MAINTENANCE AND REPROGRAMMING OF IMMUNOGLOBULIN E MEMORY

MAINTENANCE AND REPROGRAMMING OF IMMUNOLOGICAL MEMORY IN LIFELONG FOOD ALLERGIES

By KELLY BRUTON, B.Sc.

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Author:	Kelly Bruton, B.Sc. *Hons* (Brock University)
Supervisor:	Dr. Manel Jordana
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Lay Abstract

Food allergies, mediated by IgE antibodies arising from B cells, have the potential to last for a lifetime. Currently, the standard of care is strict allergen avoidance and use of rescue epinephrine upon anaphylaxis. The absence of disease-transformative treatments can largely be attributed to an inadequate understanding of the immunological mechanisms through which allergies persist. Therefore, our broad objective was to elucidate the B cell subset which maintains food allergies and the pathway through which IgE is replenished, using both human and mouse experimental systems. We identified that IgE-expressing B cells are exceedingly rare in human circulation, suggesting that allergies are maintained by another B cell subset. Moreover, we demonstrate a critical requirement of a protein messenger (IL-4) that, when absent, prevents the regeneration of IgE and reprograms the underlying allergic characteristics. Collectively, this work uncovers a critical pathway that maintains lifelong allergies and positions IL-4 as a clinically relevant therapeutic target.

Abstract

Food allergy is an IgE-mediated disease affecting up to 10% of those in Western countries. Clinical reactivity to foods occurs following allergen cross-linking of IgE-FcεRI complexes on mast cells and basophils causing rapid degranulation and release of vasoactive mediators. While some food allergies are naturally outgrown (*e.g.* milk and egg), others typically persist for a lifetime (*e.g.* peanut). For food allergic patients, the standard of care remains as strict allergen avoidance and use of rescue epinephrine upon accidental exposures.

The broad objective of this thesis was to investigate aspects of immunological memory which perpetuate IgE-mediated allergies. In humans, we found that circulating IgE^+ memory B cells were extremely rare, positioning memory B cells of other isotypes (*e.g.* IgG1) as the primary reservoir of IgE responses. IL-4/IL-13 signaling through IL-4Rα was critically required for these non-IgE-expressing memory B cells to replenish the transient IgE pool and, as a result, sensitized mice were fully protected from anaphylaxis upon challenge. Moreover, we demonstrated that IL-4Rα blockade reduced the Th2 dominant cytokine signature while upregulating IFN-γ and IL-10. In parallel, we found that allergen-specific B cell responses could be reprogrammed away from an IgE fate. Collectively, this work elucidates key requirements for sustained immunological memory against food allergens and the potential to reprogram its pathogenic fate. These findings may aid in evolving the landscape of food allergy therapeutics.

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Abbreviations

A4RA	Anti-IL-4Rα
AIFNG	Anti-IFN-γR
ANOVA	Analysis of Variance
BCR	B Cell Receptor
FBS	Fetal Bovine Serum
CD	Cluster of Differentiation
CSR	Class Switch Recombination
CT	Cholera Toxin
CXCR	C-X-C Chemokine Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked Immune Absorbent Spot
GC	Germinal Center
GFP	Green Fluorescent Protein
IC	Isotype Control
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iLN	Inguinal Lymph Node
i.p.	Intraperitoneal
i.v.	Intravenous
KO	Knockout
MBC	Memory B Cell
MHC	Major Histocompatibility Complex
mLN	Mesenteric Lymph Node
ODN	Oligodeoxynucleotides
OIT	Oral Immunotherapy
OVA	Ovalbumin
PBS	Phosphate Buffered Saline
PC	Plasma Cell
PCR	Polymerase Chain Reaction

PN	Peanut
RNA	Ribonucleic Acid
s.c.	Subcutaneous
scRNAseq	Single-Cell RNA-Sequencing
SEM	Standard Error of Mean
TFh	T Follicular Helper
TGF	Transforming Growth Factor
Th	T Helper
Treg	Regulatory T Cell
TLR	Toll-Like Receptor
TS	Tape Strip
VDJ	Variable-Diversity-Joining
WT	Wild-Type

Declaration of Academic Achievements

Chapter 2: Human BCR analysis of single-sorted, putative IgE$^+$ memory B cells in food allergy

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RJ-S, YE, and KB conceived and designed the study. RJ-S, YE, and KB performed experiments with aide from PS. RJ-S, YE, and KB analyzed data. DS, HL, and SW assisted in patient recruitment and biological sample collection. RJ-S, YE, and KB wrote the manuscript. SW, SP, WS, and MJ raised funding and reviewed the manuscript. MJ supervised the project.

Chapter 3: Interrupting reactivation of immunologic memory diverts the allergic response and prevents anaphylaxis

***Bruton K**, *Spill P, Vohra S, Baribeau O, Manzoor S, Gadkar S, Davidson M, Walker T, Koenig J, Ellenbogen Y, Florescu A, Wen J, Chu D, Waserman S, Jiménez-Saiz R, Epelman S, Robbins C, Jordana M. *Journal of Allergy and Clinical Immunology.* 2021; 147(4). ***equal contributions**

KB and PS conceived and designed the study. KB and PS performed experiments with aide from OB, SM, SG, MD, TW, JK, YE, and AF. KB and PS analyzed data. SV performed transcriptomic analysis. JW provided a key reagent. KB and PS wrote the manuscript. DC, SW, RJ-S, SE, CR, and MJ provided feedback and reviewed the manuscript. SW and MJ raised funding. MJ supervised the project.

Chapter 4: Reprogramming of pathogenic IgE memory with IL-4Rα blockade

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Chapter 1: Background

1.1 Preface

Food allergy is defined as a deleterious immune response to innocuous food antigens. Major food allergens, as defined by the U.S. Food and Drug Administration, include cow's milk, egg, fish, shellfish, tree nuts, wheat, peanuts, and soybeans.[1] At the most fundamental level, food allergy may be considered as any adverse gastrointestinal, respiratory, cutaneous, and/or cardiovascular reaction occurring upon consumption of a particular food.[2] In its most severe form, allergen exposures cause life-threatening systemic shock, termed anaphylaxis. The clinical diagnosis of food allergy is more complex than medical history and usually includes serological testing, a skin prick test, and/or – the gold standard – an oral food challenge.[1]

In Canada, 6% of the population (6.7% children, 5.9% adults) have one or more food allergies.[3] This is consistent with estimates from other Western countries where the prevalence of childhood food allergies range from 3.6% to 10.8%, with self-reported estimates averaging 60% higher.[2–4] From 1997 to 2011 the prevalence in children grew by 50% in the U.S.[5] While historically considered a disease of higher socioeconomic countries, food allergy appears to be rising even in developing nations.[6] Of all food allergens, egg, tree nuts, and peanuts are amongst the most common, with tree nuts and peanuts eliciting the most severe clinical reactions and the highest rate of hospital admission.[1,3,7]

Coping with one or more food allergies has been found to provoke profound economic and psychosocial burden. Nearly a decade ago, the annual economic cost of food allergy was estimated at over $20 billion in the U.S.[8] These expenses primarily arise from both emergency and non-emergency medical visits and the purchase of speciality allergen-free foods.[8] Canadian households with one or more food-allergic persons encounter a 20% increase in related expenses (*e.g.* medical expenses, food purchases, etc.).[9] Beyond financial strain, food allergies have a significant negative influence on quality of life in both the allergic individual and their families arising from dietary vigilance and fear of accidental allergen consumption.[10] The negative consequences of food allergy urge for better disease management and therapeutic strategies.

1.2 Immunological principles underlying clinical reactivity to foods

1.2.1 Primary Th2 sensitization

Allergic sensitization refers to the process by which an immunoglobulin (Ig) E-driven immune response is initiated against innocuous antigen. In humans, allergies usually remain undiagnosed until after a clinical reaction has occurred. For example, 72% of peanut-allergic infants/children experience a clinical reaction upon their first known exposure.[11] Thus, allergic sensitization proceeds as a clinically silent process where clinical reactivity is only gained upon repeated allergen exposure, creating obvious challenges in studying the initiation of allergies directly in humans.[12] As a result, insight into the immunological mechanisms of allergic sensitization are primarily derived from studies in animal models.[13]

As food allergens are not inherently immunogenic, the initiation of a pathogenic, rather than tolerogenic response, requires a concomitant insult to disrupt homeostasis that results in the release of alarmins at either mucosal surfaces or the skin.[13] In mice, allergy-inducing tissue perturbations can be facilitated by various adjuvants such as cholera toxin (CT), aluminum hydroxide (alum) administered systemically, and tape-stripping. The latter involves the induction of localized inflammation through disruption of the outermost epidermal layer (*stratum corneum*) and topical application of allergen extracts.[14–16] Skin/epicutaneous sensitization is a plausible mechanism through which food allergy may be initiated in humans. A longitudinal cohort study identified significant associations between peanut allergy and incidence of skin rashes, as well as the use of peanut-containing ointments for treatment of said rashes.[17] Moreover, compromised skin barrier integrity through loss-of-function mutations in the filaggrin gene are associated with development of food allergy.[18] In Chapters 3 and 4 a more procedural description of tape-stripping is included, as the majority of the murine studies conducted in this thesis employed a model of epicutaneous sensitization.

