Allergy

WILEY

Allergy EUROPEAN JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY EAACI

ORIGINAL ARTICLE OPEN ACCESS

Basic and Translational Allergy Immunology

Allergic Reactivity and Memory Occur Independently of Sequential Switching Through IgG1

Joshua F. E. Koenig[1,2] | Adam K. Wade-Vallance[1,2] | Rodrigo Jiménez-Saiz[1,2,3,4,5] | Kelly Bruton[1,2] | Siyon Gadkar[1,2] | Emily Grydziuszko[1,2] | Tina D. Walker[1,2] | Melissa E. Gordon[1,2] | Amy E. Gillgrass[2] | Justin J. Taylor[6,7,8] | Susan Waserman[1] | Manel Jordana[1,2]

[1]Schroeder Allergy and Immunology Research Institute, Department of Medicine, Faculty of Health Sciences, McMaster University, Hamilton, Canada | [2]McMaster Immunology Research Centre, Department of Medicine, Faculty of Health Sciences, McMaster University, Hamilton, Canada | [3]Department of Immunology, Instituto de Investigación Sanitaria Hospital Universitario de La Princesa (IIS-Princesa), Universidad Autónoma de Madrid (UAM), Madrid, Spain | [4]Department of Immunology and Oncology, Centro Nacional de Biotecnología (CNB)-CSIC, Madrid, Spain | [5]Faculty of Experimental Sciences, Universidad Francisco de Vitoria (UFV), Madrid, Spain | [6]Beirne B. Carter Immunology Center, University of Virginia, Charlottesville, Virginia, USA | [7]Division of Infectious Diseases & International Health, Department of Medicine, University of Virginia, Charlottesville, Virginia, USA | [8]Department of Microbiology, Immunology, and Cancer Biology, University of Virginia, Charlottesville, Virginia, USA

Correspondence: Joshua F. E. Koenig (koenigjf@mcmaster.ca) | Manel Jordana (jordanam@mcmaster.ca)

Received: 13 August 2024 | **Revised:** 15 November 2024 | **Accepted:** 6 December 2024

Funding: This work was supported by Schroeder Foundation (J.F.E.K., S.W., and M.J.), Food Allergy Canada (J.F.E.K., S.W., and M.J.), ALK Abello A/S (J.F.E.K. and M.J.), Zych Family (S.W. and M.J.), Satov Family (S.W. and M.J.), Canadian Allergy Asthma and Immunology Foundation (M.J. and J.F.E.K.), Canadian Society of Allergy and Clinical Immunology (M.J.), Canadian Institutes of Health Research (J.F.E.K.), Canadian Institutes of Health Research Doctoral Foreign Study Award DFD-17076 (A.K.W.V.). European Social Fund and Instituto de Salud Carlos III through a Miguel Servet grant CP20/00043 (R.J.S.). Canadian Institutes of Health Research Doctoral Research Award & Fellowship (K.B.).

Keywords: IgE | IgG1 | memory | sensitization | sequential switching

ABSTRACT

Allergic reactions to foods are primarily driven by allergen-binding immunoglobulin (Ig)E antibodies. IgE-expressing cells can be generated through direct switching from IgM to IgE or a sequential class switching pathway where activated B cells first switch to an intermediary isotype, most frequently IgG1, and then to IgE. It has been proposed that sequential class switch recombination is involved in augmenting the severity of allergic reactions, generating high affinity IgE, differentiation of IgE plasma cells, and in holding the memory of IgE responses. We directly tested these possibilities by comparing the allergic immunity of wild-type and IgG1-deficient (hMT) mice. We found that sequential switching through IgG1 was not required to maintain the binding capacity of IgE nor for its ability to promote degranulation and elicit anaphylaxis against *bona fide* food allergens. Furthermore, the absence of sequential switching modestly impacted IgE affinity and clinical reactivity against hapten antigens, suggesting that the nature of the antigen impacts the requirement for sequential switching. At a cellular level, the capacity to undergo sequential switching through IgG1 provided no competitive advantage for subsequent IgE expression among germinal center B cells or plasma cells. Furthermore, the recall of allergic immunity at memory timepoints was preserved in the absence of sequential switching through IgG1, a finding that corresponded with intact type 2 memory B cell polarization. Together, these data demonstrate that sequential switching through IgG1 is redundant in sensitization, anaphylaxis, and the persistence

Abbreviations: BSA, bovine serum albumin; CSR, class switch recombination; GC, germinal center; MBC, memory B cell; NP, 4-hydroxy-3-nitrophenyl; OVA, ovalbumin; PC, plasma cell; RBL, rat basophil leukemia-2H3; RT, room temperature; WT, wildtype.

Joshua F. E. Koenig, Adam K. Wade-Vallance, and Rodrigo Jiménez-Saiz contributed equally to this work.

of allergy, ultimately revealing that IgE derived from any switching source should be targeted by novel therapeutics seeking to ameliorate allergic diseases.

1 | Introduction

Food allergy is an immunological disorder associated with a significant health, quality of life, and financial burden [1, 2]. The production and persistence of allergen-specific IgE determines allergic reactivity to foods. Therefore, understanding the requirements for IgE production of sufficient binding capacity to trigger allergic reactivity is critical for the development of preventative and curative treatments in food allergy.

The classical pathway for B cells to attain high affinity and longevity is through the germinal center (GC) [3–6]. B cells augment their affinity for antigen in the GC through iterative rounds of *Ig* gene mutation and positive selection. These positively selected GC B cells are a critical source of long-lived cells, including memory B cells (MBCs) and plasma cells (PCs). Early work which imaged IgE-expressing cells in mice argued that IgE B cells are absent from the GC [5]. Later work revealed that IgE B cells constitute a rare fraction of the early GC but are rapidly eliminated as the reaction progresses [3, 7]. Congruent with these findings, IgE B cells are severely or even completely constrained in MBC and long-lived PC differentiation [3, 8]. These observations contrast with lifelong reactivity in most patients allergic to peanuts, tree nuts, fish, and shellfish, as well as with the detection of high affinity IgE clones in some allergic patients [9].

A suggested resolution for these discordant observations is the sequential switching hypothesis, which argues that IgE-secreting cells emerge from B cells that previously underwent class switch recombination (CSR) to a non-IgE isotype, usually IgG1. This hypothesis originated from early work which found that remnants of the IgG1 switch region were present in the genomic switch regions of some IgE-expressing B cells stimulated in vitro or from parasitized mice [10, 11]. This pathway purportedly overcomes the aforementioned limitations of IgE B cells: IgG1-expressing B cells can gain high affinity mutations in the GC prior to sequential CSR to IgE, and IgG1 MBCs can undergo sequential CSR to IgE upon allergen re-exposure to maintain IgE titers [5, 12, 13].

Despite conflicting evidence, sequential CSR through IgG1 to IgE is commonly believed to be important for IgE production, affinity, and recall responses. As direct comparisons of wild-type (WT) mice versus those unable to undergo CSR to IgG1 revealed similar levels of IgE [14], it was proposed that sequential CSR through IgG1 was required for the generation of high-affinity IgE, rather than for IgE *per se* [5, 15]. This was supported by data from a hapten-based immunization system, where IgG1-deficient (IgG1-def) mice had normal levels of specific IgE, but with impaired binding affinity [15]. Given reported differences in affinity maturation between hapten and complex antigens, it is unclear whether these findings apply to IgE responses against allergens [16]. Genomic evidence of IgG1 (γ1) switch remnants in IgE PCs prompted the suggestion that IgE PCs with longer lifespans are derived from IgG1 precursors [17]. Yet, in peanut-allergic patients avoiding foods, many IgE-bound epitopes were not targeted by IgG1 [18]; suggesting that a cellular IgG intermediate may not exist for those IgE-producing cells. Ultimately, the role of sequential CSR through IgG1 in IgE affinity, function, and recall remains unclear.

To address these gaps, we compared the allergic reactivity of IgG1-def and WT mice in well-established mouse models of food allergy at sensitization and at recall. We found that IgG1-def mice had similar levels and polyclonal affinity of allergen-specific IgE, and similar severity of anaphylaxis upon allergen challenge. However, IgG1-def mice produced lower affinity anti-hapten IgE and exhibited reduced clinical reactivity upon challenge with hapten conjugates, suggesting that different types of antigens have different CSR requirements. Furthermore, IgG1-def and WT mice mounted similarly robust recall IgE responses at memory timepoints that drove severe anaphylaxis upon challenge. IgG1-def mice generated type 2 polarized MBCs (MBC2s) at a similar frequency to WT mice, suggesting that MBC isotype is not the primary determinant of their capacity to contribute to IgE recall responses. Finally, using mixed bone marrow chimeras, we demonstrated that the capacity to undergo sequential CSR through IgG1 provided no advantage for subsequent IgE expression among GC B cells or PCs. Altogether, we find no essential role for sequential CSR through IgG1 in the major mechanisms governing IgE production, function, and recall.

2 | Results

2.1 | Sequential Switching Through IgG1 Is Redundant for IgE-Mediated Reactivity to Food Allergens

To evaluate the contribution of sequential CSR through IgG1 to allergic responses to food allergens, we utilized hMT mice (hereafter referred to as IgG1-def) which lack the IL-4 sensitive elements of the Iγ1 promoter region, thereby preventing CSR to IgG1 [19]. We sensitized WT and IgG1-def mice to the egg allergen and model antigen ovalbumin (OVA) or to peanut (PN), which contains several allergens, using a well-characterized intragastric model of food allergy [20, 21] (Figure 1A). This regimen yields allergen-specific IgG1 and IgE which mediate anaphylactic reactions upon allergen challenge. In mice, anaphylaxis is measured by a drop in core body temperature, increased hematocrit (indicative of vascular leakage), and clinical signs ranging from inner ear scratching to seizure (see Materials and Methods) [20, 21]. IgG1-def mice had an expected absence of serum allergen-specific IgG1 following sensitization with either OVA or PN (Figure S1A). IgG1-def mice also generated fewer allergen-specific Igs (Figure S1B), indicating that the absence of IgG1 was not fully compensated by other isotypes. However, the absence of sequential CSR through IgG1 did not impact the magnitude of allergen-specific IgE production (Figure 1B,C), consistent with previous reports [14, 15]. Upon allergen challenge, IgG1-def and WT mice experienced a similar drop in

