a much more consistent temperature drop was observed for both control and Cγ1-cre Bcl6-fl/fl mice, and importantly no difference was seen in the degree of temperature drop and hematocrit between control and Cγ1-cre Bcl6-fl/fl mice (Fig. 7E, F). These data therefore support our ELISA data in Figure 5 showing that in the food allergy response, high affinity IgE can be generated in the absence of IgG1+ GC B cells.

## Discussion

In this study, we have used a novel mouse model, Cγ1-cre Bcl6-fl/fl mice, to explore the importance of the indirect IgE switching pathway in the generation of high affinity IgE. The current model for how high affinity pathogenic IgE develops is that due to the intrinsic instability of IgE+ GC B cells, IgE-expressing B cells must go through a IgG1+ B cell phase in order to undergo affinity selection in the GC so they can eventually undergo a secondary Ig isotype switch and express high affinity IgE (14, 16, 17, 19). While there is strong data that supports this model (16, 36), there has been little exploration of alternative developmental pathways for high affinity IgE+ B cells. For instance, high affinity IgE+ B cells may develop from unswitched IgM+ B cells that go through affinity selection in the GC prior to switching signals to switch to IgE. Additionally, there may be certain types of immune responses that allow for affinity selection of IgE in the GC.

Here, we have used Cγ1-cre Bcl6-fl/fl mice to show that in a food allergy model, high affinity IgE responses can develop from an IgG1-independent GC pathway. We show here that IgG1 affinity selection is clearly impaired in Cγ1-cre Bcl6-fl/fl mice at the same time that high affinity IgE responses develop. Furthermore we show in a systemic OVA-Alum immunization model, similar to what was used to test the role of IgG1 in the IgE switching pathway and the generation of high affinity IgE (16, 36), that the IgG1 GC pathway is still

important for the generation of high affinity IgE. Taken together our data show that there is a novel pathway, possibly unique to the generation of IgE in the food allergy model, that allows for high affinity IgE responses to develop by an IgG1-independent GC pathway.

Our results here give rise to several questions to pursue in future research. First, what is the specific pathway that high affinity IgE responses develop by in the food allergy model? There are three possibilities here. First, high affinity IgE+ B cells develop from IgM+ B cells after they've gone through affinity selection in the GC. Second, high affinity IgE+ B cells develop via an indirect switch pathway but with a different Ig switch than IgG1 and where the GC B cell is selected via a BCR with this alternative Ig, e.g. IgG2b. Third, high affinity IgE+ B cells develop from IgE+ GC B cells that are able to be selected efficiently in the GC than previously known. A pathway of initial affinity selection on IgM+ GC B cells is very feasible and we have no evidence against this pathway. We cannot rule out an indirect switch pathway using an alternative Ig, but we have no evidence of other types of Ig remnants in the switch regions we analyzed in Figure 1/Table 2. However, indirect switching to other Ig isotypes has been observed for human IgE responses (46, 47).

Direct affinity selection of IgE+ GC B cells is unlikely from studies on systemic IgE responses (14, 18, 19, 27), but we cannot rule out that there is something unique about the gut environment or food allergy response that allows for better selection of IgE+ GC B cells than in non-gut-associated lymphoid tissue. What we can say for certain is that the high affinity IgE generated in our food allergy model involves the GC response, as we showed previously that the IgE response in this model is completely lost if TFH cells or GC B cells are genetically ablated (33). Consistent with this data, we have also observed significant numbers of IgE+ GC B cells in this food allergy response (35). We should also note here that while recent evidence supports the idea that Ig class switch recombination occurs outside the GC, this has not yet been demonstrated for the IgE response (48).

Another question is whether there is something unique about the food allergy model that allows a different pathway other than the IgG1 indirect pathway for high affinity IgE responses to develop. We showed previously that food allergy IgE responses rely on TFR cells for their development, in part through the production of IL-10 (33), whereas TFR cells are repressive for IgE responses in airway allergy and systemic IgE responses (31, 34, 49). Thus the gut IgE response is unique and TFR cell-mediated help may allow for a more stable development of IgE+ GC B cells. Additionally we have found that the gut IgE responses requires precise Ag priming conditions, and we were able to track the expansion of IgE+ GC B cells in this response after priming (35). It is feasible that in the right GC environment, IgE+ GC B cells can go through significant affinity selection to generate high affinity pathogenic IgE.

Although the indirect IgM-IgG1-IgE switching pathway has become the major paradigm for how high affinity IgE responses develop, many questions remain unanswered about this pathway. Most notable is the question of what signals drive the IgG1+ GC or memory B cell to switch secondarily to IgE. Is it simply an extra strong Th2 environment and subsequent IL4/IL13-Stat6 signal? Or is some other signal involved? Another question relates to the Tfh13 subset and their ability to promote high affinity IgE responses (29). Do Tfh13 cells

act on IgG1+ GC or memory B cells or do they directly help and select IgE+ GC B cells? If Tfh13 cells are found in the GC, they likely promote high affinity IgE responses by acting on IgE+ GC B cells. However, we have been unable to detect Tfh13 cells in our food allergy model (data not shown), however we cannot rule out a low level of these cells.