Analogous to the response against a foreign pathogen, the initiation of a pathogenic immune response to food allergens involves production of alarmins and dendritic cell activation, yielding a directed (*i.e.* polarized) T and B cell response.[19,20] With regard to allergies, the polarization is towards a type 2 response involving allergen-specific T helper 2 (Th2) cells and IgE. Canonically, the cytokine profile of Th2 cells includes interleukin (IL)-4, IL-5, and IL-13. While these cytokines all have defined roles in allergic disease, only IL-4 appears to be critical for allergic sensitization.[21]

IL-4 has two principal roles in allergic sensitization. Firstly, IL-4 steers the polarization of naïve CD4 T cells to a Th2 phenotype. In response to epithelial-derived alarmins (*e.g.* IL-33), dendritic cells upregulate the costimulatory molecule OX40L; subsequent engagement with naïve CD4 T cells through OX40 drives autocrine IL-4 production, prompting Th2 polarization.[20,22] Its second function in allergic sensitization is the initiation of IgE class switch recombination (CSR) in allergen-specific naïve B cells. The promoter region of the Cε gene contains IL-4-responsive regions, facilitating germline transcription upon IL-4 stimulation.[23] The critical requirement of IL-4 in broadly initiating type 2 responses has been demonstrated experimentally using IL-4 knockout (KO) mice and through monoclonal antibody-mediated blockade. In IL-4-deficient mice, oral sensitization with peanut and CT failed to elicit an IgE response and mice were clinically unresponsive upon allergen challenge.[22] Similarly, prophylactic monoclonal antibody-mediated blockade of IL-4 or the IL-4 receptor α chain (IL-4Rα) prevented typical IgE responses to helminth infection.[24,25] In contrast, IL-13-deficient mice exhibit uncompromised IgE production in the context of helminth infection.[21] Dampened allergen-specific IgE responses are also observed in IL-4 haploinsufficient mice, suggesting that even partial impairment to IL-4 production cannot be compensated for by other Th2 cytokines.[26]

Despite Th2 cells being a primary source of IL-4 during allergic sensitization, their contribution to IgE CSR appears negligible. Instead, T follicular helper (TFh) cells appear to be critically required for primary IgE responses. TFh cell-deficient mice ($Bcl6^{fl/fl}$ Cd4-Cre) are unable to generate an IgE response upon intranasal immunization to ovalbumin (OVA), despite having an intact Th2 cell compartment and Th2 cell-derived cytokine

response.[27] Likewise, TFh cell-derived IL-4 was necessary to initiate IgE responses during enteric helminth infection.[28] Considering that IL-4 production is a feature of all TFh cells regardless of disease context, it is suspected that TFh cells attain further polarization by the dominant effector Th subset. Indeed, single-cell RNA-sequencing (scRNA-seq) revealed heterogeneity within murine TFh cells including a cluster unique in allergic disease characterized by high *Il4, Il13, Il5* transcript expression and low *Il21*.[29]

Overall, the proposed model of allergic sensitization would involve the following five steps: 1) tissue perturbation eliciting secretion of alarmins, 2) dendritic cell migration and activation, including OX40L upregulation, 3) naïve CD4 T cell activation and polarization to Th2 through autocrine/paracrine IL-4 signaling, 4) Th2-skewing of allergen-specific TFh cells, and 5) B cell activation with IgG1/IgE CSR directed by Th2-skewed TFh cells.

1.2.2 Mechanisms of the effector phase

In 1921, Prausnitz and Küstner famously discovered that allergic reactivity is facilitated by a mediator in plasma. Performing what is now known as passive sensitization, they transferred plasma from a fish-allergic to a non-allergic person and found that subsequent cutaneous injection of fish extract would cause a localized wheal-and-flare in the non-allergic.[30] Nearly 50 years later, the discovery of IgE – the ominous plasma mediator in Prausnitz and Küstner's experimentation – was made by T. and K. Ishikaka.[31,32]

IgE exerts its functions through FcεRI expressed on tissue-resident mast cells and circulating basophils.[33] FcεRI binds to IgE with very high-affinity such that, even in the

absence of allergen, mast cells and basophils can remain coated with IgE. Upon allergen exposure, antigenic crosslinking of IgE-FcεRI complexes occurs provoking rapid degranulation and release of vasoactive mediators (*e.g.* histamine, proteases, etc.). The biologic activity of these mediators ultimately elicits acute clinical reactivity, which can range in severity from mild localized effects to life-threatening systemic shock (anaphylaxis).

1.3 IgE memory

1.3.1 Properties of classical (protective) immunological memory

At its core, immunological memory is understood as "the ability of the immune system to respond more rapidly and effectively to pathogens that have been encountered previously".[34] An early observation of immunological memory was made when studying eighteenth and nineteenth century measles outbreaks in the Faroe Islands. Researchers noted that those who contracted and survived primary measles infection did not become ill during a later outbreak.[35] Consistent with observations made in the Faroe Islands, immunological memory serves to protect against secondary antigen exposure – the basis of vaccination. The modern understanding of immunological memory is the generation of clonally expanded antigen-specific T cells, B cells, and plasma cells (PCs) that persist beyond antigen clearance.

Long-lived PCs provide a first line of defense through production of antigen-specific Igs (primarily IgG) that rapidly neutralize infiltrating pathogens. In a longitudinal analysis of

human serum samples, the estimated half-life of antigen-specific antibody responses following vaccination ranged from 11 years for tetanus to over 200 years for measles, mumps, and Epstein-Barr virus.[36] Given, however, that each of these viruses are still in circulation globally, it is plausible that natural exposure boosts immune memory, seemingly extending the longevity of protection. Unconfounded observations of humoral memory duration can, however, be made with regards to smallpox (vaccinia vaccination), which was declared eradicated in 1980. In a cohort of vaccinees, serum antibody titers against vaccinia were found to be maintained for at least 75 years.[37] Thus, while the rate at which antibody titers wane differs between antigens, IgG humoral memory typically persists for years, if not a lifetime.

The second line of defense against reinfection is provided by memory T cells and memory B cells (MBCs). As these cells persist in a quiescent state, they do not provide any immediate protection but, upon reinfection, can rapidly be reactivated to provide effector functions. Despite longevity being a core characteristic of immunological memory, modern evidence suggests that not all memory T and B cells are inherently long-lived.[38] Instead, regular turnover and differentiation of naïve cells serves to renew the memory populations.[39,40] With this understanding, estimates of immune memory longevity provided by memory T and B cells are not necessarily indicative of cellular lifespan, but rather population lifespan. Much the same as PC-mediated protection, vaccinia-specific memory provided by T and B cells has been documented to remain stable for over 50 years post-vaccination.[41]

The ongoing COVID-19 pandemic has provided an unprecedented opportunity to study longevity of human immunological memory following natural infection. In agreement with vaccine-induced immunological memory discussed above, SARS-CoV-2 infection establishes anti-spike IgG titers detectable beyond 11 months.[42,43] Likewise, the frequency of spike-specific MBCs is maintained beyond eight months, while the persistence of CD4 and CD8 memory T cells within the same time frame remains contentious.[42–44] Beyond detection in peripheral blood, these subsets of immune memory were detected in various lymphoid and non-lymphoid tissues six months post-infection.[45]

This preamble has discussed immunological memory in its conventional evolutionary role – protection of the host. While the inherent longevity of immune memory is particularly advantageous for protection against infectious disease, it is the central impediment in the resolution of allergies.

1.3.2 Humoral IgE memory

The pervasive availability of food allergens creates an intrinsic difficulty in studying the longevity of allergen-specific IgE titers in humans. Indeed, each year 14% of food-allergic individuals experience accidental allergen exposures, with an even higher likelihood of subclinical exposures.[46] Consequently, IgE titers against food allergens tend to be sustained for years. One such observation was made in a population-based birth cohort that measured no significant decline in peanut-specific IgE levels from 4 to 16 years of age.[47] Similarly, placebo control groups in therapeutic clinical trials (*e.g.* allergen immunotherapy) tend to exhibit no decline in allergen-specific IgE throughout the study period (up to three

years).[48–51] As PCs perpetually secrete antibodies and the half-life of serum IgE is short (less than three days in humans), serum IgE levels are a compelling surrogate measure for the longevity of PCs.[52] Thus, it can be inferred that food-specific IgE^+ PCs are long-lived; however, this conclusion is, as mentioned earlier, confounded by the high chance of allergen exposure which may replenish IgE^+ PCs.

Examination of IgE titers to other (non-food) allergens, where exposure can be tightly regulated, provides relevant insight as to the longevity of human IgE^+ PCs. For example, the fish parasite *Anisakis* can elicit IgE sensitization upon consumption of infected fish. As the life cycle of *Anisakis* takes place in fish and crustaceans, strict avoidance of raw seafood consumption adequately prevents allergen re-exposure. In this context, *Anisakis*-specific IgE titers drastically decline over 12 months of avoidance (100 to 3 kU/L with 0.7 kU/L as the threshold for sensitization).[53] A similar observation was made in the context of mammalian meat allergy, involving sensitization to galactose-α-1,3-galactose (α-Gal; a carbohydrate in red meat) following tick bites.[54] With avoidance of subsequent tick bites and red meat consumption, the median decline in α-Gal-IgE titers was 27% per year.[55] Lastly, the cyclical nature of environmental aeroallergens (*e.g.* birch pollen) mediates a comparable phenomenon wherein serum levels of aeroallergen-specific IgE increase and decrease in concordance with on- and off-season allergen peaks.[56]

Allergen exposure can be fully regulated in murine food allergy models, uniquely permitting the investigation of food allergen-specific IgE^+ PC longevity. In a model of oral sensitization to peanut, serum IgE became undetectable following six months without

allergen exposure, coinciding with ameliorated clinical reactivity upon challenge.[57] Despite the resolution of pathogenic IgE responses, peanut-specific IgG1 persisted beyond 15 months post-sensitization. Should long-lived IgE^+ PCs exist, the expectation would be to find them in the bone marrow, where survival is mediated by a specialized microenvironment. Mirroring the serum Ig data, IgG1, but not IgE, PCs were detected in the bone marrow of sensitized mice nine months later.[57] Similarly, intraperitoneal (i.p.) immunization with 4-hydroxy-3-nitrophenylacetyl (a model antigen) and alum did not yield any detectable IgE^+ PCs in the bone marrow, despite robust IgE^+ germinal center (GC) activity and generation of long-lived $IgG1^+$ PCs.[58] These data not only confirm observations made in humans, but also illustrate that short-lived humoral responses are not broadly characteristic of Th2 immunity, but rather are a unique feature of IgE.

1.3.3 B cell-mediated IgE memory

The transient nature of humoral IgE responses intimates an imperative role of MBCs in maintaining IgE responses. Classically, the MBC pool mirrors that of the humoral response with regards to isotype. For example, SARS-CoV-2 vaccination predominately induces IgG antibodies, which is reflected in the dominance of IgG-expressing MBCs.[59] Allergy, however, appears to be at odds with this convention. Evaluation of the allergen-specific MBC repertoire has revealed an alternative isotype distribution, with IgG and IgA presiding over other isotypes.[60,61] In fact, the mere existence of IgE MBCs in humans remains contentious.