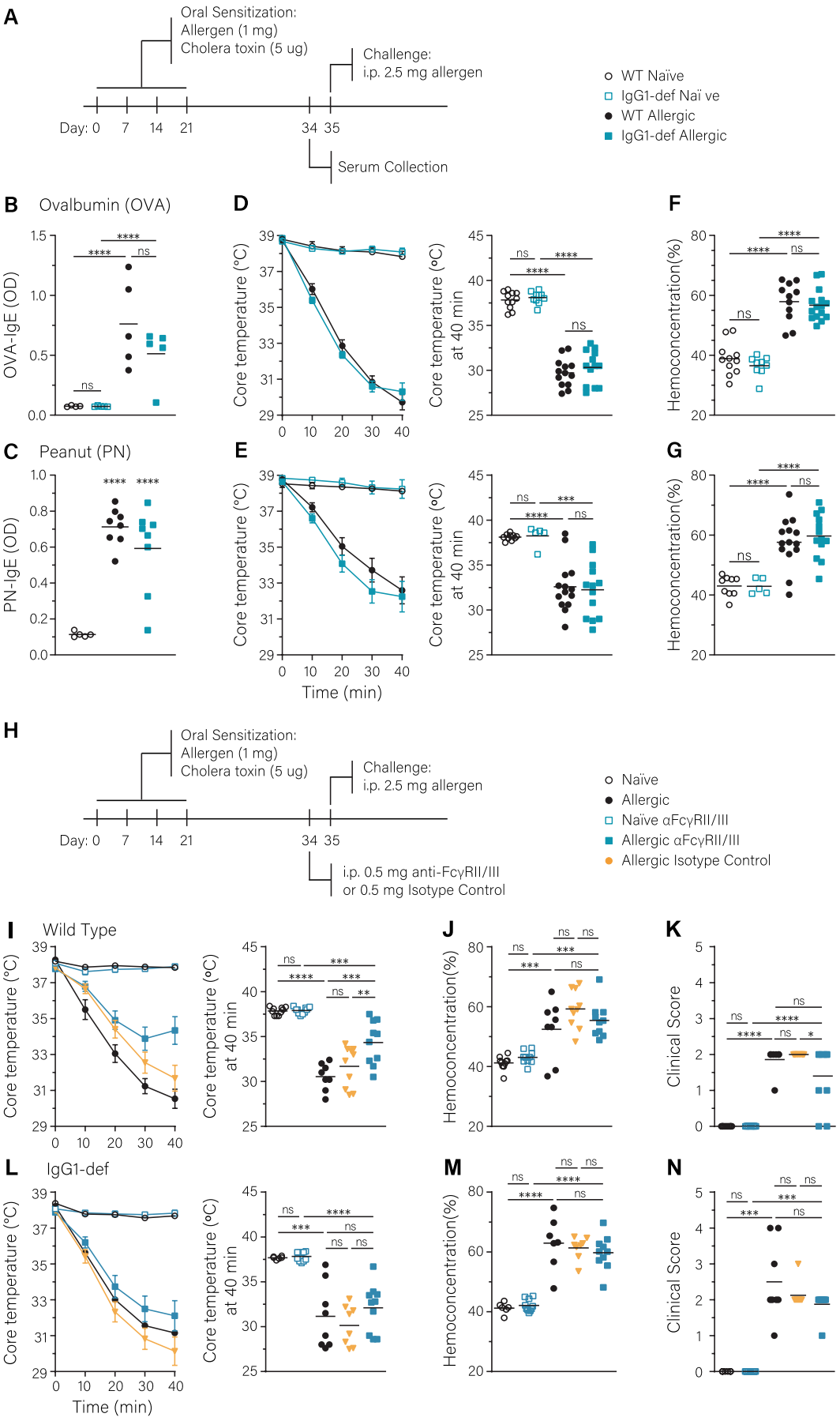

FIGURE 1 | Legend on next page.

FIGURE 1 | Sensitized IgG1-deficient and WT mice have similar IgE-mediated allergic reactivity to foods. (A–G) Wild-type (WT) and IgG1-deficient (IgG1-def) mice were sensitized to either ovalbumin (OVA; top row) or peanut (PN; bottom row) by 4 intragastric exposures of allergen + cholera toxin, as described in Materials and Methods, or were untreated (naïve). (A) Schematic of the experiment. (B and C) Quantification of serum allergen-specific IgE in sera by ELISA at 450 nm. Two weeks following sensitization mice were challenged by intraperitoneal injection of food allergen. (D and E) Depicted are measurements of core temperature over 40 min following challenge by rectal probe (left) with statistical analysis (right) performed at 40 min post-challenge. (F and G) Quantification of hematocrit in blood collected at 40 min post-challenge. (H–N) Naïve and PN-sensitized mice were left untreated or were treated with anti-FcγRII/III or isotype control 1 d prior to challenge. (I and L) Measurement of core temperature change over time following intraperitoneal allergen challenge (left) with statistical analysis at 40 min post-challenge (right). (J and M) Quantification of hematocrit in blood collected at 40 min post-challenge. (K and N) Assessment of clinical symptoms of anaphylaxis in the 40 min following allergen challenge. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ (one-way ANOVA with Tukey's post-test comparing the mean of each group with the mean of every other group). Statistical notations atop bars indicate comparisons between the bridged groups. Statistical notations in (C) indicate comparisons between the indicated group and the naïve control group, whereas notations atop bars indicate comparisons between the bridged groups. Dots represent group means (D, E, I, L) or samples from individual mice or samples from individual mice (all others). Bars within groups represent the mean. Data are representative (B and C) or pooled from two (H–N) or three (A–G) independent experiments.

core body temperature (Figure 1D,E), increase in hematocrit (Figure 1F,G), and clinical signs (Figure S1C). Altogether, these results demonstrate that sequential CSR through IgG1 is dispensable for IgE production and clinical reactivity against food allergens.

The classical pathway of anaphylaxis involves the allergen-mediated cross-linking of IgE bound by FcεRI on the surface of mast cells and basophils, resulting in the release of allergic mediators. However, an "alternative pathway" driven by IgG-allergen immune complexes, which ligate FcγRII/III on the surface of macrophages, can also contribute to anaphylaxis [22]. To confirm that the anaphylaxis experienced by IgG1-def mice was IgE-mediated, we blocked the alternative pathway by the well-established approach of administering an anti-FcγRII/III antibody prior to challenge (Figure 1H) [22, 23]. FcγRII/III-blockade issued a 50% reduction in the level of hypothermia in allergic WT mice following allergen challenge (Figure 1I), consistent with our previous report [22]. We did not detect a difference in hemoconcentration (Figure 1J), though FcγRII/III-blockade did partially protect WT mice against clinical symptoms of anaphylaxis relative to isotype control-treated animals (Figure 1K). Conversely, IgG1-def mice were not significantly protected from anaphylaxis when treated with anti-FcγRII/III (Figure 1L–N), indicating that they underwent IgE-mediated anaphylaxis. Therefore, sequential CSR through IgG1 is not required for IgE-mediated anaphylaxis against food allergens.

2.2 | Sequential Switching Through IgG1 Contributes to IgE-Mediated Reactivity to Haptens

Our observation that IgG1-def mice generated anaphylactic IgE antibodies appears to contrast with a study reporting that sequential CSR through IgG1 was required for high affinity IgE production upon systemic sensitization with the hapten 4-hydroxy-3-nitrophenyl (NP) [15]. We hypothesized that the different routes of sensitization used (systemic vs. intragastric) might explain these discordant results, so we sensitized mice intragastrically with NP conjugated to OVA (NP-OVA). To measure allergic reactivity against NP rather than OVA, we challenged mice with NP conjugated to bovine serum albumin (NP-BSA). Consistent with our previous experiments, IgG1-def mice lacked NP-specific IgG1, had reduced total NP-specific Igs, and had similar production of NP-specific IgE relative to WT animals (Figure S2A–C). We compared the polyclonal binding affinity of NP-IgE between WT and IgG1-def mice by calculating the ratio of serum IgE binding to lowly versus highly conjugated NP-BSA [24]. IgE affinity was significantly reduced in hapten-sensitized IgG1-def mice compared to WT, as had been previously described in the systemic immunization model (Figure S2C,D). Concordantly, IgG1-def mice were partially protected from hypothermia and vascular leakage and exhibited less severe clinical signs compared to WT mice (Figure S2E–G). Together with our earlier results, these findings reveal a fundamental difference in the contribution of sequential CSR through IgG1 to IgE-mediated allergic reactivity to haptens compared to proteins, independent of sensitization route.

2.3 | Sequential Switching Through IgG1 Is Redundant for the Functional Polyclonal Affinity of Food Allergen-Specific IgE

Given the divergent importance of sequential CSR through IgG1 in hapten- and food allergen-mediated anaphylaxis, we next asked whether IgE affinity was impaired against food allergens in the absence of sequential CSR through IgG1. Firstly, we reasoned that potent clinical reactivity driven by high affinity antibodies would be required in the context of limiting allergen challenge doses. Indeed, adjusting the challenge dose from 0.25 mg (Figure 2A) to 2.5 mg (Figure 1B) resulted in dose-dependent changes in the reactivity of WT mice. Across all challenge doses, IgG1-def mice experienced equivalent or greater hypothermia (Figure 2A,B) and hematocrit (Figure 2C) compared to WT mice, suggesting equivalent or greater polyclonal IgE affinity. However, the greater reactivity in IgG1-def mice could also result from their reduced total allergen-specific Ig (Figure S1B) producing a weaker allergen-blocking effect, allowing for increased allergen-IgE crosslinking on effector cells. To isolate the function of IgE, we evaluated the capacity of IgE from IgG1-def and WT mice to induce degranulation in vitro of rat basophil leukemia-2H3 (RBL) cells, a common model for mouse mast cells [25]. RBL cells were incubated with serum from sensitized IgG1-def or WT mice, washed to remove unbound

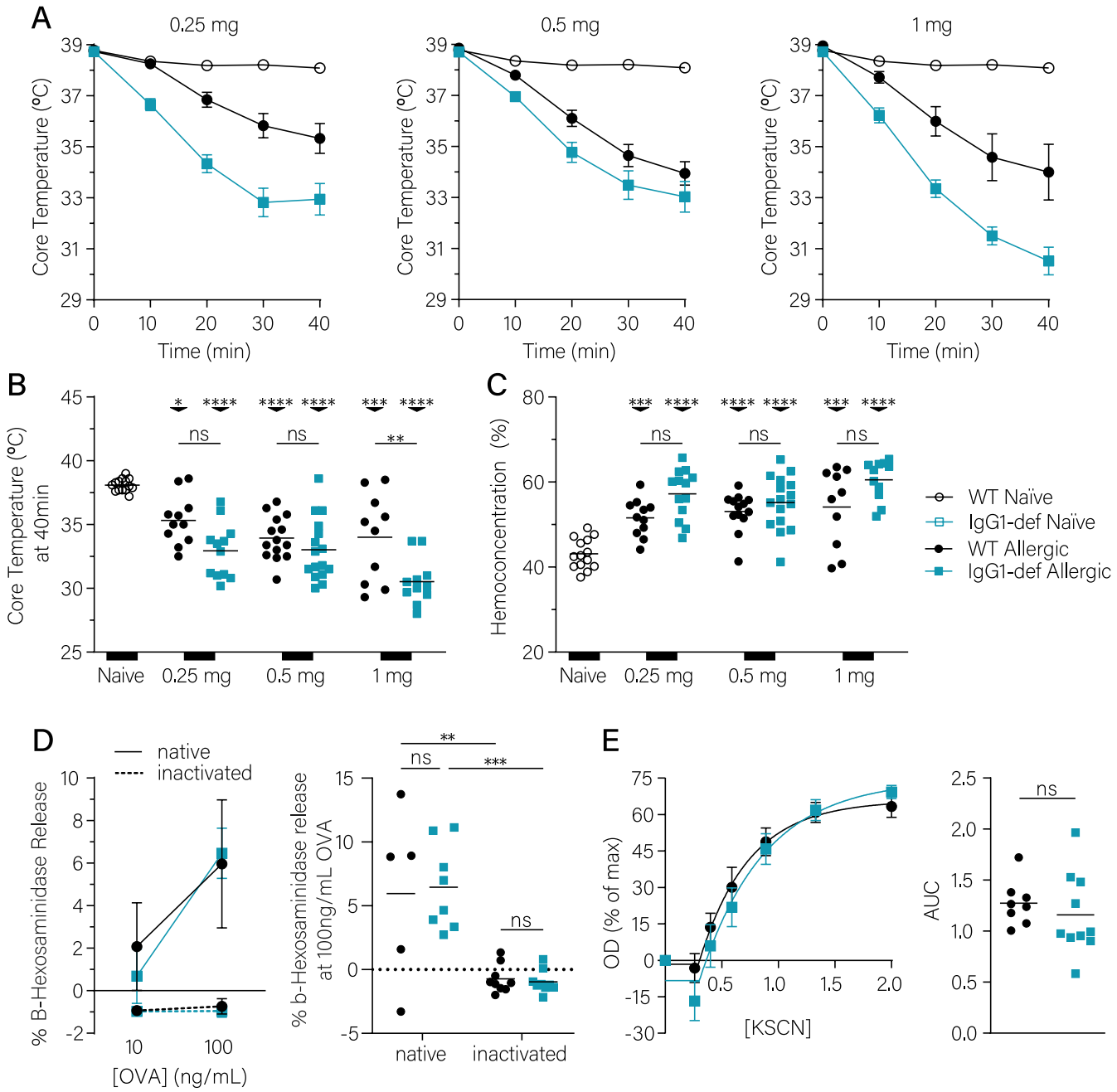

FIGURE 2 | IgG1-def and WT mice generate allergen-specific IgE of similar functional affinity. (A–E) Wild-type (WT) and IgG1-def mice were sensitized to either peanut (PN) or ovalbumin (OVA) or were left naïve. (A) Measurement of core body temperature over time by rectal probe following PN challenge at one of three doses: 0.25 mg (left), 0.5 mg (middle), and 1 mg (right). (B) Statistical analysis of data presented in Panel A at the 40 min post-challenge. (C) Quantification of hemoconcentration in blood collected at 40 min post-challenge. (D) β-hexosaminidase release from RBL-2H3 cells challenged with OVA after incubation with serum from sensitized mice. Statistical analysis for the 100 ng/mL dose is shown in the right plot. (E) Average curve of the % reduction in OVA-IgE ELISA signal as a function of increasing KSCN concentration (left), and quantification of the area under the curve (AUC) for individual samples (right). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ (one-way ANOVA with Tukey's post-test comparing the mean of each group with the mean of every other group [A–C]). Dots represent the group mean (A, left panels of D and E), readings from individual mice (B) or measurements of samples from individual mice (C, right panels of D and E). Statistical notations accompanied by downward arrows indicate comparisons between the indicated group and the naïve control group, whereas notations atop bars indicate comparisons between the bridged groups. Bars within groups represent the mean. Data are pooled from (A–C) or representative of (D–E) three independent experiments.