Finally, a critical question is what combination of signals induces IgE switching *in vivo*, and what signals induce the formation of IgE+ GC B cells? We know the major signals that promote IgE switching such as IL-4 and CD40 (13), little is known about other signals that promote IgE responses and that might aid in the selection of IgE+ GC B cells. We have identified IL-10 as a novel positive regulator of IgE responses (33), and *Fgl2* and *Entpd1* as novel negative regulators of IgE responses (35), but much remains unknown about how IgE responses are regulated *in vivo*. Despite extensive research on IgE B cell responses, the mechanisms of how high affinity IgE responses are initiated and selected *in vivo* remain poorly understood.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

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**Key Points:**

- In systemic responses, high affinity IgE develops via an IgG1+ GC B cell stage
- In a food allergy response, high affinity IgE doesn't need an IgG1+ GC B cell stage

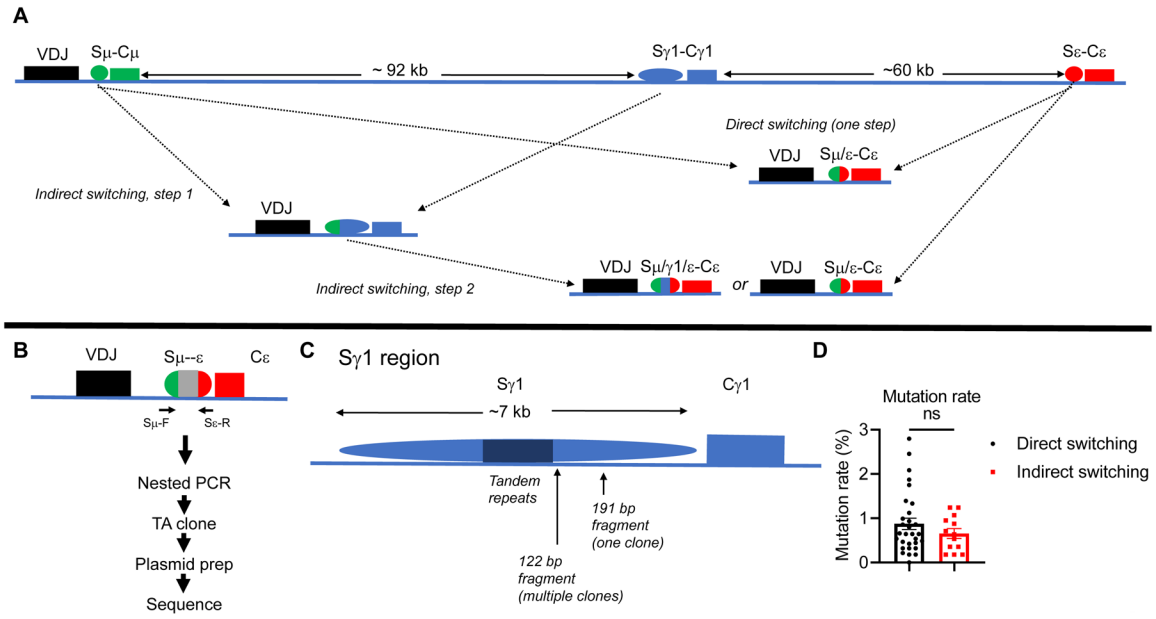


**Figure 1.**

Low frequency of Sγ1 remnants in mouse IgE switch regions. (A) Model of direct class-switching and indirect class-switching to IgE. (B) Protocol used to clone Sμ-Sε regions and sequencing. B cells were isolated after either Ova-Alum i.p. immunization or peanut + cholera toxin (PCT) i.g. priming and then genomic DNA was extracted. Nested PCR reactions were used to amplify regions between Sμ and Sε. PCR products were cloned into plasmids and used to transform bacteria. Independent clones were picked for sequencing. (C) Schematic of the Sγ1 switch region and location of mapped remnants in Sμ-Sε switch region clones combined from both types of responses. The tandem repeat region within Sγ1 is shown as a dark blue box. Sμ-Sε switch region clones with a Sγ1 remnant fell into two types: a 122 bp Sγ1 remnant observed in multiple unique clones after both OVA-Alum immunization and PCT priming, and a 191 bp Sγ1 remnant in one unique clone in the OVA-Alum response. (D) Mutation rates at the Sμ-Sε junctions (direct switching) and Sμ-Sγ1-Sε junctions (indirect switching). The sequences of these clones were aligned with germline sequences, and the mutation rate (# of mutated nucleotides divided by # of total nucleotides) was calculated. Significance was determined by t-test. ns, not significant.