In mice, IgE-expressing B cells are detected during active immune responses, but not thereafter. Following helminth infection or Th2 adjuvanted immunization, IgE B cells participate in early GC activity, though their presence is transient.[58,62] Upregulation of the transcriptional repressor, Blimp-1, is proposed to mediate the early evacuation of IgE B cells from the GC and their predisposition towards a plasmablast fate.[58] Moreover, constitutive autonomous B cell receptor (BCR) signaling appears to prompt plasmablast differentiation and apoptosis in IgE B cells.[62–64] This seemingly inherent predisposition for plasmablast differentiation and/or apoptosis has precluded detection of IgE^+ MBCs in mice, including in circulation, lymphoid-, and non-lymphoid tissues.[65]

In contrast to the high likelihood that IgE^+ MBCs are absent in mice, the presence of IgE^+ MBCs in humans appears enigmatic. In non-allergic individuals, one study reported that IgE^+ MBCs comprise approximately 0.25% of circulating B cells.[66] This is quite surprising given that non-allergic individuals often have undetectable levels of total serum IgE. In allergic asthmatics, putative IgE^+ MBCs were present at a frequency of 0.3 and 0.7% of B cells in blood and tonsils, respectively.[67] However, technical limitations (elaborated upon in Chapter 2) prohibit strong conclusions from either study and neither address the presence of these cells in food allergy. In peanut-allergic humans, IgE^+ plasmablasts, but not MBCs, were detected in gastric and duodenal biopsies.[68] The apparent absence of these cells at inductive tissue sites supports the notion that these cells would also be absent in circulation.

Should IgE^+ MBCs be present in humans, it is apparent that they are extraordinarily rare and are, therefore, unlikely to be the primary reservoir of humoral IgE responses. Unlike

classical immune responses to pathogens where CSR to the appropriate isotype is achieved through a *direct* pathway, expression of IgE primarily occurs through *sequential* CSR.[69] This indicates that rather than naïve IgM B cells switching directly to IgE, there is often an intermediate isotype that is functionally expressed prior to subsequent IgE CSR. A nearest neighbour analysis demonstrated that all upstream isotypes in humans (IgM, IgG3, IgG1, IgA1, IgG2, and IgG4) can give rise to IgE, though the most dominant intermediate was IgG1.[70] In mice, IgG1 appears to be the only intermediate expressed.[65] Upon allergic sensitization in both mice and humans allergen-specific IgG1 titers precede that of IgE, further supporting a sequential evolution of IgE responses.[16,60,71] This apparent critical role of $IgG1^+$ MBCs in the maintenance of IgE memory has since been substantiated in mice. Both adoptive transfer and long-term studies have demonstrated that $IgG1^+$ MBCs are not only sufficient, but are the primary cellular reservoir which maintain lifelong IgE responses.[57,72]

1.3.4 T cell-mediated IgE memory

A unique subset of Th2 cells, termed "Th2A", is proposed to mediate the persistence of allergic diseases in humans. While originally discovered in the circulation of individuals with food and aeroallergies, Th2A cells have since been identified as a tissue-resident population in the epidermis of atopic dermatitis patients.[73,74] This cell subset is distinct from Th2 cells in their co-expression of CRTH2 (prostaglandin D2 receptor), CD161 (C-type lectin-like receptor), and CD49d (α4 integrin subunit).[73] Th2A cells are multi-cytokine producing, with nearly 40% expressing at least three of the following cytokines: IL-4, IL-

5, IL-9, and IL-13; less than 10% of conventional Th2 counterparts produced three or more, suggestive of a more highly differentiated phenotype in Th2A cells.[73,75,76] In agreement, these cells exhibit low CD45RB and absent CD27 expression suggestive of a terminally differentiated phenotype. Th2A cells, but not Th2 cells, were observed to increase in frequency following allergen exposures.[73] While functional studies are lacking, the purported proliferation following allergen stimulation advocates for specialized role in allergic pathology. A summary of the immunological components of IgE memory is provided in Figure 1.

1.3.5 Regenerating IgE responses

Given that IgE^+ PCs are short-lived, lifelong IgE responses must be sustained through reactivation of MBCs. In general, MBCs are poised for rapid reactivation attributable to 1) an increased frequency over naïve counterparts and having previously undergone 2) affinity maturation and 3) CSR. Allergen-specific MBCs conform to two of these criteria. Firstly, the frequency of Ara h 2 (major peanut allergen)-specific B cells, for example, was found to be 8-fold higher in allergic compared to non-allergic donors (0.029% *versus* 0.0038%).[77] In mice, where allergen exposure is controlled, the increase in OVA-specific B cell frequency between immunized and unimmunized (naïve) groups is over 60-times.[78] Regarding affinity maturation, VDJ sequencing in mice and humans confirmed that the allergen-specific MBC compartment is highly mutated.[60,72,77] Indeed, the majority of allergen-specific $IgG1^+$ MBCs in mice co-express CD80 and CD73, indicative of a high mutational load and predisposition towards PC differentiation.[72,79] Thus, the preeminent

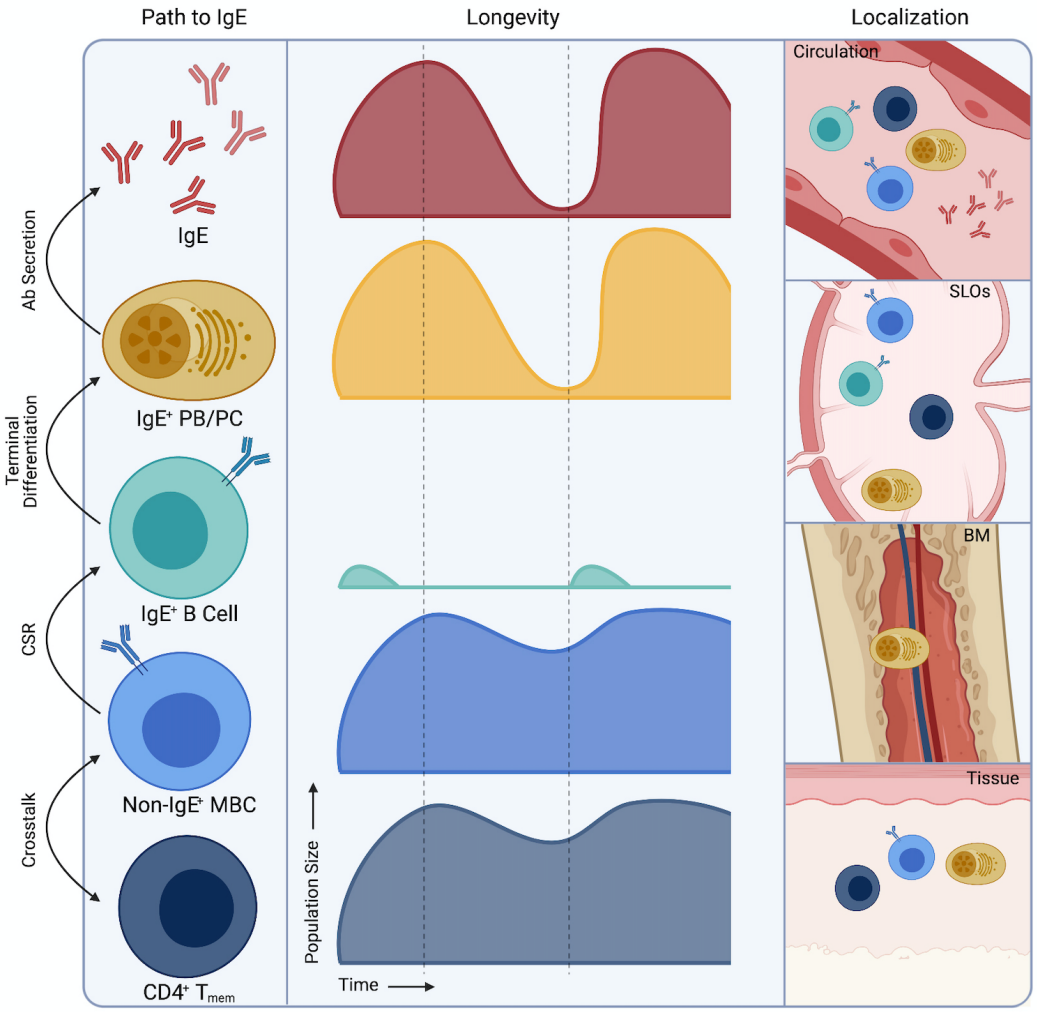

Figure 1. Key features of immunological memory mediating lifelong food allergies. Ab, antibody; CSR, class switch recombination; SLOs, secondary lymphoid organs; BM, bone marrow; PB, plasmablast. Space between dashed lines represents periods without allergen exposure.

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discrepancy between MBCs in allergy and in non-Th2 instances is the requirement of a secondary CSR event.

Both with and without the need for subsequent CSR, MBC reactivation requires cognate interactions with CD4 T cells. It has recently been demonstrated that the majority of CSR occurs prior to GC formation with aide from pre-TFh cells.[80] Accordingly, the dominant $CD80^+$ $CD73^+$ $IgG1^+$ MBC population produces early IgE^+ plasmablasts without re-entry into GCs.[72] Cognate interactions with pre-TFh cells are, therefore, likely to replenish the IgE^+ plasmablast pool, though the specific signals exchanged are not well-defined. For example, the requirement of T cell-derived IL-4 for IgE recall responses is unknown. It is plausible that the IL-4 requisite for IgE CSR in a primary response persists in a recall response; however, allergen-specific $IgG1^+$ MBCs express ε germline transcripts, suggestive of a pre-existing intrinsic commitment to an IgE fate.[60,81] Moreover, a Th2-polarized TFh cell subset produces IL-13 which was found necessary for the generation of high-affinity, anaphylactic-inducing IgE upon intranasal immunization with *Alternaria*.[29] Considering that $IgG1^+$ MBCs are already highly mutated and most do not re-enter GCs, IL-13 signaling may be irrelevant in recall responses. Comprehensively defining the cellular and molecular mechanisms through which IgE responses are replenished will be instrumental for the development of novel therapeutics.

1.4 Reprogramming adaptive immunity

1.4.1 Overview of plasticity

As implied by the name, a central property of *adaptive* immunity is to appropriately tailor – or adapt – a response dependent on the characteristics of the insulting antigen. Most often, the characteristics of a primary response are also suited for recall responses to enable efficient re-establishment of homeostasis; allergy is one exception. A deleterious response to innocuous antigen is produced upon allergic sensitization, the properties of which are maintained in subsequent exposures. This emphasizes the merit in modifying or *reprogramming* the existing adaptive response, such that the unnecessarily harmful reactivity is lost. The plasticity of said response underpins the ability for functional reprogramming.

Cell plasticity, defined as "the ability to reversibly assume different cellular phenotypes", is central to multiple instances of human health and disease, from neuroregeneration to malignancy.[82] Within the immune system, plasticity has perhaps been best studied within T cells. In response to microenvironmental cues, naïve CD4 T cells are polarized to various subsets, including Th1, Th2, Th9, Th17, Th22, TFh, and regulatory T cells (Treg). Classically, each subset has been defined by expression of a master transcriptional regulator and the profile of secreted cytokines; TFh and Th2 cells, for example, are identified through expression of Bcl6 + IL-21 and GATA3 + IL-4, respectively. It is evident that substantial heterogeneity exists in CD4 T cell responses, including allergy where all Th subsets have been identified, but the frequency and function of Th2 cells presides.[83] What remains of

interest is whether sufficient plasticity exists at the population or single-cell level, which may overtake the driving Th response. In allergy, this may occur through an outgrowth of existing non-Th2 cells or a direct reprogramming of Th2 cells to a non-pathogenic phenotype.