antibody, and, then, challenged with allergen. RBL cells sensitized with serum from IgG1-def and WT mice degranulated to an equivalent extent when incubated with allergen (Figure 2D). To confirm that RBL degranulation was IgE-mediated, we heat-inactivated the serum at 56°C prior to incubation with the RBL cells. Unlike other antibody isotypes, IgE is irreversibly denatured at 56°C due to a unique heat-labile domain [26, 27]. Following heat-inactivation, RBL cells did not degranulate when

sensitized with either IgG1-def or WT serum, confirming that the reactivity seen in this system was IgE-mediated (Figure 2D). Altogether, these results support that IgG1-def and WT mice produced IgE of similar polyclonal affinity.

To strengthen this conclusion, we directly compared the allergen-binding capacity of allergen-specific IgE from IgG1-def mice and WT mice using an affinity-dependent ELISA technique. Briefly, we adapted our allergen-specific IgE ELISA to include an elution step using the chaotrope thiocyanate which interrupts the allergen-IgE interaction in a dose-dependent manner. High-affinity antibodies are eluted at higher concentrations of chaotrope, allowing polyclonal binding affinity to be evaluated using elution curves [28]. Allergen-specific IgE from IgG1-def and WT mice eluted at similar concentrations of thiocyanate (Figure 2E), indicating that they had similar polyclonal binding affinity for allergen. Cumulatively, these data indicate that IgE produced against food allergens in the absence of sequential CSR through IgG1 has intact functional polyclonal affinity for allergen.

2.4 | GC Isotype Composition Shifts in the Absence of IgG1

Given its central role in the production of affinity-matured B cells, we reasoned that alterations in the GC of IgG1-def mice could explain their intact IgE affinity and reactivity against food allergens. To investigate the allergen-specific GC response, we detected OVA-specific GC B cells (defined as $CD19^+GL7^+CD95^+$) 6 days post-sensitization, which is the previously reported peak of the primary response [29] in the mesenteric lymph nodes using monomeric OVA-FITC (Figure 3A). IgG1-def mice had a lower proportion and number of OVA^+ GC B cells compared to WT mice (Figure 3B), suggesting incomplete compensation for the absence of IgG1 GC B cells, which constitute ~90% of OVA-specific GC B cells in WT mice (Figure 3C). While the overall GC response of IgG1-def mice was reduced, they exhibited an increased frequency and absolute number of OVA-specific IgE-, IgM-, and IgG3-expressing GC B cells compared to WT mice (Figure 3D–F).

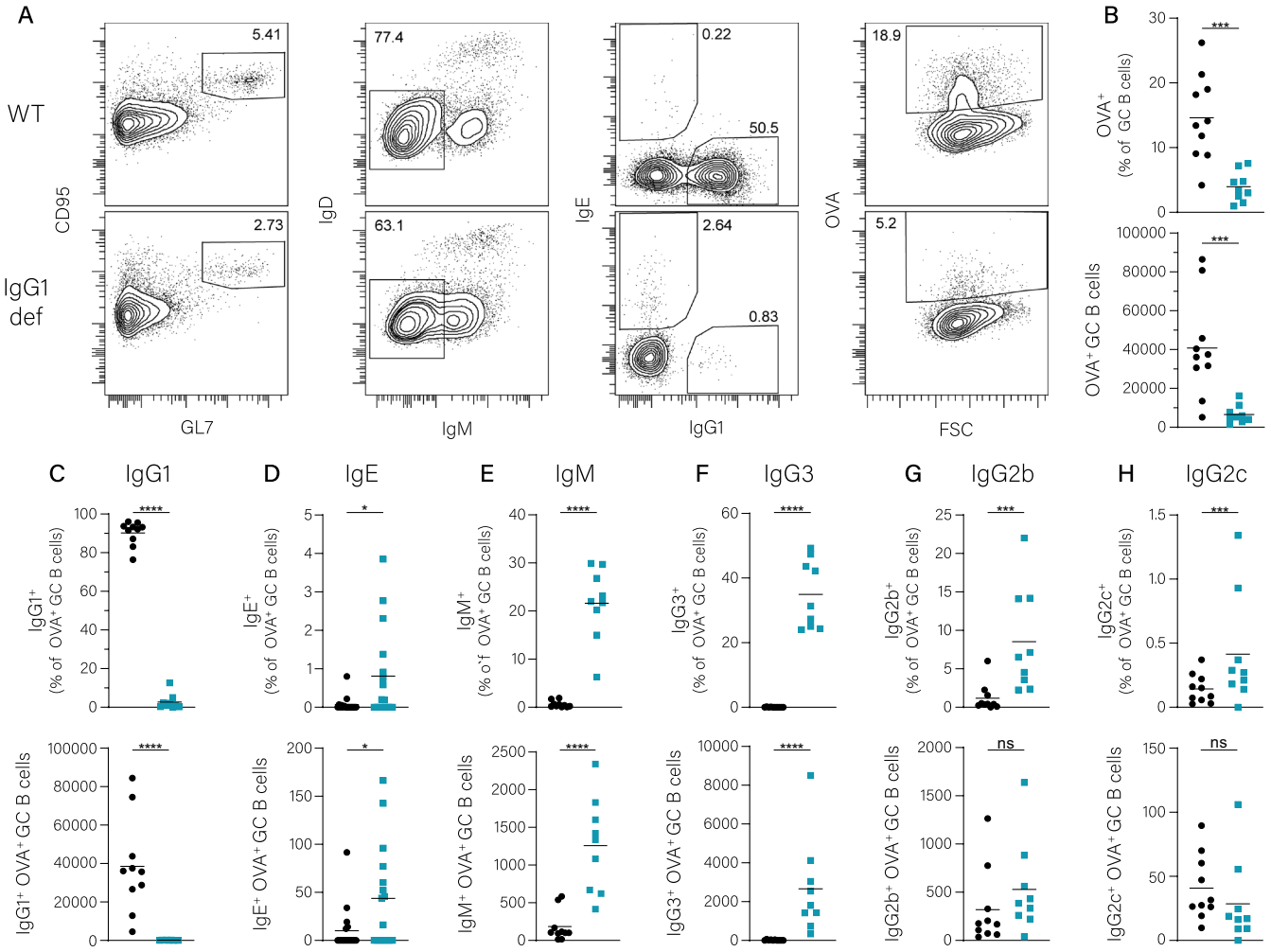

FIGURE 3 | Isotype compensation in the IgG1-def GC. (A–H) IgG1-def and wild-type (WT) mice were sensitized to ovalbumin (OVA) and the germinal center (GC) response in the mesenteric lymph nodes were assessed by flow cytometry 6 days later. (A) Representative contour plots depicting the hierarchical gating strategy used to identify allergen-specific GC B cells in WT (top) and IgG1-def (bottom) mice. Cells were pre-gated as $CD19^+$ live singlets, then as $CD95^+$ $GL7^+$ GC B cells, IgM^-IgD^-, by isotype (shown for IgE and IgG1), and as allergen binding using a monomeric OVA-FITC probe. (B–H) The proportion (top) and total counts (bottom) of total allergen-specific GC B cells (B) or of allergen-specific GC B cells of the indicated isotype (C–H). $^*p < 0.05$; $^{***}p < 0.001$; $^{****}p < 0.0001$ (unpaired t test). Dots represent samples from individual mice and bars within groups represent the mean. Data are representative of (A) or pooled from (B–H) two (E–H) or three (B–D) independent experiments.

They also had a comparable or slightly elevated frequency and number of OVA-specific IgG2b- and IgG2c-expressing GC B cells (Figure 3G,H). Therefore, IgG1 CSR constrains the participation of other isotypes in the GC, especially IgE, IgM, and IgG3, which might otherwise serve as intermediates for the subsequent differentiation of IgE-secreting cells.

2.5 | Sequential CSR Through IgG1 Provides no Intrinsic Advantage for IgE GC B Cell or PC Formation

As our data indicated sequential switching through IgG1 was not a requirement to generate anaphylactic IgE, we next questioned whether the capacity to undergo sequential CSR through IgG1 provided any intrinsic advantage for subsequent IgE expression as a GC B cell or PC. Toward this end, we generated chimeras using a 50:50 mixture of bone marrow from IgG1-def (CD45.2) and WT (CD45.1) mice. After bone marrow reconstitution, chimeras were intragastrically sensitized to OVA. We reasoned that, if a B cell's ability to undergo sequential CSR through IgG1 provided an advantage to become an IgE GC B cell and/or PC, then the WT bone marrow would be overrepresented in these compartments. Following immunization, the naïve B cell populations in WT:WT and WT:IgG1-def chimeras were evenly distributed between CD45.1 and CD45.2 marrow (Figure 4A). In WT:IgG1-def chimeras, IgG1 GC B cells and PCs were derived exclusively from the WT bone marrow, as expected (Figure 4B–D, Figure 3A). However, the majority of IgE and IgM GC B cells, as well as the majority of IgM PCs, were derived from the IgG1-def bone marrow, while the IgE PC compartment was evenly divided (Figure 4C,D). These data indicate that, relative to wild-type cells, B cells unable to undergo IgG1 CSR have a competitive advantage at forming IgE GC B cells and are equivalently effective at forming IgE PCs. To understand the differences in switch preferences of wild-type versus IgG1-def cells that might drive this effect, we analyzed the intrinsic CSR patterns of OVA$^+$ GC B cells and PCs derived from wild-type versus IgG1-def bone marrow (Figure 4E). This analysis revealed that IgG1-def cells expressed IgE and IgM at a higher rate than WT cells, consistent with our observations in non-chimeric animals (Figure 4E, Figure 3D,E). Strikingly, in some chimeras up to 25% of IgG1-def OVA$^+$ PCs expressed IgE (Figure 4E). These data demonstrate that the capacity to undergo sequential switching through IgG1 does not provide a competitive advantage to become an IgE-expressing cell and may even reduce the likelihood of subsequent IgE expression.

2.6 | IgG1 Expression Provides a Competitive Advantage for Representation in the GC B Cell and PC Compartments

This bone marrow chimera system also provided the opportunity to examine whether the dominance of IgG1 among OVA-specific GC B cells and PCs in non-chimeric mice (Figures 3C and 4E) was reflective of greater competitive fitness. As in non-chimeric mice, IgG1-switched cells made up 90% of WT OVA$^+$ GC B cells and the large majority of OVA$^+$ PCs in both WT:WT and WT:IgG1-def chimeras (Figure 4F). In control WT:WT chimeras, OVA-specific PCs and GC B cells were equally derived from CD45.1 and CD45.2 marrow, indicating the absence of intrinsic bias in favor of either strain (Figure 4G,H). However, in the WT:IgG1-def chimeras, the large majority of OVA-specific GC B cells and PCs were derived from the WT bone marrow (Figure 4G,H). Further illustrating this bias, > 75% of all OVA$^+$ GC B cells in both WT:WT and WT:IgG1-def bone marrow chimeras expressed IgG1 (Figure 4I). Together these data indicate that cells capable of expressing IgG1 have a competitive advantage to enter or be retained in the GC and PC compartments.