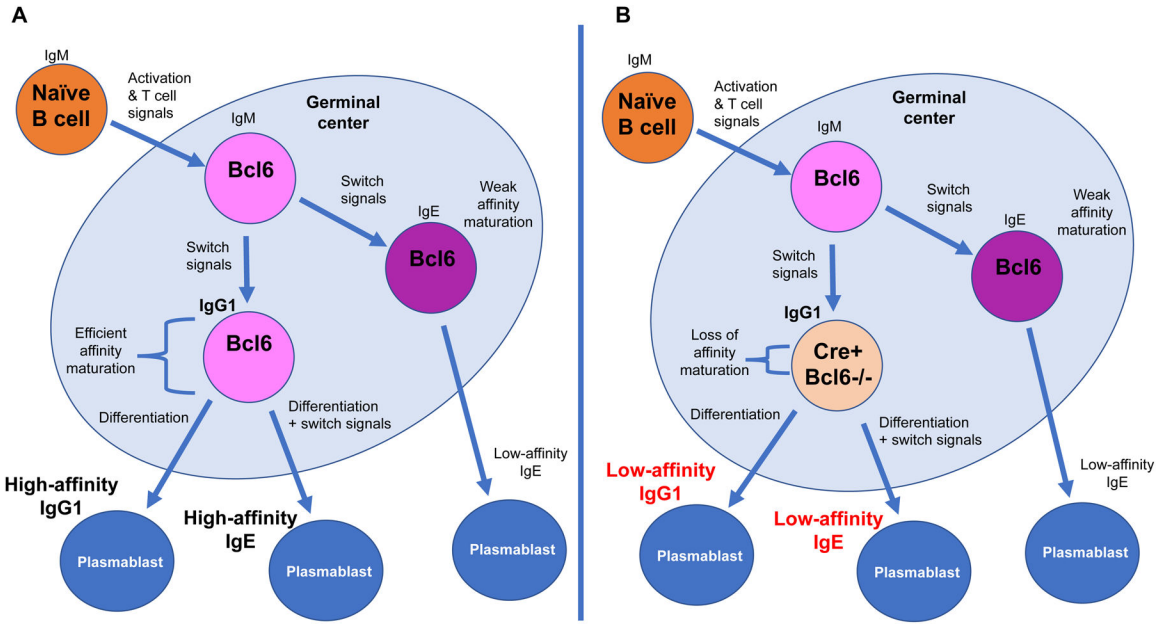


**Figure 2.**

Deletion of IgG1-expressing germinal center (GC) B cells in novel mouse model. (A) Model for sequential IgG1-IgE switching in the germinal center and generation of high affinity IgE via an IgG1+ intermediate GC B cell. (B) Prediction for GC B cell differentiation and affinity of IgG1 and IgE in Cγ1-Cre Bcl6-fl/fl mice according to sequential switching model.