1.4.2 Plasticity of Th2 immunity

In our current understanding, the functions of MBCs are directly instructed by CD4 T cells, without capacity for autonomous fate decisions.[84] Phenotypic and/or isotypic reprogramming in the B cell arm must, therefore, arise from CD4 T cell reprogramming. Hence, an appraisal of CD4 T cell plasticity may provide insight as to the possibility of altering the fate of allergen-specific $IgG1^+$ MBCs.

Upon its discovery, Th2 polarization was largely regarded as irreversible; however, with further studies it has become apparent that CD4 T cell plasticity exists on a spectrum and is conditioned by many factors.[85] *In vitro,* Th2 cells can be reprogrammed into putative Th1 and Th9 cells with the addition of anti-IL-4 + IL-12 and transforming growth factor (TGF)-β, respectively.[86,87] Whether this phenomenon holds true *in vivo* remains to be seen. In contrast, the plasticity of inducible Tregs has been widely shown *in vivo.* In allergic disease, Tregs acquire a pathogenic Th2-skewed phenotype, which is ameliorated during oral immunotherapy (OIT).[88,89] In essence, an inverse relationship seems to exist between function and plasticity – the more highly specialized a cell becomes, the less phenotypic flexibility that persists. In this regard, the highly specialized Th2A cell subset is not likely

to exhibit phenotypic plasticity and, therefore, would not be amenable to reprogramming; however, it may be possible to reprogram their less differentiated precursors.

Naturally- and therapeutically acquired "tolerance" in food-allergic humans provides further evidence of immunological Th2 plasticity. For example, allergen immunotherapy (elaborated upon in section 1.5) promotes the production of Treg cells and allergen-specific IgG4 with a concurrent decrease in Th2 cells and serum IgE.[90,91] An increase in allergen-responsive Tregs is also observed in children who outgrow milk allergy.[92] In the rare instance of outgrowing peanut allergy, Th1 skewing is observed.[93] Unfortunately, it is not well understood how outgrowth is facilitated, but the mere occurrence suggests that, at least in young children, pathogenic responses to foods are malleable. In both allergen immunotherapy and allergy outgrowth, it remains unclear if the shift in T and B cell responses arises from changes at the population level or a direct reprogramming of the pathogenic allergen-specific memory cells. In OIT patients, 40% of peptides recognized by the induced peanut-specific IgG4 repertoire are *not* recognized by the peanut-specific IgE repertoire, suggesting that both *de novo* B cell responses and a redirection of $IgG1^+$ MBC fate towards IgG4 take place.[94] From a therapeutic standpoint, it is not necessarily relevant whether direct versus population reprogramming occurs, so long as any persisting pathogenic cells are outcompeted by the reprogrammed progenies.

1.5 Treatment of lifelong food allergies

1.5.1 Current landscape of food allergy treatments

The current standard of care for food allergies is strict allergen avoidance. Upon accidental allergen exposures, H1- and H2-antihistamines are indicated for treatment of mild, non-life-threatening reactions (*e.g.* urticaria).[1] The first-line treatment for food-induced anaphylaxis is rescue epinephrine. With this management approach, 200,000 Americans seek urgent medical care annually due to severe allergic reactions, equating to one emergency room intake every three minutes.[95]

The leading treatment option under investigation for food allergy is OIT. The U.S. is the first and only country to have approved the use of OIT for the treatment of peanut allergy (Palforzia), while in Canada and elsewhere in the world OIT remains experimental. In brief, OIT involves the consumption of a food allergen at gradually increasing amounts until reaching a predetermined dose, which is continued indefinitely. The primary objective of OIT is to induce desensitization, a state of clinical unresponsiveness to oral food challenge while maintaining daily low-dose allergen consumption. OIT has proven efficacious in inducing desensitization, though the induction of sustained unresponsiveness (maintained clinical unresponsiveness following treatment cessation) is rare with clinical reactivity returning in over 50% of patients one month post-treatment and 85% by three months.[96,97] While OIT is undisputedly effective at inducing desensitization, it comes at the cost of increased allergic reactions over the course of treatment.[98]

Despite its widespread investigation in clinical trials, the immunological mechanisms that facilitate desensitization in OIT remain speculative. Immunological features associated with successful desensitization include a decreased frequency of allergen-reactive Th2 and Th2A cells, with a concomitant increase in Tregs lacking pathogenic Th2-skewing.[99] On the B cell side, desensitized patients exhibit decreased allergen-specific IgE and increased IgG4 and IgA. How these immunological changes come about is largely unknown, though is proposed to occur through an increase in IL-10-secreting Tregs and/or regulatory B cells.[99]

1.5.2 Towards disease-transformative therapies

In the context of food allergy, a disease-transformative therapy would require a modification to the underlying immunological processes leading to pathogenic IgE production. As discussed in Section 1.3, evidence in mice and humans have persuasively established that allergen-specific memory cells, but not long-lived PCs, maintain lifelong allergies. Thus, a disease-transformative therapy should seek to disable, eliminate, or reprogram allergen-specific memory cells. This may be accomplished using biologics which deprive cells of signals required for their maintained pathogenic profile or, reversely, administering therapeutics which actively upregulate tolerogenic/non-pathogenic signals.

1.6 Project rationale and objectives

1.6.1 Rationale

Progress in therapeutic development is obstructed by an incomplete understanding of the mechanisms through which IgE responses are regenerated. Extensive research in mouse models has revealed key molecules for the process of allergic sensitization, such as IL-33, OX40L, and IL-4. The requirement of these molecules in a primary response, however, does not substantiate their requirement in a recall response. To date, very little of what we know to be true in allergic sensitization has been investigated in the context of allergic recall responses. While learning how to prevent allergic sensitization is imperative, it does not address the millions of patients worldwide currently living with food allergies. Moreover, mechanistic food allergy studies have been heavily reliant on mouse models. While a critical assessment of these models found them to be highly valid for translational research, there are of course, inherent limitations.[13]

This body of research broadly seeks to investigate the maintenance and potential reprogramming of immunological memory to food allergens. In Chapters 2 and 3 an emphasis was placed on developing novel methodologies which permitted the investigation of allergic recall responses with human cells. The role of IL-4 signaling in the recall response was central to Chapters 3 and 4. We hypothesized that, given the need for sequential CSR from IgG1 to IgE, IL-4 would remain critical in the recall response; but above and beyond CSR, IL-4 facilitates Th2 polarization and thus, loss of IL-4 signaling may dampen the overall Th2 signature.

1.6.1 Specific aims

1) Investigate the existence of IgE^+ MBCs in peanut-allergic and atopic humans (Chapter 2).
2) Determine the requirement of IL-4/IL-13 signaling for the regeneration of IgE responses to food allergens (Chapter 3).
3) Investigate the plasticity of allergen-specific MBCs and the potential to divert the response upon allergen recall (Chapter 4).

Chapter 2: Human BCR analysis of single-sorted, putative IgE$^+$ memory B cells in food allergy

Rodrigo Jiménez-Saiz*, Yosef Ellenbogen*, **Kelly Bruton***, Paul Spill, Doron D. Sommer, Hermenio Lima, Susan Waserman, Sarita U. Patil, Wayne G. Shreffler, Manel Jordana

***Equal contributions**

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MAIN TEXT

The biology of IgE$^+$ memory B cells (MBCs) remains enigmatic. The body of data from murine models demonstrates that IgE$^+$ MBCs are extremely rare, at best, therefore suggesting that IgE-mediated recall responses are derived from non–IgE-expressing MBCs, particularly IgG1.[1] In stark contrast, several human studies have claimed that a population of IgE$^+$ MBCs can be identified in PBMCs of healthy donors, atopic donors, and donors with food allergy,[2-4] as well as in the sputum of asthmatic patients.[5,6] Moreover, the presence of these cells in the circulation has been proposed as a prognostic marker of allergy.[2]

Divergent findings on the existence of IgE-expressing MBCs between mice and human subjects could be partly due to the precision of techniques used in their quantification. While the identification of IgE-expressing MBCs in mice has been comprehensive, identification of these cells in human subjects has relied on flow cytometry.[1] In light of this, we sought to establish a method to validate the flow cytometric identification of IgE^+ MBCs through genetic analysis of the BCR isotypes from single-sorted cells.

We modified a single-cell nested PCR amplification method[7] to amplify the variable region of IgE transcripts with primers shown specific to the 5′ end of IgE heavy chain constant region (Fig E1, *A*).[3] Then amplicons underwent Sanger sequencing, and resulting sequences were aligned to human heavy chain constant region alleles of all antibody isotypes to assess homology. To evaluate that the PCR technique amplifies IgE transcripts with high specificity and sensitivity, we single-sorted a human IgE-expressing cell line and performed IgE RT-PCR according to the same methodology (Fig E1, *B*).

Our protocol demonstrated high sensitivity, amplifying, on average, more than 90% of the cells tested, sequences of which all aligned to IgE constant region (IGHE) alleles (Fig E1, *C*). Additionally, we generated a DNA vector containing a human IgE backbone (IGE_v) for use as a positive control. To determine that our technique specifically amplifies IgE transcripts, we single-sorted peripheral blood B cells ($CD20^+$) that did not stain for IgE (Fig E1, *D*). Using the same RT-PCR strategy as in Fig E1, *A*, none of the IgE^- B cells were amplified (Fig E1, *E*), indicating that the technique is both sensitive and specific. Together,

these data demonstrate that this system accurately amplifies rearranged IgE heavy chain variable sequences, specifically in single-sorted IgE-expressing cells.

Reported frequencies of IgE-expressing MBCs in peripheral blood vary depending on the flow cytometric identification strategies.[2,3,6,8] A basic approach to identify these cells involves intracellular staining of IgE without preventing staining of cytotropic IgE.[5,6] A more stringent detection method involves the stepwise exclusion of each BCR isotype through extracellular staining.[3] With our single-cell IgE amplification protocol, we sought to validate the frequency of peripheral blood IgE-expressing MBCs in donors with peanut allergy using these previously reported flow cytometric approaches.