2.7 | Type 2 Polarization of Memory B Cells Does Not Rely on IgG1 Isotype Expression

Multiple reports in both mice and humans have implicated sequential switching of IgG1$^+$ MBCs to IgE in maintaining persistent titers of allergen-specific IgE [12, 29–32]. We and others have recently described a novel phenotype of type 2 polarized MBCs (MBC2), identified based on high expression of IL-4Rα and CD23, that holds the memory of allergen-specific IgE responses [31–33]. The MBC2 population is enriched in IgG1-expressing MBCs, which is putatively consistent with the proposed importance IgG1 MBCs in holding the memory of IgE responses. However, it has not been assessed whether IgG1 expression is a necessary feature of the MBC2 program, nor whether sequential CSR through IgG1 is necessary for IgE recall responses.

To evaluate the role of IgG1 in IgE recall responses, we first measured the frequency and number of allergen-specific MBCs in IgG1-def and WT mice at one-month post-sensitization. Due to the rarity of allergen-specific MBCs at this timepoint, we used a validated antigen-tetramer enrichment approach to detect and enumerate these cells [34]. Allergen-specific B cells were considered those which bound an OVA-APC tetramer, but not a tetramer loaded with an irrelevant protein, which ensured the exclusion of cells that were specific for the tetramer backbone. This strategy revealed that the number of OVA-specific MBCs was greater in WT mice than IgG1-def mice (Figure 5A,B), consistent with their larger GC responses (Figure 3B). IgG1 dominated the WT OVA-specific MBC compartment, comprising > 70% of OVA-specific MBCs in most mice (Figure 5C,D). IgG1-expressing MBCs were expectedly absent in IgG1-def mice (Figure 5C,D). Further mirroring our results in the GC, OVA-specific MBCs from IgG1-def mice contained a higher frequency and number of IgG3$^+$ cells (Figure 5C,E). While the proportions of IgG2b and IgG2c MBCs were slightly elevated in IgG1-def mice, their numbers were comparable to WT mice (Figure 5F–H). Despite the difference in number and isotype, a similar proportion of the OVA-specific MBC compartment in allergic WT and IgG1-def mice expressed MBC2 markers (Figure 5I,J). A large proportion of IgG1-def MBC2s expressed IgG3, consistent with the overall increase in IgG3 in these mice (Figure 5K,L). These data indicate that IgG1 expression is not required for MBC2 polarization.

2.8 | Sequential Switching Through IgG1 Is Not Required for IgE Recall Responses

We next sought to determine whether the generation of allergen-specific IgE during a recall response was impaired in the

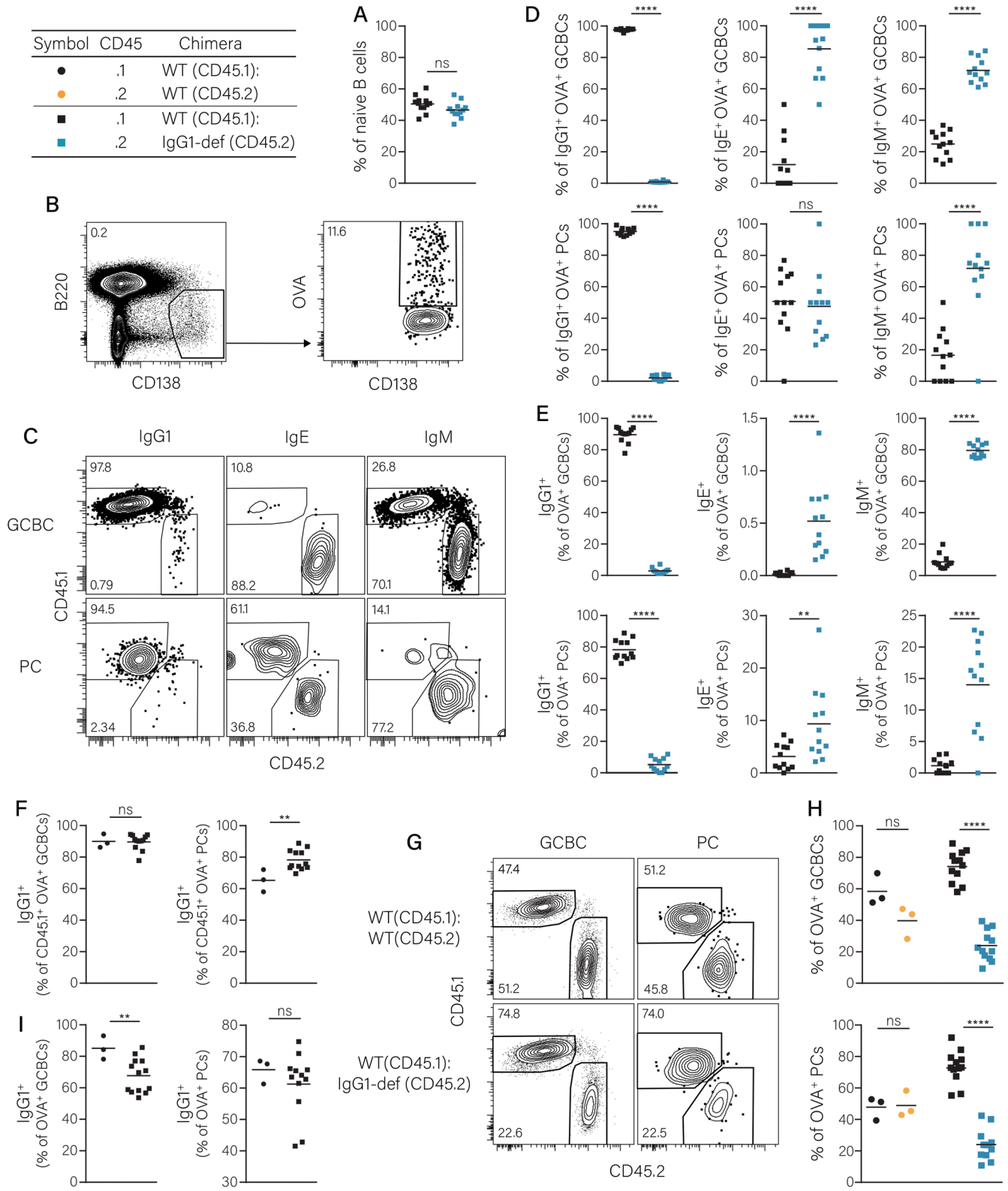

FIGURE 4 | Legend on next page.

absence of sequential switching through IgG1. We sensitized IgG1-def and WT mice and waited until clinical reactivity waned at 10 months post-sensitization (Figure 6A). We have previously reported that oral exposure to allergen without adjuvant at this timepoint results in a robust secondary response that replenishes allergen-specific IgE titers and allergic reactivity [29]. Titers of allergen-specific IgE in IgG1-def mice were no longer detectable above background at 20 weeks post-sensitization, while most WT mice had detectable allergen-specific IgE until 45 weeks post-sensitization (Figure 6B). Consistent with our previous report, WT and IgG1-def mice lost the majority of their clinical reactivity after 10 months (Figure 6C). Upon

FIGURE 4 | Sequential switching through IgG1 provides no competitive advantage for subsequent IgE expression. (A–I) Following reconstitution, bone marrow chimeras were intragastrically sensitized to ovalbumin (OVA) as described in Methods, and mesenteric lymph nodes (mesLN) were collected for analysis. (A) Quantification of chimerism as the representation of CD45.1 and CD45.2 cells among naïve B cells in the mesLN in wild-type (WT; CD45.1):IgG1-def (CD45.2) chimeras. (B) Representative flow cytometry gating strategy for OVA$^+$ plasma cells (PCs). (C–D) Concatenated flow cytometry plots (C) and frequency (D) of WT (CD45.1) and IgG1-def (CD45.2) cells among IgG1, IgE, and IgM germinal center (GC) B cells and PCs. (E) Quantification of the fraction of WT (CD45.1; black square) or IgG1-def (CD45.2; blue square) GC B cells or PCs that expressed IgG1, IgE, or IgM. (F) Quantification of the proportion of CD45.1$^+$ OVA$^+$ GC B cells or PCs in WT: WT (black circle) or WT: IgG1-def (black square) chimeras that expressed IgG1. (G,H) Representative flow cytometry plots (G) and quantification (H) of the proportion of CD45.1 and CD45.2 cells within the GC and PC compartments of WT: WT or WT: IgG1-def chimeras. (I) Quantification of the proportion of all OVA$^+$ GC B cells or PCs that expressed IgG1 in WT: WT (black circle) or WT: IgG1-def (black square) chimeras. ns, not significant; $^{**}p < 0.01$; $^{****}p < 0.0001$ (unpaired *t* test). Dots represent samples from individual mice and bars represent the mean.

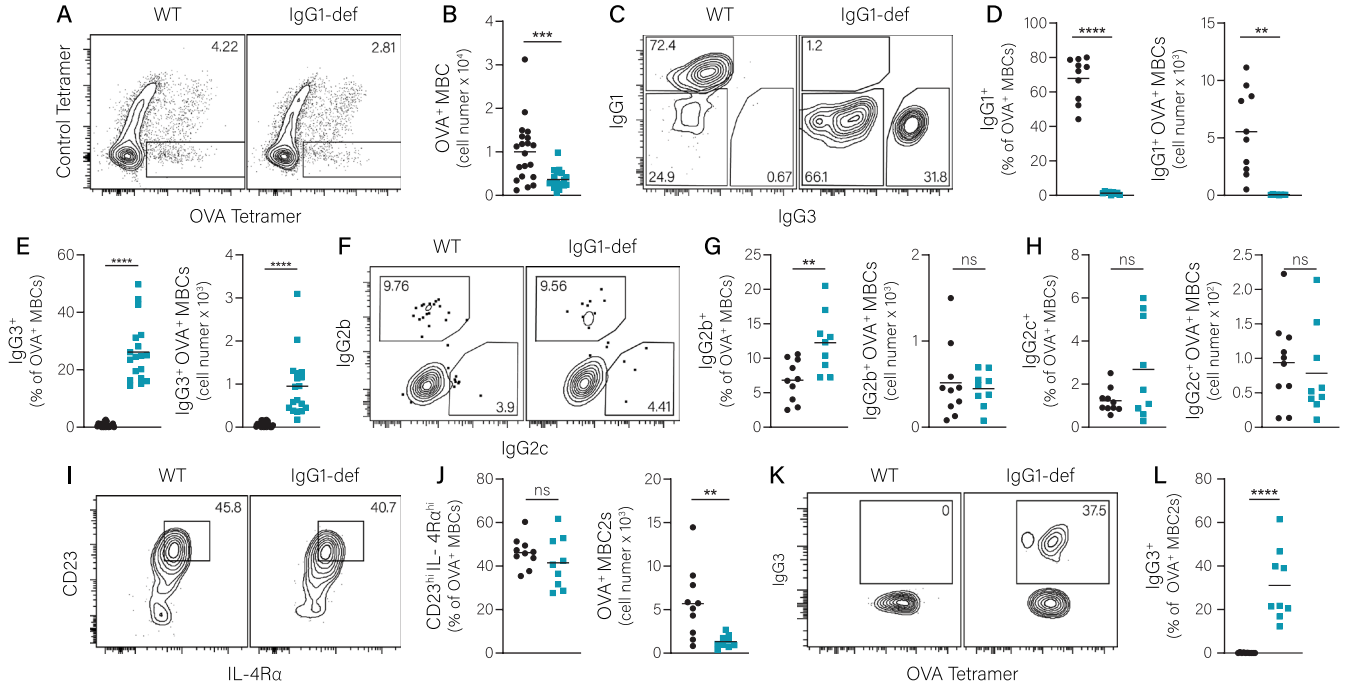

FIGURE 5 | Memory B cells retain type 2 polarization in the absence of IgG1 expression. (A–L) IgG1-def and wild-type (WT) mice were sensitized to ovalbumin (OVA) and memory B cell (MBC) number and phenotype were assessed 1 month later. Spleen and mesenteric lymph node samples were pooled prior to OVA-tetramer enrichment. (A and B) Representative flow cytometry plots and number of OVA-specific isotype switched MBCs. (C–H) Representative flow cytometry plots depicting IgG subtype expression (C and F) and plots summarizing the frequency (left) and number (right) of IgG MBCs (D and E, G and H). Pre-gate: B220$^+$ CD3$^-$ F4/80$^-$ OVA Tetramer$^+$ Control Tetramer$^-$ GL7$^-$ CD38$^+$ IgM$^-$ IgD$^-$. (I and J) Representative flow cytometry plots of CD23 and IL-4Rα expression (I) and summary plots (J) depicting the frequency and number of MBC2s. Pre-gate: B220$^+$ CD3$^-$ F4/80$^-$ OVA Tetramer$^+$ Control Tetramer$^-$ GL7$^-$ CD38$^+$ IgM$^-$ IgD$^-$. (K and L) Representative flow cytometry plots (K) and summary plot of frequency (L) of IgG3 expression among MBC2s. Pre-gate: B220$^+$ CD3$^-$ F4/80$^-$ OVA Tetramer$^+$ Control Tetramer$^-$ GL7$^-$ CD38$^+$ IgM$^-$ IgD$^-$ CD23^{hi} IL-4Rα^{hi}. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ (unpaired *t* test). Dots represent samples from individual mice and bars within groups represent the mean. Data are pooled from two independent experiments.