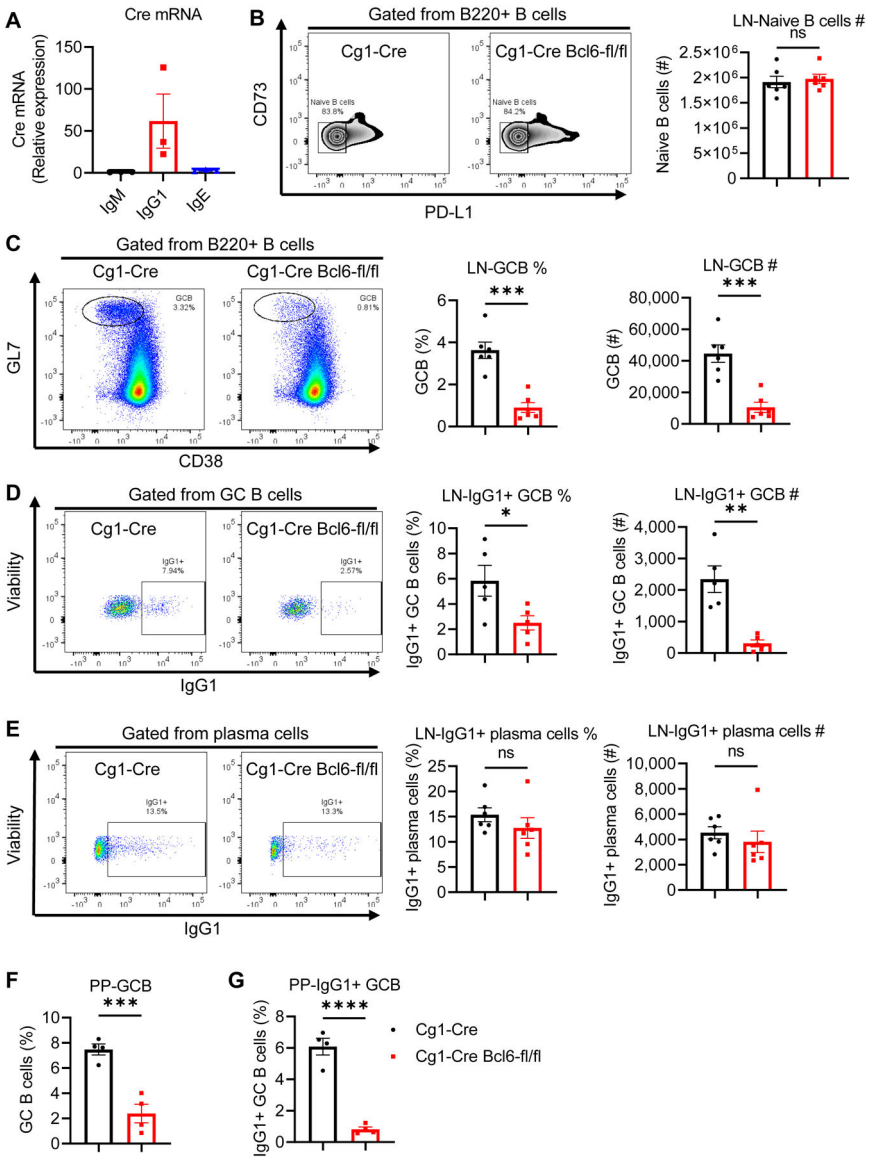


**Figure 3.**

Altered B cell responses in Cγ1-Cre Bcl6-fl/fl mice after food allergy priming. (A) mRNA expression of Cre in mice. Cγ1-Cre Verigem+ mice were i.g. sensitized with PCT. IgM+, IgG1+, and IgE+ B cells were sorted from mesenteric lymph node (mLN). mRNA level of Cre was measured using QPCR. n = 3. (B-G) Cγ1-Cre and Cγ1-Cre Bcl6-fl/fl mice were sensitized with PCT (i.g.) at day 1 and day 8. Mice were analyzed at day 15, one week after the last priming. Percent and cell number of naïve B cells (B), GC B cells (C), IgG1+ GC B cells (D), and IgG1+ plasma cells (E) were measured in mLN from Cγ1-Cre and Cγ1-Cre Bcl6-fl/fl mice using flow cytometry. Gating used for flow cytometry analysis was shown in Supplemental Fig. 1. Percentages of GC B cells (F) and IgG1+ GC B cells (G) were analyzed in Peyer's patch in Cγ1-Cre and Cγ1-Cre Bcl6-fl/fl mice using flow cytometry and similar gating as in (C) and (D). n = 4–6. Data are representative of two independent

experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001; **** P < 0.0001 by t-test. ns, not significant.