MBCs were identified as live singlet $CD20^{+}CD38^{lo\text{-}med}CD27^{+}$ and $IgD^{-}IgM^{-}$ cells, and IgE^{+} MBCs were further identified through basic IgE staining or the stepwise exclusion approach (Fig E2, *B*). Twelve putative IgE-expressing MBCs were single-sorted from each staining technique for subsequent single-cell nested PCR. Basic IgE staining revealed a 3.4% frequency of IgE^{+} MBCs from B cells. However, no cells were amplified with IgE-specific RT-PCR (Fig E2, *C*). The stepwise exclusion approach reported a frequency of putative IgE-expressing MBCs 20 times lower (0.17%), but likewise, none of the cells were amplified with IgE-specific RT-PCR (Fig E2, *D*).

To delineate the BCR identity of the spurious cells that fell into the IgE gate, we generated a cocktail of primers specific to IgG, IgA, and IgM heavy chain regions (GAM). By using our single-cell nested PCR strategy, more than 90% of the sorted cells were amplified with GAM primers, and the rest did not amplify (<10%). Amplicons were confirmed to align

predominantly with IGHG and to a minor extent with IGHA or IGHM through Sanger sequencing. These data demonstrate that previously reported IgE^+ MBC flow cytometry detection protocols result in a high rate of false-positive events.

The demonstration that previously reported flow cytometric methods for IgE^+ MBC identification are faulty prompted us to ascertain the true frequency of these cells. Because the stepwise exclusion method generated a substantially lower number of spurious events than the basic IgE staining method, we sought to modify the former to remove contamination from non–IgE-expressing cells, which largely originated from IgG^+ MBCs carrying cytotropic IgE (data not shown). This enhanced protocol consisted of purifying B cells from PBMCs and a stepwise exclusion of IgD^+, IgM^+, IgA^+, and IgG^+ cells (Fig 1, *A*). Furthermore, use of a polyclonal anti-IgG antibody markedly contributed to resolving the double-negative population of MBCs ($IgD^-IgM^-IgG^-IgA^-IgE^-$) compared with the previous stepwise exclusion method (3.8% vs 58.6%). Because B cells canonically express at least 1 BCR isotype, we reasoned that the population arose from B cells with a low BCR surface density, in which use of a polyclonal (rather than monoclonal) antibody to stain for surface IgG was superior. Notably, the improved staining resulted in a significantly lower frequency of cells in the IgE gate, at 0.006% of total B cells, which was considered background as it was comparable to the frequency observed in the fluorescence minus one control (0.01% of total B cells; Fig 1, *A*).

To validate that our enhanced staining technique was capable of detecting IgE^+ MBCs, we stimulated PBMCs in culture with IL-4 plus anti-CD40. As expected, culturing under these

conditions resulted in the robust emergence of IgE-secreting cells and IgE, as detected by using total IgE ELISpot and ELISA, respectively (Fig 1, *B*). A population of putative IgE^+ MBCs was observed through the enhanced stepwise exclusion method (Fig 1, *C*), and their IgE identity was confirmed through single-cell nested RT-PCR and Sanger sequencing (Fig 1, *D* and *E*). Further validation was carried out in PBMCs from 4 patients with atopic dermatitis and serum IgE levels of between 2370 and 6350 kIU/L. *Bona fide* IgE^+ MBCs, confirmed with Sanger sequencing, were identified in 2 of these 4 patients at a frequency of 0.0015% from total B cells (Fig 1, *F* and *G*).

With our enhanced detection method, we conducted analyses on PBMCs of 20 donors, which included patients with peanut allergy (n = 9; mean serum total IgE level, 196 kIU/L [11-890 kIU/L]) and nonallergic subjects (n = 10, see Table E1 in this article's Online Repository at www.jacionline.org). We detected similar frequencies of putative IgE^+ MBCs (percentage from total B cells: 0.0019 for patients with peanut allergy and 0.0046 for nonallergic subjects). However, in all instances there was no IgE amplification (Table I).

To ensure that IgE^+ MBCs were not being missed through our exclusion of $CD27^-$ cells, we sorted $CD27^-$ IgE^+MBCs because it has been speculated that MBCs arising extrafollicularly[3] might not gain CD27, the canonical MBC marker. Similarly, no PCR amplification occurred with IgE primers (data not shown).

Furthermore, we investigated the possibility that the polyclonal anti-IgG antibody could bind nonspecifically to IgE^+ MBCs because of serum IgG or IgA bound to MBCs, thus

masking IgE^+ MBCs cells in the IgG^+ or IgA^+ populations. Here we stained for IgA and IgG on the same fluorophore and flow-sorted class-switched MBCs that were positive for IgE (Fig E3, *A* and *B*). The frequency of this population was 0.074%, and genetic analysis demonstrated that these MBCs were of a non-IgE identity that presumably bound secreted IgE. Additionally, we single-sorted class-switched MBCs from the IgG, IgA, and (residual) double-negative gate. There was no PCR amplification with IgE primers but with GAM (Fig E3, *C* and *D*), thus supporting that the methodology used did not underestimate the frequency of IgE^+ MBCs.

The value of scientific knowledge relies to a large extent on the fidelity of the tools used to generate such knowledge. In this context, we provide a validated method to identify *bona fide* IgE^+ MBCs. Our data demonstrate the extreme rarity of these cells in the circulation of allergic patients, at least orders of magnitude lower than previously reported,[2,3] and are in agreement with human genetic studies that reported few IgE transcripts in the circulation but without unambiguously defining the B-cell phenotype (eg, MBCs and plasmablasts).[9] This finding strengthens the concept that the reservoir of IgE-secreting cells resides in MBCs of a non-IgE isotype and, as such, informs future research directions. Of note, it is possible that tissues from allergic subjects could harbor IgE^+ MBCs; this remains both a challenge and a prime venue for future efforts. Nevertheless, it is evident that the proposal that circulating IgE^+ MBCs could be a clinical marker for allergic disease is unproved.

METHODS

Flow cytometry

Antibodies were obtained from BioLegend (San Diego, Calif), Columbia Biosciences (Frederick, Md), BD Biosciences (San Jose, Calif), Miltenyi Biotec (Bergisch Gladbach, Cologne, Germany), eBioscience (Carlsbad, Calif), or Thermo Fisher Scientific (Waltham, Mass): CD38–phycoerythrin (PE)-Cy7 (clone HIT2); IgE-allophycocyanin (Columbia Biosciences SKU: D3-110-E); IgG-PE (clones G18-145 and HP6017 and Thermo Fisher Scientific Catalog #12-4998-82); IgA-PE (clone IS11-8E10); IgA-allophycocyanin (clone IS11-8E10); IgM–Brilliant Violet 510 (clone MHM-88); IgD–Brilliant Violet 421 (clone IA6-2); IgG-biotin (Thermo Fisher Scientific Catalog #A18815); CD20–Alexa Fluor 700 (clone 2H7); and CD27–fluorescein isothiocyanate (clone O323). In all assays 1×10^6 cells were first incubated with Human TruStain FcX (Fc Receptor Blocking Solution, BioLegend) or anti-human CD32 (FcγRII Blocker; STEMCELL Technologies, Vancouver, British Columbia, Canada) for 15 minutes on ice to block nonspecific staining and then incubated with fluorochrome-conjugated antibodies for 30 minutes on ice and protected from light. When IgG-biotin was used to label IgG^+ cells, cells were incubated for an additional 30 minutes with streptavidin-PE (BioLegend) on ice and protected from light. Dead cells were excluded by using the fixable viability dye eFluor780 (eBioscience) and by gating on singlets. Fluorescence minus one was used for gating. Data were acquired on a Fortessa (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, Ore), and single cells were sorted on a MoFlo XDP Cell Sorter (Beckman Coulter, Fullerton, Calif).

PCR amplification

Single cells were sorted into 96-well PCR plates (Thermo Fisher) containing 20 U of RNasin Ribonuclease Inhibitors (Promega, Madison, Wis), 2 μL of First Strand buffer (Thermo Fisher), and nuclease-free water to a volume of 10 μL per well. The cells were centrifuged at 4°C and 550*g* for 1 minute and immediately placed in a −80°C freezer. Next, heat lysis was performed by adding 3 μL of Nonidet P-40 Substitute (G-Biosciences, St Louis, Mo) and 150 ng of random hexamers (Thermo Fisher). The reaction was performed at 65°C for 10 minutes and then 25°C for 3 minutes. All thermocycler reactions were done with the Mastercycler Pro S (Eppendorf). cDNA was synthesized, as follows: 2 μL of 5× First Strand buffer (Thermo Fisher), 2 μL of 0.1 mol/L dithiothreitol (Qiagen, Hilden, Germany), 1 μL of 10 mmol/L of each dNTP, and 0.5 μL of SuperScript III (Thermo Fisher) were added to the plate containing the heat lysis contents (final volume, 19.5 μL). Reverse transcription was performed at 37°C for 1 hour and then 70°C for 10 minutes.

IgH amplification was accomplished by using a 2-step nested PCR, as previously described. Briefly, a mix of 6 forward primers[E1] and either a reverse primer specific to *IGHE*[E2] (first 5′-CATCACCGGCTCCGGGAAGTAG-3′ and second 5′-GTTTTTGCAGCAGCGGGTCAAG-3′) or a pool of reverse primers specific to *IGHM*, *IGHA*, and *IGHG* were used (*IGHM*: first 5′-CAGGAGACGAGGGGGAAAAG-3′ and second 5′-GAAAAGGGTTGGGGCGGATGC-3′; *IGHA*: first 5′-GCTCAGCGGGAAGACCTT-3′ and second 5′- GACCTTGGGGCTGGTCGGGGA-3′; and *IGHG:* first 5′-GCCAGGGGGAAGACSGATG-3′ and second 5′-GACSGATGGGCCCTTGGTGGA-3′).[E3] The first PCR reaction contained 8 μL of cDNA mixture, 1 U of HotStar Plus Taq

(Qiagen), 200 nmol/L of each primer, 400 μmol/L dNTP (Thermo Fisher), 5 μL of 10× PCR buffer (Qiagen), and nuclease-free water to a final volume of 50 μL. The reaction was performed starting with 3 cycles of preamplification of 95°C for 45 seconds, 45°C for 45 seconds, and 72°C for 45 seconds, followed by 30 cycles of 94°C for 45 seconds, 50°C for 45 seconds, and 72°C for 1 minute and 45 seconds and a final extension of 72°C for 10 minutes. The second PCR reaction contained 4 μL of PCR 1 product, 5 μL of 10× Pfu buffer (Agilent, Santa Clara, Calif), 1.25 μL of 10 mmol/L dNTP (Thermo Fisher), 400 nmol/L of each primer, 1.25 U of Pfu polymerase (Agilent), and nuclease-free water to a final concentration of 50 μL. The reaction was performed for 30 cycles at 94°C for 45 seconds, 50°C for 45 seconds, and 72°C for 1 minute and 45 seconds, followed by a final extension of 72°C for 10 minutes.