re-exposure, sensitized IgG1-def and WT mice exhibited a similar regeneration of allergen-specific IgE (Figure 6D). Challenging re-exposed allergic mice with allergen revealed comparable hypothermia (Figure 6C), hematocrit (Figure 6E), and clinical signs (Figure 6F) between IgG1-def and WT mice. These data demonstrate that sequential CSR through IgG1 is not a critical pathway for IgE recall responses nor allergic memory.

3 | Discussion

Here, we have shown that mice incapable of CSR to IgG1 can undergo severe, IgE-mediated allergic reactions against food allergens. Whereas sequential CSR through IgG1 contributes to allergic reactivity and IgE affinity to haptens, it plays no mandatory role in allergic reactivity or IgE affinity to food allergens. At the cellular level, the absence of IgG1 B cells alters GC composition (most notably by increasing IgE, IgM, and IgG3 GC B cells) in a B cell-intrinsic manner, providing a rationale for the equivalent affinity of IgE produced by IgG1-def mice. MBCs are a major output of the GC, and we found changes in the isotype composition of IgG1-def MBCs which mirrored the alterations we observed in the isotype composition of the GC. However, the polarization of IgG1-def MBC2s was intact, providing a pathway for IgE production and reactivity following allergic recall. Overall, our findings indicate that sequential CSR through IgG1

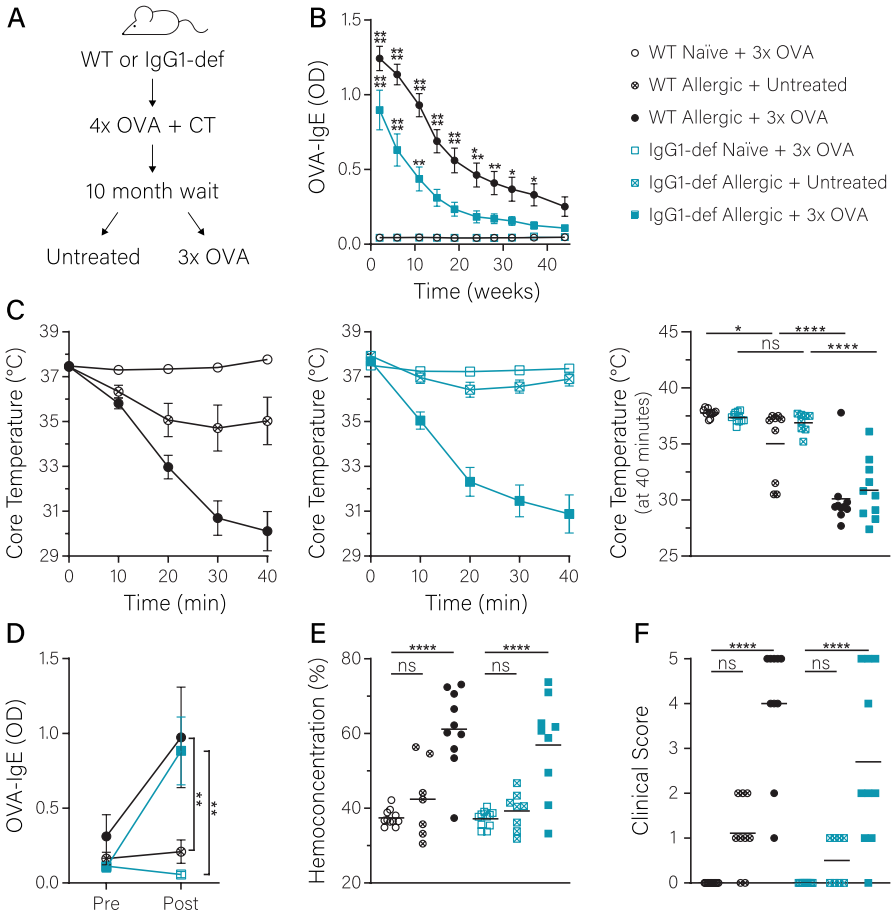

FIGURE 6 | IgG1-def and WT mice have equivalent allergic memory. (A) Schematic depicting the allergic memory model used in panels (B–F). Mice were left naïve (hollow symbols) or sensitized with ovalbumin (OVA) + cholera toxin (CT). Following sensitization, allergic reactivity was allowed to wane over a 10-month period, after which one group of allergic mice was given three oral re-exposures to allergen prior to challenge (solid symbols) whereas the other was not hollow symbols with cross. (B) Quantification of OVA-specific IgE versus weeks following sensitization. Statistical notations indicate comparisons between the below allergic mice and the naïve controls of the same genotype. When notations are not provided the comparisons are ns. (C) Core temperature over time following challenge of wild-type (WT; left) and IgG1-def (center) mice with accompanying statistical analysis (right). (D) Serum OVA-specific IgE measured by ELISA at 450 nm before (Pre) and after (Post) the oral re-exposures at 10 months post-sensitization. Statistical notations indicate comparisons between the bridged groups at the "Post" timepoint. (E) Hematocrit in blood collected at 40 min post-challenge. (F) Clinical signs of anaphylaxis assessed over 40 min following allergen challenge. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ (two-way ANOVA comparing the mean of each group with the mean of every other group [A], one-way ANOVA comparing the mean of each group with the mean of every other group [C, right]), one-way ANOVA comparing the mean of the indicated pairs of groups [D, E, F]. Dots represent the group mean (B, C left and center panels, D), readings from individual mice (C right panel, F) or measurements of samples from individual mice (E). (C–F) Statistical notations atop bars indicate comparisons between the bridged groups. Bars within groups represent the mean. Data are representative of (B, D) or pooled from (C, E, F) two independent experiments.

is redundant for two core features of allergic responses: the production of anaphylactic IgE and allergic recall responses. That expression of IgG1 per se does not inherently pre-dispose to pathogenic IgE production suggests that transformative allergy therapies should target allergen-specific B cells of all isotypes upstream of IgE.

Our observation that IgG1 is not required for IgE-mediated reactivity to food allergens is at variance with classical associations linking IgE and IgG1 responses. One possible explanation is that IgE responses are less dependent on IgG1 than previously appreciated. The original observations that formed the basis for the IgG1 sequential CSR hypothesis were the shared induction of IgG1 and IgE by IL-4 and the frequent identification of γ1 switch remnants in IgE B cells [11, 35, 36]. However, we have since uncovered mechanisms which uniquely restrict IgE and not IgG1 responses (e.g., IL-21) [7, 37, 38], exposing that IgE and IgG1 may be less tightly linked than once thought. Furthermore, it is generally assumed that the rate at which switch remnants are identified represents the minimal possible contribution of IgG1 intermediaries because only a portion of switching events leave these remnants [35]. However, switch remnants can be present on the productively recombined IgH allele or on the allele lacking a recombined VDJ [39], and many published analyses do not discriminate between the two. Further, the recent discovery of a "reverse sequential switching pathway" in which the IgG1 and IgE loci recombine first, prior to recombination the IgE and IgM switch regions, suggests that some fraction of reported γ1 switch remnants do not indicate a true,

cellular, IgG1-expressing intermediate [40]. Therefore, switch remnant-based analyses can both under-estimate and over-estimate IgG1 B cell ancestry and, thus, do not provide conclusive evidence for understanding the CSR history of a cell.

A recent study sought to selectively deplete IgG1 GC B cells to test whether high affinity IgE could be produced in their absence [41]. However, the genetic and experimental systems employed in this study preclude it from addressing this question. Cγ1-cre Bcl6$^{fl/fl}$ mice were used with the intention of restricting GC B cell differentiation in IgG1-expressing cells, however, Cγ1-cre is known to be transcribed in most activated B cells regardless of their isotype; most investigators use the strain to drive cre recombinase expression broadly in GC B cells [42, 43]. In fact, recent work has demonstrated that non-IgG1 MBCs continue to transcribe Cγ1-cre even at memory timepoints, further demonstrating the poor specificity of the allele for the intended purpose [44]. Consistent with the poor specificity of the allele, Chen et al. reported a near-complete ablation of the GC in their model [41], and therefore, their conclusions cannot be applied to sequential CSR through IgG1. While Cγ1-cre Bcl6$^{fl/fl}$ mice did not have impaired high-affinity NP-IgE production, we found an impact on both NP-IgE affinity and clinical reactivity to NP in IgG1-def mice, consistent with previous work [15]. These divergent observations may be explained by the low frequency of IgG1 (< 10%) among GC B cells observed in WT mice in their model, which would limit the opportunity for sequential CSR through IgG1 even without genetic manipulation. In contrast, despite our model inducing a much greater frequency of IgG1 (~90%) among OVA-specific GC B cells in WT mice, we saw no impact on the allergen-specific IgE response and clinical reactivity upon allergen challenge in IgG1-def mice. Our work extends beyond IgE affinity to several other elements of the IgE response where sequential CSR through IgG1 is thought to play a role, like in the recall IgE response and in IgE PC differentiation.

Our results revealed that IgG1-def cells could form IgE GC B cells and PCs and, in some cases, had an advantage relative to WT cells in doing so. We propose two mechanisms for this advantage. First, we hypothesize that IgG1-switched B cells possess an intrinsic competitive advantage over IgE GC B cells. Therefore, in a GC without IgG1 GC B cells, such as is present in IgG1-def mice, relaxed competition may enable greater retention of IgE GC B cells. Such competition has been previously reported; the IgG BCR constant region provides a competitive advantage in the GC relative to that of the IgM BCR even when the variable region is fixed [45]. Further consistent with this notion, both IgM and IgE are lost from IgG1-competent GCs over time and prior direct comparisons of IgG1 and IgE GC B cells revealed that IgG1 cells cycle more quickly and gather more antigen [3, 4, 45]. Evidence presented here also supports this competition hypothesis: IgG1 dominates within the WT GC (Figure 3C) and is overrepresented in the GC of WT:IgG1-def chimeras (Figure 4F). However, relaxed competition cannot fully explain the increased representation of IgE among IgG1-def cells, as even when competition was restored due to the presence of WT cells in WT:IgG1-def chimeras, the IgG1-def cells retained higher rates of IgE expression (Figure 4D,E). These findings, instead, suggest that the ability to switch to IgG1 intrinsically reduces IgE CSR. Our observation that IgG1 and IgM are the most commonly expressed isotypes among allergen-specific WT and IgG1-def cells, respectively, indicates that the most common CSR instruction for an allergen-specific B cell to receive in our model is to switch to IgG1 or to not switch at all. However, the B cell-intrinsic increase in IgE and IgG3 among IgG1-def cells indicates that a subset of B cells is licensed to undergo CSR without being absolutely committed to IgG1 expression. This implies that when the IgG1 switch locus is available for targeting, it dominates, perhaps by preferentially recruiting CSR machinery. However, when the IgG1 switch region is unavailable, some CSR-licensed B cells reroute to switch to a different isotype, such as IgE or, more commonly, IgG3. Whether such IgG3-expressing B cells might then contribute to the IgE pool by sequential class-switching remains unclear, although it was previously reported that, in IgG1-deficient mice, sequential switch remnants were absent from IgE-expressing cells [15]. We propose that these two mechanisms together account for the increased representation of IgE among IgG1-def cells.