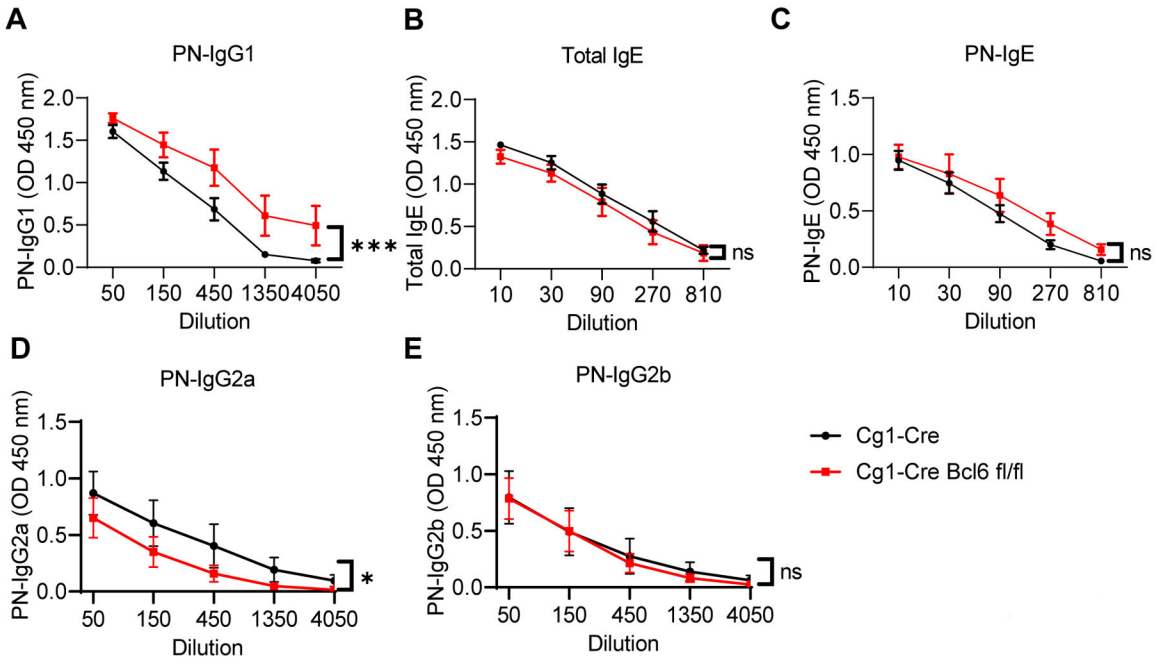


**Figure 4.**

Altered IgG1+ antibody responses in Cγ1-Cre Bcl6-fl/fl mice. Cγ1-Cre and Cγ1-Cre Bcl6-fl/fl mice were sensitized with PCT. Sera were collected at day 36, 4 weeks after the last priming, similar to Xie et al (33). The levels of peanut-specific (PN-) IgG1 (A), total IgE (B), PN-IgE (C), PN-IgG2a (D), and PN-IgG2b (E) in sera were measured at different dilution points. Data were presentative of two independent experiments. n = 7. *, $P < 0.05$; ***, $P < 0.001$ by two-way ANOVA. ns, not significant.