The second PCR product was visualized on a 1.5% agarose gel, and the expected band size was approximately 400 bp. Amplified IgH sequences were enzymatically purified by using ExoSAP-IT PCR Product Cleanup Reagent (Thermo Fisher) and subsequently Sanger sequenced by using the forward and reverse primers (GENEWIZ). The sequences were analyzed by using IMGT/HighV-QUEST (http://imgt.org/HighV-QUEST) for V, D, and J sequences with the highest identity, as well as nucleotide and amino acid mutations from their germline sequences.

Generation of a DNA vector containing a human IgE backbone

To generate a human IgE, we started with a heavy chain IgG1 vector (gifted by Michel C. Nussenzweig) previously modified to include an Ara h 2 variable chain.[E4] The human ε

constant region was amplified from an anti-OVA human IgE vector[E5] with primers (5′-TTTTGTCGACGGCGCACCA-3′ and 5′-TTTTAAGCTTCTCAATGGTGGTGATGTTTA-3′) to add flanking *Sal*I and *Hind*III restriction enzyme sites. The human ε constant region then replaced the γ1 constant region under the CMV provider by using the *Sal*I and *Hind*III restriction enzyme sites. Sanger sequencing confirmed successful insertion of the ε constant region.

Study population

A cohort of 10 blood donors with peanut allergy and 10 nonallergic blood donors were recruited from McMaster University (Hamilton, Ontario, Canada). Allergy to peanut was ascertained by using a peanut-specific IgE ImmunoCAP performed at LRC Hamilton (McMaster Children's Hospital) and by using skin prick testing. Patients with peanut allergy were considered for inclusion with peanut-specific serum IgE levels of greater than 0.35 kU/L and skin prick test responses of 3 mm or more larger than those elicited by the saline control. Total serum IgE levels were quantified by using the IMMAGE 800 (Beckman Coulter) performed at LRC Hamilton for a general measure of atopy. We recruited an additional 4 participants with total serum IgE levels of greater than 2300 kIU/L and received 3 tonsil discards from subjects undergoing routine tonsillectomies. Exclusion criteria for all recruited donors included the following: allergen immunotherapy, previous or current omalizumab (Xolair) treatment, other systemic immunomodulatory treatments (ie, rituximab), or autoimmune/immunodeficiency diseases. Patients' demographics and

allergic indicators are summarized in Table E1. All donors were recruited with written consent and ethical approval from Hamilton Integrated Research Ethics Board.

Mononuclear cell isolation and B-cell enrichment

Up to 80 mL of peripheral blood was collected into heparinized tubes (BD), and tonsils were crushed into a single-cell suspension. PBMCs were isolated through Ficoll-Paque (GE Healthcare, Chicago, Ill) density gradient centrifugation. Immediately afterward, B cells were isolated from PBMCs by using a negative selection magnetic separation kit (19054; STEMCELL Technologies) with at least 70% purity.

PBMC culture

PBMCs were cultured in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% human AB serum (Corning, Corning, NY), 10 mmol/L HEPES, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 55 μmol/L 2-mercaptoethanol, 1% l-glutamine, and 1% penicillin-streptomycin. Cells were plated at a density of 1.5×10^6 cells/mL in 24-well plates. Stimulated cells were treated with 68.7 ng/mL (8000 IU) IL-4 (Sigma-Aldrich, St Louis, Mo) and 5 μg/mL anti-CD40 (Bio X Cell, West Lebanon, NH) on day 1. Cells were incubated at 37°C and 5% CO_2 for the duration of culture. On days 4 and 8 of culture, 1 mL of cell-free supernatant was withdrawn and replaced with fresh media. Supernatants were also withdrawn on day 11 of culture and stored at −80°C for later analysis of total IgE by means of ELISA. After 11 days in culture, cells were harvested, and IgE-secreting cells were quantified by using ELISpot.

ELISA and ELISpot

For total IgE ELISA, MaxiSorb plates (Thermo Fisher Scientific) were coated with 0.5 μg/mL anti-human IgE (555894; BD PharMingen, San Jose, Calif) in carbonate-bicarbonate buffer overnight at 4°C. Coated wells were blocked with 5% skim milk in PBS for 2 hours at room temperature, followed by 3 washes (1× PBS and 0.05% Tween 20). Cell-free supernatant samples and a serial dilution of purified human IgE (401152; Calbiochem, San Diego, Calif) were incubated overnight at 4°C. Wells were washed 3 times and incubated with 1 μg/mL biotinylated anti-human IgE (A18803; Invitrogen, Carlsbad, Calif) in 1% skim milk for 2 hours at room temperature. Subsequently, wells were washed 3 times and incubated with alkaline phosphatase streptavidin for 1 hour at room temperature. Plates were developed with *p*-nitrophenyl phosphate, and the reaction was stopped with 2N NaOH. ODs were measured at 405 nm (Multiskan FC; Thermo Scientific).

A commercially available ELISpot kit (3810-2H; Mabtech, Stockholm, Sweden) was used for detection of IgE-secreting cells. On day 11 of culture, samples were plated in duplicates at 4×10^6 cells/mL. Plates were imaged with the ImmunoSpot S6 Analyzer, and spots were counted independently by 2 blinded investigators.

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TABLES & FIGURES

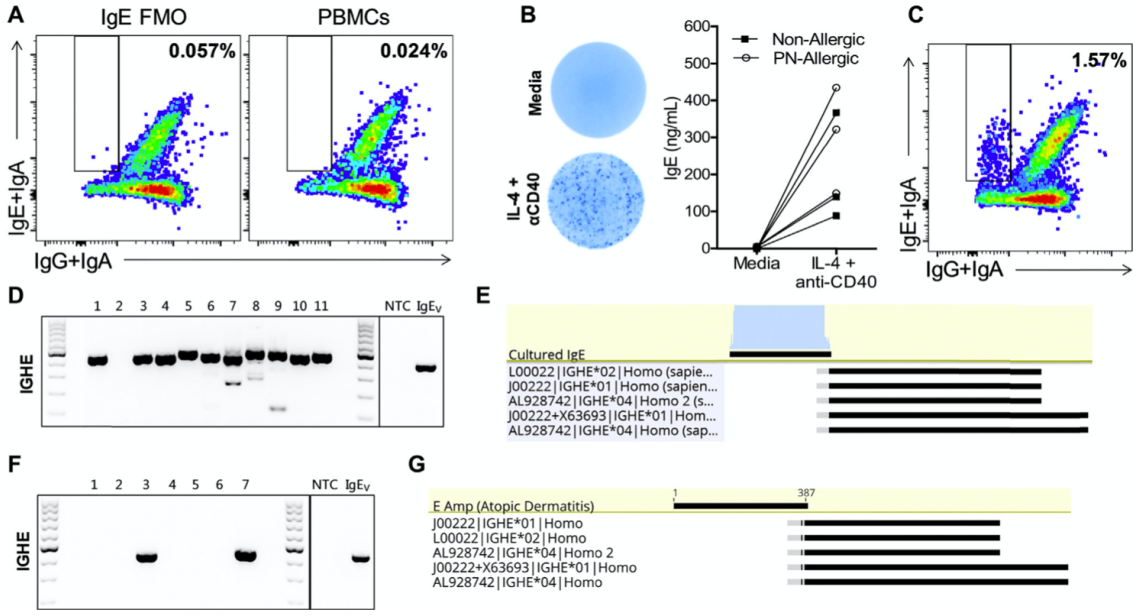

Figure 1. Enhanced flow cytometric method for detection of IgE$^+$ MBCs. **A**, Cytometric detection of IgE$^+$ MBCs. FMO, Fluorescence minus one control. **B** and **C**, PBMCs cultured with IL-4 plus anti-CD40 were analyzed by means of ELISpot and ELISA (Fig 1, B) and single-sorted (Fig 1, C). PN, Peanut. **D-G**, Amplification of IgE transcripts of single-sorted cells from positive control (Fig 1, D and E) or patients with atopic dermatitis (Fig 1, F and G) and alignment to the constant region of IGHE. Data are representative of 2 independent experiments (1-2 donors per experiment and 12-24 single-sorted cells per donor).

TABLE I. Quantification of IgE^+ MBCs in healthy and allergic donors

Tissue	Donor ID	Allergic status	Mononuclear cells	Purified B cells	Events in $CD20^+$ $CD38^{lo\text{-}med}$ gate	Events in IgE gate	Sorted cells	IGHE amplification
Blood	P001	–	250,000,000	9,540,000	600,304	21	6	0
	P003	–	123,000,000	8,640,000	169,267	29	12	0
	P007	–	125,000,000	2,685,000	645,055	5	3	0
	P009	–	98,600,000	4,140,000	325,734	12	12	0
	P014	–	109,000,000	2,820,000	605,535	8	8	0
	P021	–	173,000,000	26,000,000	440,963	2	1	0
	P025	–	86,600,000	1,401,000	246,090	20	20	0
	P026	–	94,200,000	1,494,000	250,018	14	10	0
	P030	–	78,800,000	1,128,000	132,157	4	4	0
	P031	–	85,400,000	945,000	172,260	4	4	0
	P008	Peanut	125,000,000	8,160,000	335,641	20	20	0
	P011	Peanut	250,000,000	7,150,000	520,021	6	3	0
	P013	Peanut	210,000,000	5,450,000	690,015	13	12	0
	P016	Peanut	125,000,000	1,068,000	143,759	5	5	0
	P017	Peanut	125,000,000	1,467,000	213,345	11	10	0
	P020	Peanut	124,000,000	3,900,000	537,856	4	1	0
	P024	Peanut	71,800,000	1,026,000	166,313	3	1	0
	P028	Peanut	94,800,000	1,440,000	157,340	1	1	0
	P029	Peanut	54,000,000	1,467,000	277,359	2	2	0
Tonsils	TP-9	–	10,000,000	–	204,125	10	7	0
	TP-10	–	10,000,000	–	68,461	15	12	0
	TP-11	–	10,000,000	–	56,127	6	6	0