Our findings demonstrate the importance of investigating food allergy using *bona fide* food allergens. This is evident in the differential requirement for CSR through IgG1 for robust allergic reactivity against haptens versus allergens. This difference may be explained by the observation that immunization with haptens induces a GC which purifies for affinity-enhancing mutations, whereas complex antigen-directed GCs maintain epitope diversity at the cost of affinity enhancement [16]. In the setting of PN allergy, the epitope diversity of specific IgE is a stronger determinant of reactivity than polyclonal IgE affinity [18, 46–50], findings echoed by a recent mechanistic study of mast cell activation by PN-specific IgE [48] and by the observation that GC-deficient mice generate allergen-specific IgE and are anaphylactic upon challenge [29, 41]. Hapten-based models may amplify the minor contribution of IgE affinity to allergic reactivity because there is only one epitope available for binding, possibly amplifying competition for antigen binding between mast cell-bound IgE and blocking Ig. However, knowledge from hapten experiments may be applicable to some small molecule allergens such as galactose-α-1,3-galactose (α-gal), which is the allergen in mammalian meat allergy that occurs following bites from the lone star tick [51–53]. Subcutaneous administration of α-gal with tick extract induced α-gal-specific IgG1 and IgE and clinical reactivity upon challenge with red meat allergens in mice [54]. Based on our data, the generation of anaphylactogenic α-gal-specific IgE may have a greater dependance on sequential switching through IgG1 than complex food allergens such as peanut or egg.

Importantly, we found that the absence of IgG1 had no impact on allergic recall responses. These observations challenge the widespread notion that IgG1 MBCs act as a critical reservoir for secondary IgE responses [12, 13, 30, 55, 56]. While our data could be interpreted to mean that IgG1 MBCs do not contribute to IgE recall responses in the WT setting, clonal connections in humans and adoptive transfer experiments in mice suggest that they likely do contribute [12, 31, 57, 58]. Since B cells are unable to switch to IgG1 and appear to be diverted to other isotypes in our model, it is possible that depleting established allergen-specific IgG1 MBCs could abort the recall IgE response. In favor of this hypothesis, the observation is that

replacing the extracellular domain of IgG1 with IgE in $IgE^{ki/ki}$ mice results in poor secondary IgE responses to helminths [59], since tonic signaling of IgE BCR restricts MBC longevity [4, 7, 60]. However, B cell receptor repertoire analysis has found that all upstream isotypes are clonally related to IgE in circulation and along the gastrointestinal tract [57, 58, 61], and we have found an overrepresentation of $IgG2^+$ MBCs that are clonally related to IgE PCs during a recall response [31]. These data, instead, strongly support a role of non-IgG1 MBCs in human IgE recall responses. Our approach of preventing IgG1 CSR demonstrates that expression of the IgG1 BCR itself does not improve the ability of an MBC to give rise to IgE during recall. An alternate hypothesis consistent with our findings is that MBC phenotype, rather than isotype, is the primary determinant of its tendency to contribute to IgE recall responses. In support of this, MBC2s were found to be the primary clonal relative to IgE PCs following chronic allergen exposure in humans [31–33]. While MBC2s are enriched in IgG1-expressing cells, our data demonstrate that IgG1 expression itself is not a defining feature of an MBC that later gives rise to IgE-secreting cells [31–33].

Overall, we found that sequential switching through CSR is not required for IgE production, function, and recall, despite what might have been predicted from previous studies. Our findings have important implications for therapeutic strategies aiming to eliminate IgE by targeting B cells. Future research will be required to elucidate the mechanisms that govern these processes to identify novel targets in allergic disease.

4 | Materials and Methods

4.1 | Mice

Age-, sex-, and vendor-matched control mice were used for all experiments. Mice were maintained in biohazard specific pathogen-free conditions on a 12-h light–dark cycle with low-fat food and water *ad libitum*. Where possible, littermate controls were used. C57Bl/6 mice were purchased from Charles River Laboratories. IgG1-deficient (Ighg1-tm3Cgn, Igh^{hMT}) mice were provided by Dr. Amy Kenter (University of Illinois) [19]. All animal procedures were approved by McMaster University's Animal Research Ethics Board.

4.2 | Intragastric Model of Sensitization

Mice were sensitized with either 3.75 mg All-Natural Smooth Peanut Butter (Kraft Heinz, Northfield, USA), 1 mg ovalbumin (OVA) (MilliporeSigma, A5378), or 1 mg 4-hydroxy-3-nitrophenyl (NP)-OVA (LGC Biosearch Technologies, N-5051-100) by oral gavage with 5 μg cholera toxin (List Biological Labs, 100B) in 0.5 mL PBS. Gavages were performed once weekly for 4 weeks. Serum was collected from retro-orbital bleeds between 2 weeks and 12 months post-sensitization for serum Ig analyses. Two weeks following the last gavage, mice were challenged intraperitoneally with 0.25–2.5 mg OVA or crude peanut extract (CPE; Stallergenes Greer USA, XPF171D3A25). In NP sensitization experiments, NP-BSA (LGC Biosearch Technologies, N5050H-100) was used for challenge to measure reactivity specific to NP, rather than the OVA-conjugate used for sensitization. Antigens used for sensitization and challenge are noted in the figure legend. Core temperature was measured at 10-min intervals for 40 min post-challenge using a rectal probe (VWR International, 23,226–656). Hemoconcentration was measured using the HemataSTAT II centrifugation device (VWR International, 14,221–620). Clinical signs were scored by a blinded research technician; scoring criteria are: 0 = no clinical signs, 1 = hind leg scratching in the ear, 2 = reduced movement, 3 = motionless, 4 = no response to whisker stimuli, 5 = moribund, seizure, or death. The NP:carrier ratio of NP-conjugated reagents depended on availability from the supplier; NP-OVA ranged from 16 to 22 NP moieties/molecule, and NP-BSA ranged from 27 to 30. To block IgG-mediated anaphylaxis, 500 μg anti-CD16/32 (Clone 2.4G2; Bio X Cell, BE0307) in 500 μL PBS was administered by intraperitoneal injection 24 h prior to challenge.

For memory experiments, mice were sensitized as above and then left to desensitize over 10 months. Memory responses were induced by re-exposing the mice with 3 gavages of 1 mg OVA alone (no adjuvant), 1 week apart.

4.3 | Antigen-Specific Ig ELISAs

Blood was collected by retro-orbital bleed and centrifuged at ≥9000 g to separate serum and cellular fractions. Serum was removed and stored at −20°C for ELISA.

ELISAs were performed in a 96-well flat bottom polystyrene plate (VWR, 4394554). Plates were washed using a Tecan Hydroflex Plate Washer and read using a Thermo Scientific Multiskan FC. No-sample controls were included in all assays. The average optical density of the no-sample control wells was subtracted from all samples prior to plotting. In cases where subtraction would result in negative values for some samples, instead the largest value that would yield an OD of at least 0.01 would be subtracted to ensure compatibility with log transformation (see statistical analysis below).

4.3.1 | IgG1, IgG3, and Total Ig

Plates were coated with either 4 μg/mL OVA, NP-BSA, or CPE in 100 μL of carbonate bicarbonate buffer (Sigma, C-3041), sealed with an adhesive cover, and incubated overnight in the fridge. The next day, plates were blotted entirely and blocked for 2 h at room temperature (RT) with 100 μL of 5% skim milk powder or 1% BSA dissolved in PBS. Plates were washed 3 times with 0.05% Tween 20 in PBS (PBST) and incubated with 50 μL of serum diluted to the indicated concentrations in 1% skim milk or 0.3% BSA in PBS overnight in the fridge. The next day, plates were washed 3 times and incubated with 50 μL of either 0.25 μg/mL biotinylated anti-mouse IgG1 (Southern Biotech, 1070–08) or biotinylated anti-mouse IgG (H + L) (Poly4053, Biolegend, 405,301) for 2 h at RT. Plates were then washed 3 times, incubated with 50 μL streptavidin-alkaline phosphatase (ThermoFisher, 434,322) diluted 1:1000 in 0.3% BSA-PBS for 1 h

at RT, and covered from light. After 3 washes, plates were developed using 50 μL 4-nitrophenyl phosphate (Sigma, N-9389) dissolved in 1x diethanolamine substrate buffer (ThermoFisher, 34,064) and stopped using 25 μL of 1 N NaOH. Plates were read at 405 nm.

4.3.2 | IgE

Plates were coated with 50 μL of 2 μg/mL anti-mouse IgE (R35-72, BD, 553413) in PBS, sealed with an adhesive cover, and incubated overnight at 4°C. The next day, plates were washed 3 times and blocked for 1 h at 37°C with 5% skim milk in PBS. Plates were washed 3 times and incubated with 50 μL of serum diluted to the indicated concentrations in 1% skim milk or 0.3% BSA in PBS overnight at 4°C. The next day, plates were washed 5 times, incubated with 50 μL of 300 ng/mL OVA, 150 ng/mL CPE, or 300 ng/mL NP-BSA conjugated to digoxigenin following supplier recommendations (ANP technologies, 90–1023-1KT) for 90 min at RT. Plates were washed 3 times, incubated with 50 μL of anti-digoxigenin POD fragments (Roche, 11,633,716,001) diluted 1:5000 in 0.3% BSA PBS for 1 h at RT, and covered from light. Plates were washed 5 times, then developed using 50 μL TMB (Sigma, T0440), and stopped using 25 μL 2 N H_2SO_4. Plates were read at 450 nm.

4.3.3 | Thiocyanate Elution

A thiocyanate elution step was added after sample incubation. Plates were washed 3 times, and wells were incubated for 15 min with the indicated concentrations of potassium thiocyanate (Sigma, 207,799) at RT. Plates were then washed, and the ELISA protocol continued as described above.

4.4 | Degranulation Assay

Serum samples from allergic mice were diluted to 100 ng/mL based on total IgE. Total IgE was measured by ELISA as described above; however, rather than coating the plate with antigen, the plate was coated with anti-IgE (LO-ME-3, Fitzgerald, 10R-I105A), and detection was performed using anti-IgE (23G3, Southern Biotech, 1130–08). Concentrations were determined using mouse IgE (C48-2, BD, 557080) as a standard. For heat-inactivation, serum was heated to 56°C for 1 h.

Rat basophil leukemia cells (RBL-2H3, ATCC, Cat: CRL-2256) were incubated with diluted serum samples, plated in a 96 well flat bottom plate, and equilibrated at 37°C, 5% CO_2 for 10 min. RBL-2H3 degranulation was assessed as previously described [62]. Briefly, sensitized RBL-2H3 cells were stimulated with the indicated concentrations of allergen and incubated at 37°C for 30 min. Cell-free supernatant was collected, and cells were lysed with 0.1% Triton X-100. Cell supernatants and cell lysates were incubated in p-nitrophenyl N-acetyl-β-D-glucosamide in citrate buffer for 90 min at 37°C followed by the addition of 0.4 M glycine. Optical density at 405 nm was acquired using a Thermo Scientific Multiskan FC plate reader. Percent degranulation was calculated as 100 x (supernatant content)/(supernatant+lysate content).