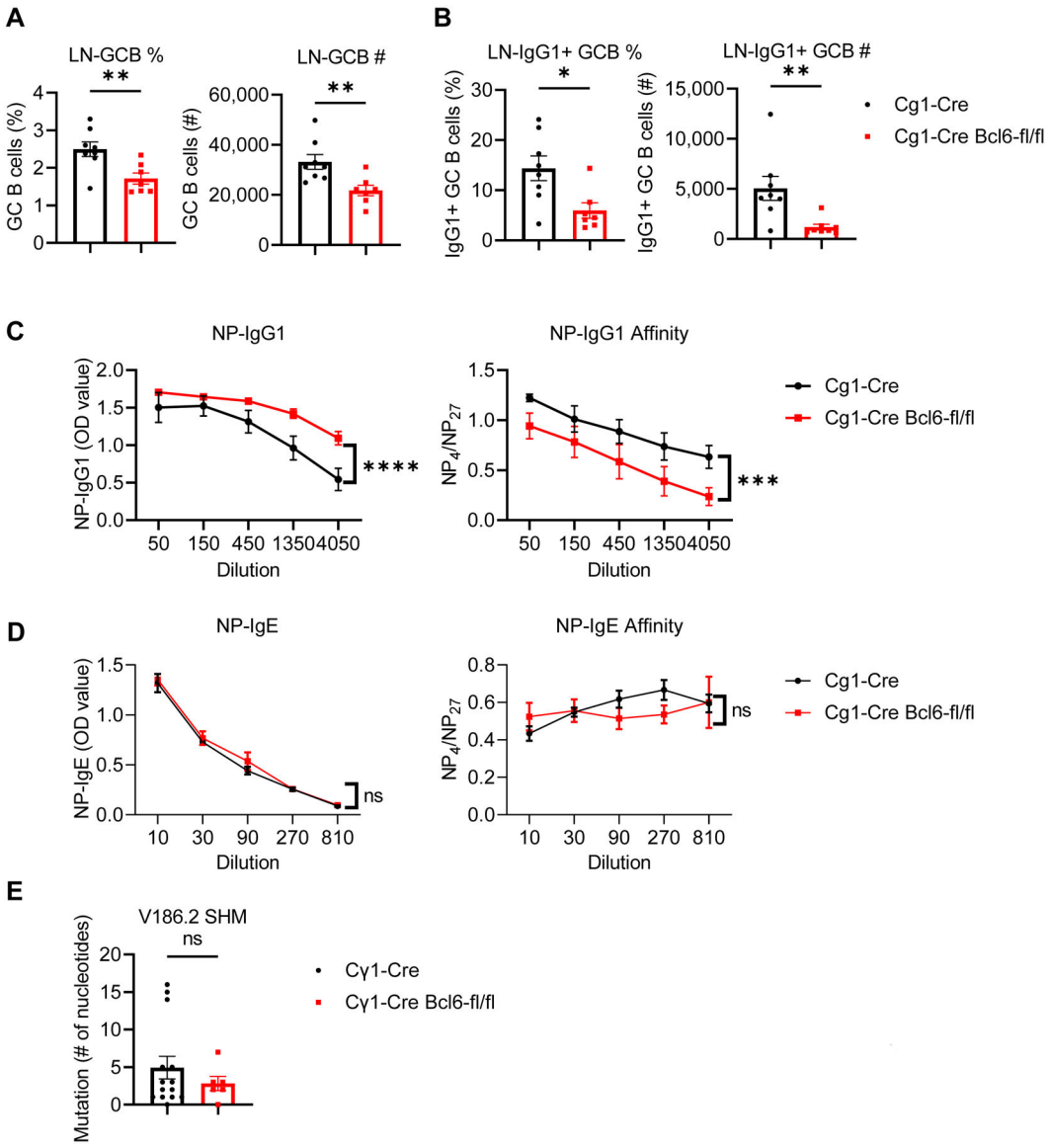


**Figure 5.**

Affinity of IgG1 but not IgE is decreased by loss of Cγ1-expressing germinal center B cells in food allergy. Cγ1-Cre and Cγ1-Cre Bcl6-fl/fl mice were sensitized with $NP_{16}$-OVA plus CT 4 times, one week apart and analyzed at day 29 (one week after the last sensitization). (A) Percent and cell number of GC B cells analyzed in mLN in sensitized mice. n = 7–8. (B) Percent and cell number of IgG1+ GC B cells were analyzed in mLN in sensitized mice. n = 7–8. (C) Titer and affinity of IgG1 were tested in sera. n = 7–8. (D) Titer and affinity of NP-specific (NP-) IgE were tested in sera. Overall titer was assessed by ELISA against $NP_{27}$ which is bound by both low affinity and high affinity NP-specific Abs. Affinity is represented as the ratio of $NP_4$ to $NP_{27}$ binding over different dilutions measured by ELISA, where $NP_4$ binding represents high affinity. n = 7–8. (E) Somatic hypermutation frequency in V186.2 gene rearrangements in control versus Cγ1-Cre Bcl6-fl/fl mice. Cγ1-Cre and Cγ1-Cre Bcl6-fl/fl mice were sensitized with $NP_{16}$-OVA plus CT. GC B cells were isolated

from mLN, RNA was extracted and cDNAs was synthesized. PCR products amplified with primers specific for V186.2 and Cε were cloned into plasmids. Independent clones were then picked for sequencing and analysis. For control Cγ1-Cre mice, 14 unique clones were obtained from 6 separate mice. For Cγ1-Cre Bcl6-fl/fl mice, 6 unique clones were obtained from 3 separate mice. (A-D) Data were representative of four independent experiments. *, $P < 0.05$; ** $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ by t-test (A, B, E) or two-way ANOVA (C, D). ns, not significant.