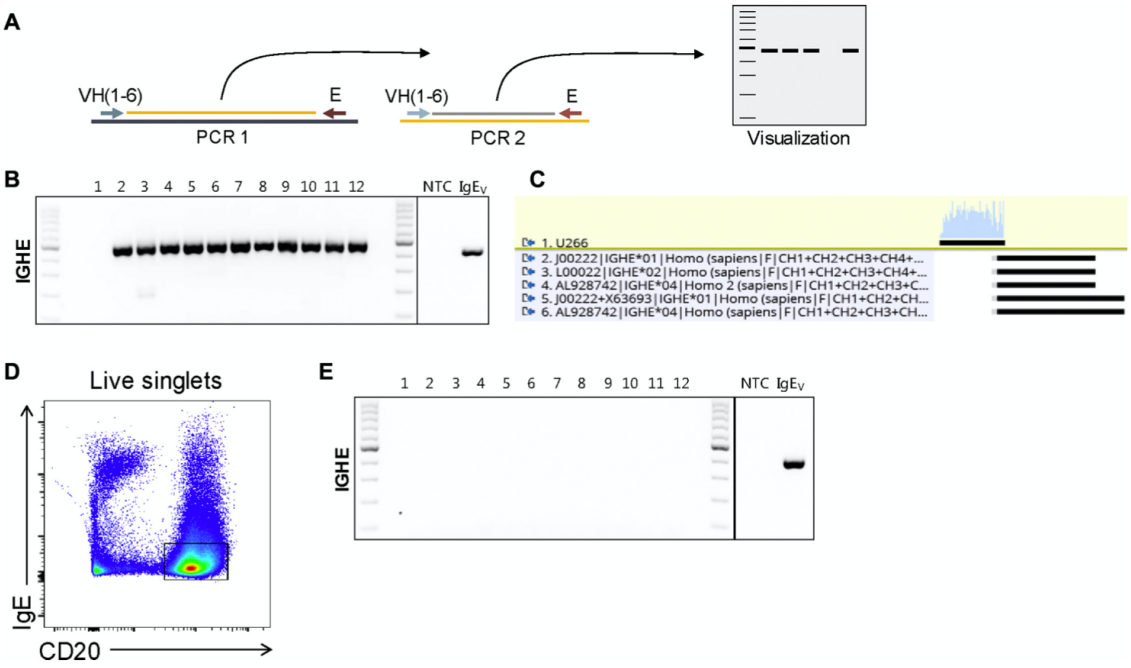

Figure E1. Methodology to genetically validate the IgE identity of single-sorted cells. **A**, Schematic of the method. **B**, Single-sorted human IgE-expressing myeloma cells were assayed by using nested PCR. **C**, IgE transcripts were shown to align with IGHE alleles. **D**, Peripheral blood $CD20^{+}$ B cells that stained negative for IgE were single-sorted. **E**, IgE transcripts did not amplify, as evidenced by using DNA gel electrophoresis.

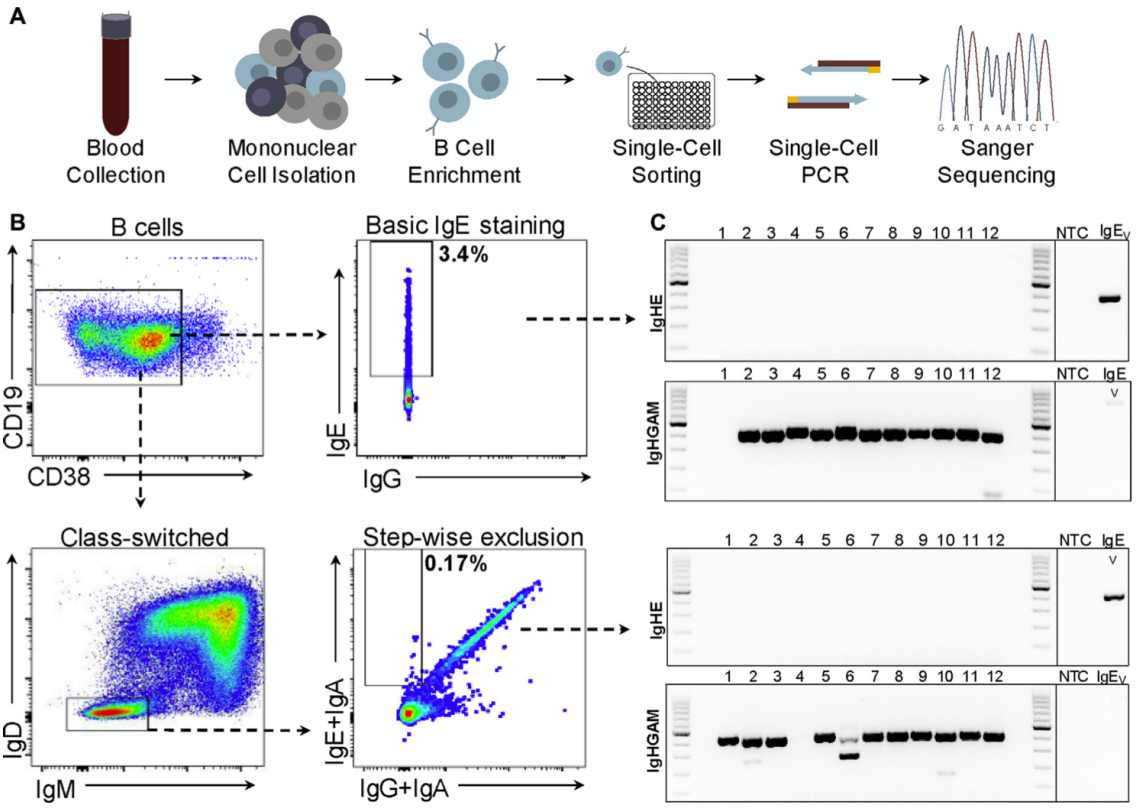

Figure E2. BCR analysis of single-sorted, putative IgE$^+$ MBCs stained with commonly used methods demonstrates a non-IgE identity. A, Schematic of the experimental design. **B**, Gating strategy used with a basic IgE staining (upper) or stepwise exclusion (lower) method. **C**, BCR amplification with primers specific for IGHE or a mix (IgHGAM). Data are representative of 5 independent experiments (1-2 donors per experiment and 12-24 single-sorted cells per donor).

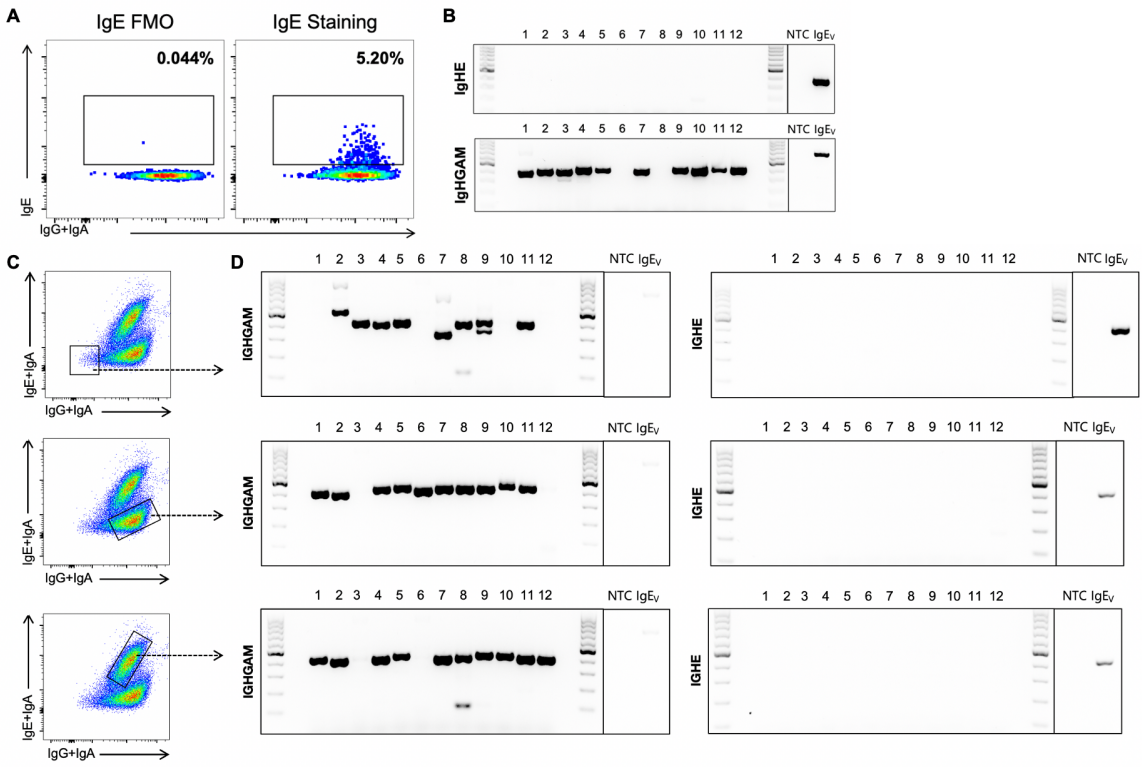

Figure E3. Assessment of cytotropic (IgG and/or IgA) and negative staining of IgE$^+$ MBCs. A and **C,** Cytometric detection and sorting of class-switched MBCs from different gates. FMO, Fluorescence minus one control. **B** and **D**, BCR amplification with primers specific for IgE (IGHE) or a mix specific of IgG, IgA, and IgM (IgHGAM) of single-sorted cells. Data are representative of 2 independent experiments (2 donors per experiment and 12-18 single-sorted cells per donor).

TABLE E1. Patients' profiles

	Donor ID	Sex	Age	Serum IgE (kIU/L)	Atopic dermatitis	Clinical reactivity to peanut	Peanut IgE (kU/L)	Skin prick test (S/H/P)
Nonallergic subjects	P001	M	20	<5	No	No	<0.1	2/5/2
	P003	M	23	96	No	No	<0.1	1/3/1
	P007	M	23	<5	No	No	<0.1	1/4/1
	P009	F	24	<5	No	No	<0.1	1/4/1
	P014	M	22	230	No	No	0.34	2/5/2
	P021	M	21	45	No	No	<0.1	1/4/2
	P025	F	34	40	No	No	<0.1	1/5/1
	P026	M	19	37	No	No	0.11	1/6/1
	P030	M	24	9	No	No	<0.1	1/4/1
	P031	M	23	12	No	No	<0.1	1/5/1
	TP09	M	2	NA	No	No	NA	NA
	TP10	M	30	NA	No	No	NA	NA
	TP11	M	5	NA	No	No	NA	NA
Patients with atopic dermatitis	P006	F	23	5430	Yes	Yes	13.48	1/4/5
	P034	F	39	3760	Yes	No	10.29	NA
	P035	M	53	2370	Yes	No	NA	NA
	P036	F	20	6350	Yes	No	NA	NA
Patients with peanut allergy	P008	M	32	130	No	Yes	15.87	1/4/7
	P011	M	19	210	No	Yes	0.66	1/5/7
	P013	M	20	890	No	Yes	28.09	1/5/4
	P016	M	21	91	No	Yes	1.66	1/4/8
	P017	M	22	120	No	Yes	43.28	1/4/10
	P020	M	26	256	No	Yes	7.56	1/4/12
	P024	M	60	37	No	Yes	5.54	1/4/10
	P028	M	25	27	No	Yes	1.11	1/4/7
	P029	F	23	11	No	Yes	3.79	1/4/6

F, Female; *M*, male; *NA*, not available; *S/H/P*, saline/histamine/peanut.