4.5 | Tissue Collection and Processing

Mesenteric lymph nodes were collected into HBSS (Fisher, SH30015.03) and kept on ice until processing. Mesenteric lymph nodes were crushed between frosted microscope slides into a single cell suspension in HBSS and passed through a 40 μm strainer (Corning Inc., 352,340). After processing, single cells were pelleted by centrifugation and re-suspended in FACS buffer (2% FBS (Gibco, F4135), 2 mM EDTA (Sigma, 324,504) in PBS) for downstream analysis. Cells were counted manually using a hemocytometer. Total cells were counted with Turk's stain.

4.6 | Flow Cytometry

All steps were performed in FACS buffer unless otherwise specified. Three million cells were plated into a 96 well U-bottom plate (Corning, 353,077) for staining. Centrifugation steps were performed at 200 g at 4°C prior to fixation, and 300 g after fixation. After each centrifugation, the supernatant was decanted. Table 1 contains a list of all antibodies used for flow cytometry staining and the dilutions they were used at, based on 50 μL final staining volume.

Cells were pelleted and resuspended in 25 μL FACS with anti-CD16/32 (Clone 93, Biolegend, 101,302), to block antibodies binding to Fcγ receptors, for 15 min on ice. In experiments where intracellular IgE was detected, unlabeled anti-mouse IgE (Clone RME-1, Biolegend, 406,902) was used to block surface IgE bound to the low affinity IgE receptor (CD23) expressed by B cells. Twenty-five μl of a mix of antibodies to label extracellular targets was added directly to blocking solution, and the cells were incubated for 30 min on ice and covered from light. After this incubation, cells were washed twice by adding 200 μL of FACS and centrifuging each time. For fixation and permeabilization, cells were resuspended and incubated in 100 μL of BD Cytofix/Cytoperm (BD, 554714) for 20 min on ice. Cells were then washed with 150 μL BD Perm/Wash (BD, 554714), pelleted, washed again with 200 μL BD Perm/Wash, and pelleted again. For intracellular staining, cells were then resuspended in 50 μL of intracellular staining cocktail in BD Perm/Wash and stained for 30–45 min on ice, protected from light. Finally, cells were washed twice with 200 μL of BD Perm/Wash and then resuspended in 200 μL of FACS. Data were collected using a BD LSRFortessa (BD, Franklin Lakes, USA) and analyzed using FlowJo (FlowJo LLC, Ashland, USA). OVA-specific cells were probed using FITC-conjugated OVA (Molecular Probes, O23020) in the extracellular staining mix. Cell numbers were obtained by multiplying the total cell count by the frequency of the target population among live single cells for each sample.

Tetramer staining was performed using the protocol described by Phelps et al. (2024) [34]. OVA-APC and control tetramers were produced as described previously [34, 63]. In brief, spleen and mesenteric lymph nodes were pooled for each mouse and stained with the control tetramer with 2% rat serum (Sigma, R9759) for 5 min at room temperature. OVA-APC tetramer was added and incubated for 25 min on ice. Samples were washed using FACS and then incubated with anti-PE microbeads

TABLE 1 | Antibody-fluorochrome conjugates and other reagents used for flow cytometry.

Marker	Clone	Company	Conjugate	Dilution	Stain	Catologue
B220	RA3-6B2	BioLegend	Alexa Fluor 700	100	Extracellular	103232
CD138	281-2	BioLegend	BV605	100	Extracellular	142516
CD19	6D5	BioLegend	PerCP-Cy5.5	100	Extracellular	115534
CD23	B3B4	BioLegend	BV421	100	Extracellular	101621
CD3	17A2	BioLegend	BV711	100	Extracellular	100241
CD38	90	BioLegend	PE-Cy7	100	Extracellular	102718
CD45.1	A20	BioLegend	APC	100	Extracellular	110714
CD45.2	104	BioLegend	BV421	100	Extracellular	109832
CD95	Jo2	BD Biosciences	PE-Cy7	100	Extracellular	557653
F4/80	BM8	BioLegend	BV711	200	Extracellular	123147
Fixable Viability Dye		ThermoFisher Scientific	Aqua	600	Extracellular	L34957
			eFluor 780	600	Extracellular	65-0865-18
GL7	GL7	BioLegend	PerCP-Cy5.5	100–200	Extracellular	144610
			Alexa Fluor 647	100–200	Extracellular	144606
IgD	11-26c.2a	BioLegend	BV510	100	Extracellular	405723
			BV605	100	Extracellular	405727
IgE	RME-1	BioLegend	PE	200–400	Intracellular	406908
IgG1	RMG1-1	BioLegend	BV421	50	Extracellular	406616
			BV650	500	Intracellular	406629
IgG2b	RMG2b-1	BioLegend	Alexa Fluor 488	100	Extracellular	406718
IgG2c		Southern Biotech	FITC	100	Intracellular	1079-02
IgG3	R40-82	BD Biosciences	BV650	100	Extracellular	744136
IgM	II/41	BD Biosciences	BV786	50	Extracellular	743328
IL-4Ra	mIL4R-M1	BD Bioscience	PE	50	Extracellular	552509
OVA		ThermoFisher Scientific	FITC	100	Extracellular or Intracellular	O23020
			Alexa Fluor 647	100	Extracellular	O34784

(Miltenyi, 130–105-639) for 15–30 min prior to positive selection using an LS column. Samples were then stained and analyzed as described above.

4.7 | Bone Marrow Chimera

C57Bl/6 mice were irradiated with two doses of 550 rads, 4 h apart. Bones from both hind legs of donor IgG1-def and CD45.1 WT (Jackson Laboratories) mice were flushed with PBS to collect bone marrow cells. Red blood cells were lysed using ACK lysis buffer and donor cells were pooled at a 1:1 ratio. 8×10^6 donor bone marrow cells were injected intravenously into the tail vein of recipient mice, which were subsequently rested for 6 weeks to allow for bone marrow reconstitution. Recipient Mice were then sensitized with OVA as described above. MesLN were taken at day 6 post-sensitization.

4.8 | Statistical Analysis

Experiments were performed with multiple biological replicates and multiple times (see figure legends) to ensure reproducibility and statistical power to detect meaningful differences. GraphPad Prism v9 was used for all statistical analyses. Data which approximated a log-normal distribution were log transformed prior to statistical testing, except for data sets which included values of 0 (e.g., clinical sign data) which could not be log-transformed. See figure legends for specific statistical tests performed.

Author Contributions

Conceptualization: J.F.E.K., A.K.W.V., R.J.S., and M.J. Formal Analysis: J.F.E.K., A.K.W.V., and K.B., Investigation: J.F.E.K., A.K.W.V., R.J.S., K.B., S.G., T.W., M.G., A.E.G., J.J.T., and E.G. Visualization: J.F.E.K. and A.K.W.V. Funding Acquisition: J.F.E.K., S.W., and M.J. Supervision: J.F.E.K., R.J.S., S.W., and M.J. Writing – Original Draft: J.F.E.K. and A.K.W.V. Writing – Review and Editing: J.F.E.K., A.K.W.V., R.J.S., M.J., and J.J.T.

Acknowledgments

We thank Hong Liang from the McMaster Flow Cytometry Core for assistance with flow cytometry experiments. We thank McMaster's Central Animal Facility for supporting animal experimentation. We thank Amy L. Kenter (University of Illinois) for generously providing hMT mice. We thank Dr. Hirohito Kita (Mayo Clinic) for critical review of the manuscript prior to publication. We thank Madeleine Lepard for support with bone marrow chimera experiments.

Conflicts of Interest

J.E.E.K., S.W., and M.J. received funding from ALK Abello A/S. M.J. is an advisor to ALK Abello A/S.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. V. Sampath, E. M. Abrams, B. Adlou, et al., "Food Allergy Across the Globe," *Journal of Allergy and Clinical Immunology* 148 (2021): 1347–1364.

2. C. M. Warren, J. Jiang, and R. S. Gupta, "Epidemiology and Burden of Food Allergy," *Current Allergy and Asthma Reports* 20 (2020): 6.

3. Z. Yang, B. M. Sullivan, and C. D. C. Allen, "Fluorescent in Vivo Detection Reveals That IgE+ B Cells Are Restrained by an Intrinsic Cell Fate Predisposition," *Immunity* 36 (2012): 857–872.

4. Z. Yang, M. J. Robinson, X. Chen, et al., "Regulation of B Cell Fate by Chronic Activity of the IgE B Cell Receptor," *eLife* 5 (2016): 1–31.

5. A. Erazo, N. Kutchukhidze, M. Leung, et al., "Unique Maturation Program of the IgE Response In Vivo," *Immunity* 26 (2007): 191–203.

6. J.-S. He, M. Meyer-Hermann, D. Xiangying, et al., "The Distinctive Germinal Center Phase of IgE+ B Lymphocytes Limits Their Contribution to the Classical Memory Response," *Journal of Experimental Medicine* 210 (2013): 2755–2771.

7. A. K. Wade-Vallance and C. D. C. Allen, "Intrinsic and Extrinsic Regulation of IgE B Cell Responses," *Current Opinion in Immunology* 72 (2021): 221–229.

8. R. Jiménez-Saiz, Y. Ellenbogen, K. Bruton, et al., "Human BCR Analysis of Single-Sorted, Putative IgE+ Memory B Cells in Food Allergy," *Journal of Allergy and Clinical Immunology* 144 (2019): 336–339.e6.

9. D. Croote, S. Darmanis, K. C. Nadeau, and S. R. Quake, "High-Affinity Allergen-Specific Human Antibodies Cloned From Single IgE B Cell Transcriptomes," *Science* 362, no. 6420 (2018): 1306–1309.

10. K. Yoshida, M. Matsuoka, S. Usuda, A. Mori, K. Ishizaka, and H. Sakano, "Immunoglobulin Switch Circular DNA in the Mouse Infected With Nippostrongylus Brasiliensis: Evidence for Successive Class Switching From Mu to Epsilon via Gamma 1," *Proceedings of the National Academy of Sciences of the United States of America* 87 (1990): 7829–7833.

11. R. Mandler, F. D. Finkelman, A. D. Levine, and C. M. Snapper, "IL-4 Induction of IgE Class Switching by Lipopolysaccharide-Activated Murine B Cells Occurs Predominantly Through Sequential Switching," *Journal of Immunology* 150 (1993): 407–418.

12. J.-S. He, S. Subramaniam, V. Narang, et al., "IgG1 Memory B Cells Keep the Memory of IgE Responses," *Nature Communications* 8 (2017): 641.

13. R. Jiménez-Saiz, K. Bruton, J. F. E. Koenig, S. Waserman, and M. Jordana, "The IgE Memory Reservoir in Food Allergy," *Journal of Allergy and Clinical Immunology* 142 (2018): 1441–1443.

14. S. Jung, G. Siebenkotten, and A. Radbruch, "Frequency of Immunoglobulin E Class Switching Is Autonomously Determined and Independent of Prior Switching to Other Classes," *Journal of Experimental Medicine* 179 (1994): 2023–2026.

15. H. Xiong, J. Dolpady, M. Wabl, M. A. Curotto de Lafaille, and J. J. Lafaille, "Sequential Class Switching Is Required for the Generation of High Affinity IgE Antibodies," *Journal of Experimental Medicine* 209 (2012): 353–364.

16. M. Kuraoka, A. G. Schmidt, T. Nojima, et al., "Complex Antigens Drive Permissive Clonal Selection in Germinal Centers," *Immunity* 44 (2016): 542–552.

17. S. Asrat, N. Kaur, X. Liu, et al., "Chronic Allergen Exposure Drives Accumulation of Long-Lived IgE Plasma Cells in the Bone Marrow, Giving Rise to Serological Memory," *Science Immunology* 5 (2020): 8402.

18. G. Chen, E. L. Shrock, M. Z. Li, et al., "High-Resolution Epitope Mapping by AllerScan Reveals Relationships Between IgE and IgG Repertoires During Peanut Oral Immunotherapy," *Cell Reports Medicine* 2 (2021): 100410.

19. M. Lorenz, S. Jung, and A. Radbruch, "Switch Transcripts in Immunoglobulin Class Switching," *Science* 267 (1995): 1825–1828.