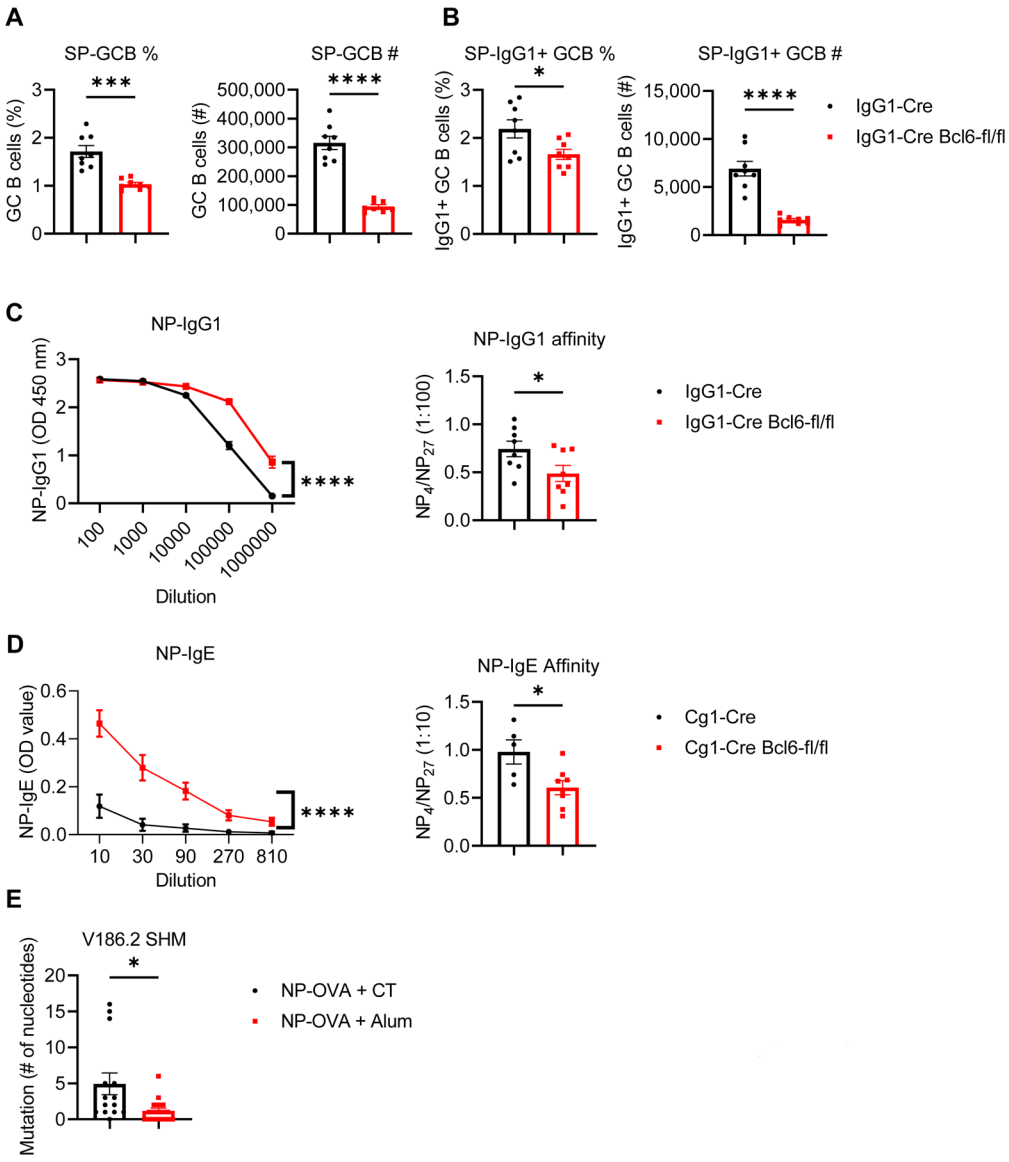


**Figure 6.**

Affinity of IgG1 and IgE is affected by loss of IgG1-expressing germinal center B cells after systemic immunization. Cγ1-Cre and Cγ1-Cre Bcl6-fl/fl mice were immunized with $NP_{16}$-Ova plus Alum (i.p.) twice, at day 1 and 8 and analyzed on day 15. (A) Percent and cell number of GC B cells were analyzed in spleen in immunized mice. n = 8. (B) Percent and cell number of IgG1+ GC B cells were analyzed in spleen in immunized mice. n = 8. (C-D) Titer and affinity of IgG1 (C) and IgE (D) in sera was measured. Overall titer was assessed by ELISA against $NP_{27}$ which is bound by both low affinity and high affinity NP-specific Abs. Affinity was represented as the ratio of $NP_4$ to $NP_{27}$ binding over different dilutions measured by ELISA, where $NP_4$ binding represents high affinity. n = 8. (E) Somatic hypermutation frequency in V186.2 gene rearrangements between $NP_{16}$-OVA + CT (i.g.) model and $NP_{16}$-OVA + Alum (i.p.) model. WT mice were sensitized with $NP_{16}$-OVA + CT (i.g.) or immunized with $NP_{16}$-OVA + Alum (i.p.). GC B cells were isolated

from mLN, RNA was extracted and cDNAs was synthesized. PCR products amplified with primers specific for V186.2 and Cε were cloned into plasmids. Independent clones were then picked for sequencing and analysis. For NP-OVA + CT, 14 unique clones were obtained from 6 separate mice (same set of clones as in Figure 5E). For NP-OVA + Alum, 17 unique clones were obtained from 3 separate mice. (A-D) Data were representative of four independent experiments. *, $P < 0.05$; *** $P < 0.001$; ****, $P < 0.0001$ by t-test (A-D) or two-way ANOVA (C, D). ns, not significant.