20. X. M. Li, B. H. Schofield, C. K. Huang, G. I. Kleiner, and H. A. Sampson, "A Murine Model of IgE-Mediated cow's Milk Hypersensitivity," *Journal of Allergy and Clinical Immunology* 103 (1999): 206–214.

21. J. Sun, K. Arias, D. Alvarez, et al., "Impact of CD40 Ligand, B Cells, and Mast Cells in Peanut-Induced Anaphylactic Responses," *Journal of Immunology* 179 (2007): 6696–6703.

22. K. Arias, D. K. Chu, K. Flader, et al., "Distinct Immune Effector Pathways Contribute to the Full Expression of Peanut-Induced Anaphylactic Reactions in Mice," *Journal of Allergy and Clinical Immunology* 127 (2011): 1552–1561.e1.

23. E. B. Brandt, R. T. Strait, D. Hershko, et al., "Mast Cells Are Required for Experimental Oral Allergen–Induced Diarrhea," *Journal of Clinical Investigation* 112 (2003): 1666–1677.

24. L. A. Herzenberg, S. J. Black, T. Tokuhisa, and L. A. Herzenberg, "Memory B Cells at Successive Stages of Differentiation. Affinity Maturation and the Role of IgD Receptors," *Journal of Experimental Medicine* 151 (1980): 1071–1087.

25. N. Sun, C. Zhou, X. Zhou, L. Sun, and H. Che, "Use of a Rat Basophil Leukemia (RBL) Cell-Based Immunological Assay for Allergen Identification, Clinical Diagnosis of Allergy, and Identification of Anti-Allergy Agents for Use in Immunotherapy," *Journal of Immunotoxicology* 12 (2015): 199–205.

26. K. J. Dorrington and H. Bennich, "Thermally Induced Structural Changes in Immunoglobulin E," *Journal of Biological Chemistry* 248 (1973): 8378–8384.

27. K. Ishizaka, T. Ishizaka, and A. E. Menzel, "Physicochemical Properties of Reaginic Antibody. VI. Effect of Heat on Gamma-E-, Gamma-G- and Gamma-A-Antibodies in the Sera of Ragweed Sensitive Patients," *Journal of Immunology* 99 (1967): 610–618.

28. G. R. Pullen, M. G. Fitzgerald, and C. S. Hosking, "Antibody Avidity Determination by ELISA Using Thiocyanate Elution," *Journal of Immunological Methods* 86 (1986): 83–87.

29. R. Jiménez-Saiz, D. K. Chu, T. S. Mandur, et al., "Lifelong Memory Responses Perpetuate Humoral TH2 Immunity and Anaphylaxis in Food Allergy," *Journal of Allergy and Clinical Immunology* 140 (2017): 1604–1615.e5.

30. I. Hoof, V. Schulten, J. A. Layhadi, et al., "Allergen-Specific IgG+ Memory B Cells Are Temporally Linked to IgE Memory Responses," *Journal of Allergy and Clinical Immunology* 146 (2020): 180–191.

31. J. F. E. Koenig, N. P. H. Knudsen, A. Phelps, et al., "Type 2-Polarized Memory B Cells Hold Allergen-Specific IgE Memory," *Science Translational Medicine* 16 (2024): eadi0944.

32. M. Ota, K. B. Hoehn, W. Fernandes-Braga, et al., "CD23+IgG1+ Memory B Cells Are Poised to Switch to Pathogenic IgE Production in Food Allergy," *Science Translational Medicine* 16 (2024): eadi0673.

33. C. J. Aranda, E. Gonzalez-Kozlova, S. P. Saunders, et al., "IgG Memory B Cells Expressing IL4R and FCER2 Are Associated With Atopic Diseases," *Allergy* 78 (2022): 752–766, https://doi.org/10.1111/all.15601.

34. A. Phelps, D. Pazos-Castro, F. Urselli, et al., "Production and Use of Antigen Tetramers to Study Antigen-Specific B Cells," *Nature Protocols* 19 (2024): 727–751, https://doi.org/10.1038/s41596-023-00930-8.

35. G. Siebenkotten, C. Esser, A. Radbruch, and M. Wabl, "The Murine IgG1/IgE Class Switch Program," *European Journal of Immunology* 22 (1992): 1827–1834.

36. L. Cameron, A. S. Gounni, S. Frenkiel, F$_s$. Lavigne, D. Vercelli, and Q. Hamid, "S S and S S Switch Circles in Human Nasal Mucosa Following Ex Vivo Allergen Challenge: Evidence for Direct as Well as Sequential Class Switch Recombination," *Journal of Immunology* 171 (2003): 3816–3822.

37. A. K. Wade-Vallance, Z. Yang, J. B. Libang, M. J. Robinson, D. M. Tarlinton, and C. D. C. Allen, "B Cell Receptor Ligation Induces IgE Plasma Cell Elimination," *Journal of Experimental Medicine* 220 (2023): e20220964.

38. Z. Yang, C. A. M. Wu, S. Targ, and C. D. C. Allen, "IL-21 Is a Broad Negative Regulator of IgE Class Switch Recombination in Mouse and Human B Cells," *Journal of Experimental Medicine* 217 (2020): e20190472.

39. A. Radbruch, W. Muller, and K. Rajewsky, "Class Switch Recombination Is IgG1 Specific on Active and Inactive IgH Loci of IgG1-Secreting B-Cell Blasts," *Proceedings of the National Academy of Sciences* 83 (1986): 3954–3957.

40. H. M. Shen, R. Wuerffel, J. F. Cantillo, et al., "Loop Extrusion Promotes an Alternate Pathway for Isotype Switching," *Cell Reports* 37 (2021): 110059.

41. Q. Chen, H. Liu, N. Luling, J. Reinke, and A. L. Dent, "Evidence That High-Affinity IgE Can Develop in the Germinal Center in the Absence of an IgG1-Switched Intermediate," *Journal of Immunology* 210 (2023): 905–915.

42. S. Casola, G. Cattoretti, N. Uyttersprot, et al., "Tracking Germinal Center B Cells Expressing Germ-Line Immunoglobulin gamma1 Transcripts by Conditional Gene Targeting," *Proceedings of the National Academy of Sciences* 103 (2006): 7396–7401.

43. T. Weber, J. Seagal, W. Winkler, T. Wirtz, V. T. Chu, and K. Rajewsky, "A Novel Allele for Inducible Cre Expression in Germinal Center B Cells," *European Journal of Immunology* 49 (2019): 192–194.

44. C. F. Díaz, E. Kabrani, W. Winkler, et al., "In Vivo Sequential Mutagenesis in Germinal Center B Cells Using a Dual-Recombinase Approach: FOXO1 Re-Expression Upon FOXO1 Knockout Rescues Class Switch Recombination," *BioRxiv* (2024), https://doi.org/10.1101/2024.05.17.592915.

45. C. Sundling, A. W. Y. Lau, K. Bourne, et al., "Positive Selection of IgG+ Over IgM+ B Cells in the Germinal Center Reaction," *Immunity* 54 (2021): 988–1001.e5.

46. F. El-Khouly, S. A. Lewis, L. Pons, A. W. Burks, and J. O. Hourihane, "IgG and IgE Avidity Characteristics of Peanut Allergic Individuals," *Pediatric Allergy and Immunology* 18 (2007): 607–613.

47. W. G. Shreffler, K. Beyer, T.-H. T. Chu, A. W. Burks, and H. A. Sampson, "Microarray Immunoassay: Association of Clinical History, In Vitro IgE Function, and Heterogeneity of Allergenic Peanut Epitopes," *Journal of Allergy and Clinical Immunology* 113 (2004): 776–782.

48. O. Hemmings, U. Niazi, M. Kwok, L. K. James, G. Lack, and A. F. Santos, "Peanut Diversity and Specific Activity Are the Dominant IgE Characteristics for Effector Cell Activation in Children," *Journal of Allergy and Clinical Immunology* 148 (2021): 495–505.e14.

49. W. G. Shreffler, D. A. Lencer, L. Bardina, and H. A. Sampson, "IgE and IgG4 Epitope Mapping by Microarray Immunoassay Reveals the Diversity of Immune Response to the Peanut Allergen, Ara h 2," *Journal of Allergy and Clinical Immunology* 116 (2005): 893–899.

50. C. Ji, Y. Huang, L. H. Yeung, et al., "Ara h 2-Specific IgE Presence Rather Than Its Function Is the Best Predictor of Mast Cell Activation in Children," *Journal of Allergy and Clinical Immunology: In Practice* 11 (2023): 1154–1161.e3.

51. S. P. Commins, H. R. James, L. A. Kelly, et al., "The Relevance of Tick Bites to the Production of IgE Antibodies to the Mammalian Oligosaccharide Galactose-α-1,3-Galactose," *Journal of Allergy and Clinical Immunology* 127 (2011): 1286–1293.e6.

52. S. P. Commins, S. M. Satinover, J. Hosen, et al., "Delayed Anaphylaxis, Angioedema, or Urticaria After Consumption of Red Meat in Patients With IgE Antibodies Specific for Galactose-Alpha-1,3-Galactose," *Journal of Allergy and Clinical Immunology* 123 (2009): 426–433.

53. J. L. Chandrasekhar, K. M. Cox, and L. D. Erickson, "B Cell Responses in the Development of Mammalian Meat Allergy," *Frontiers in Immunology* 11 (2020): 1532.

54. J. L. Chandrasekhar, K. M. Cox, W. M. Loo, H. Qiao, K. S. Tung, and L. D. Erickson, "Cutaneous Exposure to Clinically Relevant Lone Star Ticks Promotes IgE Production and Hypersensitivity Through CD4+ T Cell- and MyD88-Dependent Pathways in Mice," *Journal of Immunology* 203 (2019): 813–824.

55. J. F. Koenig, K. Bruton, A. Phelps, E. Grydziuszko, R. Jiménez-Saiz, and M. Jordana, "Memory Generation and re-Activation in Food Allergy," *ImmunoTargets and Therapy* 10 (2021): 171–184.

56. S. P. Saunders, E. G. M. E. G. M. Ma, C. J. Aranda, C. De Lafaille, and M. A. Curotto de Lafaille, "Non-classical B Cell Memory of Allergic IgE Responses," *Frontiers in Immunology* 10 (2019): 715.

57. T. J. Looney, J. Y. Lee, K. M. Roskin, et al., "Human B-Cell Isotype Switching Origins of IgE," *Journal of Allergy and Clinical Immunology* 137 (2016): 579–586.e7.

58. R. A. Hoh, S. A. Joshi, J. Y. Lee, et al., "Origins and Clonal Convergence of Gastrointestinal IgE+ B Cells in Human Peanut Allergy," *Science Immunology* 5 (2020): eaay4209.

59. A. Turqueti-Neves, M. Otte, C. Schwartz, et al., "The Extracellular Domains of IgG1 and T Cell-Derived IL-4/IL-13 Are Critical for the Polyclonal Memory IgE Response in Vivo," *PLoS Biology* 13 (2015): e1002290.

60. K. Haniuda, S. Fukao, T. Kodama, H. Hasegawa, and D. Kitamura, "Autonomous Membrane IgE Signaling Prevents IgE-Memory Formation," *Nature Immunology* 17 (2016): 1109–1117.

61. R. A. Hoh, S. A. Joshi, Y. Liu, et al., "Single B-Cell Deconvolution of Peanut-Specific Antibody Responses in Allergic Patients," *Journal of Allergy and Clinical Immunology* 137 (2016): 157–167.

62. H. S. Kuehn, M. Radinger, and A. M. Gilfillan, "Measuring Mast Cell Mediator Release," *Current Protocols in Immunology* 91 (2010): 7.38.1–7.38.9.

63. J. J. Taylor, R. J. Martinez, P. J. Titcombe, et al., "Deletion and Anergy of Polyclonal B Cells Specific for Ubiquitous Membrane-Bound Self-Antigen," *Journal of Experimental Medicine* 209 (2012): 2065–2077.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.