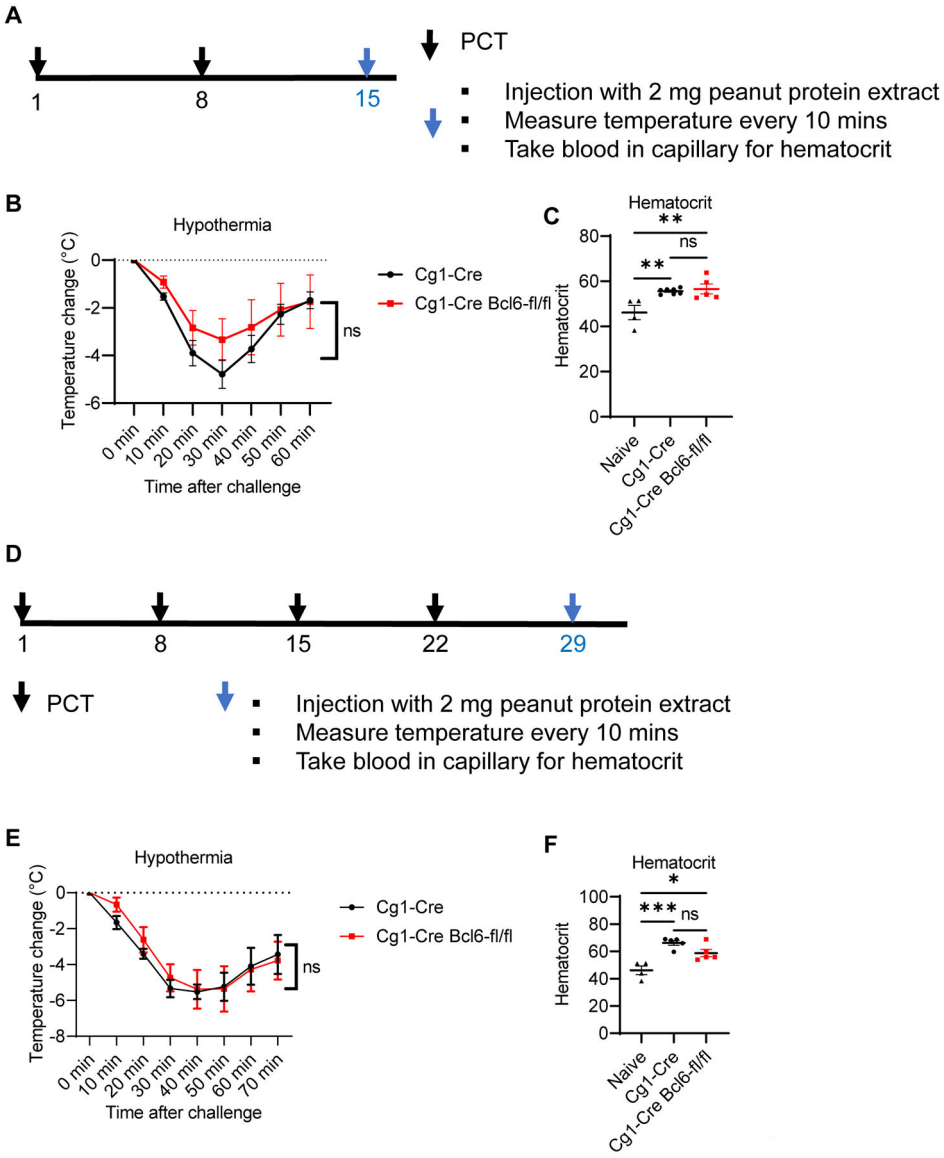


**Figure 7.**

Anaphylactic IgE can be produced without an IgG1+ stage of germinal center B cells. (A) Cγ1-Cre and Cγ1-Cre Bcl6-fl/fl mice were sensitized with PCT twice at day 1 and day 8. At day 15, these mice were injected with 2 mg peanut extract (i.p.). (B) Hypothermia (core boy temperature drop) was monitored for 60 minutes. n = 5–7. (C) Hematocrit values were measured in blood after anaphylaxis analysis. Naïve mice were used as control. n = 4–7. (D) Cγ1-Cre and Cγ1-Cre Bcl6-fl/fl mice were sensitized with PCT for four times. At day 29, these mice were injected with 2 mg peanut extract (i.p.). (E) Hypothermia (core boy temperature drop) was monitored for 70 minutes. n = 5. (F) Hematocrit values were measured in blood after anaphylaxis analysis. Naïve mice were used as control. n = 4–5. Data were representative of two independent experiments. *, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$ by two-way ANOVA (B, E) or one-way ANOVA (C, F). ns, not significant.

**Table 1.**

Oligonucleotide primers used in this study.

| Oligo primers | Sequences |
|---|---|
| Sμ-F-1 (1st PCR) | 5'-TTGGGGAAGGGAAAATAAA-3' |
| Sε-R-1 (1st PCR) | 5'-TAGGGCTGTTGGTCATAGAT-3' |
| Sμ-F-2 (2nd PCR) | 5'-CACTAGGTAAACTTGTAGCTGTGG-3' |
| Sε-R-2 (2nd PCR) | 5'-TTCTGGGTCTTGTCTTTATTCACT-3' |
| Cre-F | 5'-CCTGTTTTGCACGTTCACCG-3' |
| Cre-R | 5'-ATGCTTCTGTCCGTTTGCCG-3' |
| B2M-F | 5'-AGACTGATACATACGCCTGCAG-3' |
| B2M-R | 5'-GCAGGTTCAAATGAATCTTCAG-3' |
| V186.2-leader | 5'-AGCTGTATCATGCTCTTCTTGGCA-3' |
| V186.2-nested | 5'-CATGCTCTTCTTGGCAGCAACAG-3' |
| Cε-cDNA | 5'-ACCGAGGGCAGGGAAGTTC-3' |
| Cε-PCR | 5'-CAGTGCTCATGTTCAGGGAG-3' |

**Table 2.**

Sμ-Sε switch region segments.

| Experiment | # Unique Clones | # Clones with any Sγ1 Remnant (%) | # Clones with repeated 122 bp Sγ1 Remnant (%) |
|---|---|---|---|
| Ova/Alum #1 | 21 | 2 | 1 |
| Ova/Alum #2 | 5 | 0 | 0 |
| Ova/Alum #3 | 2 | 1 | 1 |
| All Ova/Alum | 28 | 3 (11) | 2 (7) |
| PCT #1 | 7 | 1 | 1 |
| PCT #2 | 2 | 1 | 1 |
| PCT #3 | 4 | 1 | 1 |
| PCT #4 | 5 | 0 | 0 |
| All PCT | 18 | 3 (17) | 3 (17) |

65 PCR amplified switch region clones were picked for sequencing and alignment with the mouse genome. Sequences of these clones were analyzed using NCBI Blast Genomic + transcript databases. Numbers of clones representing unique joins for each experiment and the frequency of Sγ1 remnants are shown. Supplemental Material for more detailed clone and genomic alignment information.