Chapter 3: Interrupting reactivation of immunologic memory diverts the allergic response and prevents anaphylaxis

***Kelly Bruton**, *Paul Spill, Shabana Vohra, Owen Baribeau, Saba Manzoor, Siyon Gadkar, Malcolm Davidson, Tina D. Walker, Joshua F.E. Koenig, Yosef Ellenbogen, Alexandra Florescu, Jianping Wen, Derek K. Chu, Susan Waserman, Rodrigo Jiménez-Saiz, Slava Epelman, Clinton Robbins, Manel Jordana ***Equal contributions**

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ABSTRACT

Background: IgE production against innocuous food antigens can result in anaphylaxis, a severe life-threatening consequence of allergic reactions. The maintenance of IgE immunity is primarily facilitated by IgG^+ memory B cells, as IgE^+ memory B cells and IgE^+ plasma cells are extremely scarce and short-lived, respectively. *Objective:* Our aim was to investigate the critical requirements for an IgE recall response in peanut allergy. *Methods:* We used a novel human PBMC culture platform, a mouse model of peanut allergy, and various experimental readouts to assess the IgE recall response in the presence

and absence of IL-4Rα blockade. *Results:* In human PBMCs, we have demonstrated that blockade of IL-4/IL-13 signaling aborted IgE production after activation of a recall response and skewed the cytokine response away from a dominant type 2 signature. T_H2A cells, identified by single-cell RNA sequencing, expanded with peanut stimulation and maintained their pathogenic phenotype in spite of IL-4Rα blockade. In mice with allergy, anti–IL-4Rα provided long-lasting suppression of the IgE recall response beyond antibody treatment and fully protected against anaphylaxis. *Conclusion:* The findings reported here advance our understanding of events mediating the regeneration of IgE in food allergy.

INTRODUCTION

Allergens trigger the production of IgE, the key effector molecule in allergic reactions and, therefore, a critical therapeutic target. In contrast to T_H1 immunity, wherein infection or vaccination generates antigen-specific IgG titers that typically persist for decades,[1] allergen-specific IgE levels drastically decline after extended periods without reexposure. For example, in seasonal allergic rhinitis, serum allergen-specific IgE levels are cyclic and concordant with on- and off-season allergen peaks.[2,3] A similar phenomenon has been observed in humans with allergy to the common fish parasite *Anisakis* spp after strict avoidance of fish consumption for at least 10 months.[4] This is challenging to observe in human food allergy, in which persistently increased peanut-specific IgE levels are likely the consequence of high rates of accidental exposures[5] or even higher rates of subclinical exposures.[6] In this regard, in a mouse model of peanut allergy, peanut-specific IgE titers became undetectable 6 months after sensitization.[7] Irrespective of declining or absent

allergen-specific IgE titers, the capacity of mice and humans with allergy to regenerate IgE following reexposure can remain for a lifetime, presumably via reactivation of memory B cells (MBCs) and subsequent reemergence of IgE-secreting plasma cells (PCs).

Recent evidence in mice and humans has demonstrated that IgE^+ MBCs are extremely rare.[7-9] The predisposition of IgE^+ B cells to terminally differentiate into IgE-secreting PCs, rather than quiescent MBCs, is thought to be a consequence of chronic membranous IgE signaling.[10,11] Moreover, IgE^+ PCs are shorter-lived than their IgG counterparts, thus resulting in declining serum IgE titers over time. IgE expression can be achieved through distinct pathways of class switch recombination, including direct class switching from IgM and sequential class switch from IgM to 1 or more intermediate isotypes.[12] Analyses of human immunoglobulin heavy chain (IgH) repertoires identified $IgG1^+$ B cells as the dominant intermediate of IgE^+ B cells.[13] *In vivo* experimentation corroborated a preferential switch through IgG and therefore positioned IgG^+ MBCs as the chief reservoir of IgE memory responses.[7,14-17] IgG^+ MBCs class switching to IgE occurs under the influence of type 2 cytokines; however, the critical requirements that facilitate regeneration of IgE^+ PCs remain to be fully elucidated.

IL-4 and IL-13, hallmark type 2 cytokines, are involved in T_H2 cell polarization, B-cell proliferation, and IgE class switching. Both cytokines exert their biologic activity through a shared heterodimeric receptor composed of IL-4Rα and IL-13Rα1, with IL-4 having a second signaling receptor composed of IL-4Rα and γc.[18] Whereas mouse models of Th2 cell immunity to helminth infection support a critical requirement of IL-4 signaling for

primary and secondary IgE production,[19-22] the function of IL-4/IL-13 signaling in class switching in human lymphocytes, as well as in the context of allergic (ie, nonhelminth) Th2 cell immunity, remains incompletely understood.

Here, we have used a novel model system, comprehensive protein analysis, and single-cell transcriptomic analysis to interrogate the activation of Th2 cellular and humoral immune memory in humans and mice with food allergy. We first developed a novel *in vitro* human platform with PBMCs that, for the first time, enabled joint T-cell and B-cell readouts. Our characterization of cytokine, cellular, and single-cell transcriptomic profiles in the human recall response to peanut unveiled unique populations of Th2A cells, *MAL*$^{+}$ CD4 T cells, and *IGHE*$^{+}$ MBCs that were differentially regulated by IL-4 signaling. We have also shown that these findings are clinically relevant *in vivo* because treatment of mice with peanut allergy and anti–IL-4Rα fully aborted the formation of peanut-specific IgE and prevented anaphylaxis. These effects were sustained even beyond IL-4Rα blockade, highlighting the potential to reprogram the pathogenic allergic response. Collectively, these data elucidate key events in human and mouse Th2 cell recall responses, which implicates anti–IL-4Rα as a persuasive therapeutic approach for peanut allergy.

METHODS

Study population

A cohort of 12 blood donors with peanut allergy and 5 blood donors without allergy were recruited from McMaster University and McMaster Children's Hospital (Hamilton,

Ontario, Canada). Peanut allergy was determined by peanut-specific IgE ImmunoCap performed at Laboratory Reference Centre Hamilton (McMaster Children's Hospital), saline and histamine-controlled skin prick test, and clinical history of reactivity. Individuals with peanut allergy, a peanut-specific serum IgE level higher than 0.35 kU/L, and a skin prick test result at least 3 mm greater than the saline control were considered for inclusion. All allergy testing was performed within 6 months before blood was drawn for experimentation. Exclusion criteria for all recruited donors included allergen immunotherapy; previous or current omalizumab, dupilumab, or other systemic immunomodulatory treatment; or autoimmune or immunodeficiency disease. Patient demographics and allergy indicators are summarized in Table E1. All donors were recruited with written consent and ethical approval from the Hamilton Integrated Research Ethics Board.

Mice

Female C57BL/6 mice (5-8 weeks old) were purchased from Charles River Laboratories (Wilmington, Mass). The 4get mice and KN2 mice were a generous gift from Dr Irah King (McGill University). Homozygous 4get (*Il4*^{tm1Lky})[23] and KN2 (*Il4*$^{tm1(CD2)Mmrs}$) mice on C57BL/6 backgrounds were crossbred to generate 4get/KN2 mice, which were described previously.[24] All experimentation was in compliance with the McMaster University Research Ethics Board.

Food allergy models

The mice were epicutaneously sensitized to and challenged with either crude peanut extract (CPE) (Greer, Cambridge, Mass) or ovalbumin (OVA) (Millipore Sigma, St Louis, Mo), as previously described.[16,25,26] Briefly, 200 μg of CPE or OVA was applied on the shaved and tape-stripped backs of mice for 10 days. Two weeks after the final sensitization, 100 μg of CPE or OVA was injected subcutaneously (in the lower back) 3 times in 1 week. For the anti–IL-4Rα clearance experiments, 3 additional subcutaneous injections of CPE occurred 6 weeks after anti–IL-4Rα treatment. Two weeks after the subcutaneous injections, the mice were challenged through intraperitoneal injection of 5 mg of CPE. Core body temperature and hematocrit were recorded, as previously described.[26] Clinical signs were graded on a scale of 0 to 5 (0 = no clinical signs; 1 = in-ear scratching; 2 = decreased activity; 3 = motionless; 4 = no response to whisker stimuli; and 5 = seizure, moribund, death). Retroorbital bleeds were performed to collect serum for analysis of peanut-specific antibodies by ELISA.

For intraperitoneal sensitization, 250 μL of Alhydrogel 2% (an aluminum hydroxide [alum], InvivoGen, San Diego, Calif) with 250 μg of CPE was delivered intraperitoneally twice, 1 week apart. After 2 weeks of rest, the mice were bled and challenged, as already described.

Antibody production and administration

A hybridoma secreting monoclonal rat IgG2a anti-mouse IL-4Rα (M1), generated by the Immunex Corporation (Amgen, Thousand Oaks, Calif), was generously provided by Dr Fred Finkelman (University of Cincinnati, Cincinnati, Ohio). Hybridomas were expanded

in tissue culture flasks with serum-containing media and then transferred in serum-free media to a spinner flask for up to 10 days. Antibodies were purified from culture supernatant with Protein G Sepharose (GE Healthcare, Chicago, Ill). Next, 1 mg of anti–IL-4Rα or an isotype control (IC) (rat IgG2a anti-trinitrophenol; Bio X Cell, Lebanon, NH) was injected intraperitoneally on the day before subcutaneous reexposure, or 6 weeks, 2 weeks, or 1 day before sensitization. IgG-mediated anaphylaxis was blocked with rat IgG2b anti-mouse CD16/32 (Bio X Cell), as previously published.[27] CD4 and NK1.1 depletions were carried out with rat anti-mouse CD4 (GK1.5; Bio X Cell) and rat anti-mouse NK1.1 (PK136; Bio X Cell) antibodies, respectively. Two intraperitoneal injections of 200 μg of anti-CD4 or anti-NK1.1 were administered before subcutaneous reexposure and once during reexposures.

Tissue processing

The mice were humanely killed under inhalational anesthetic by cervical dislocation, and their inguinal lymph nodes and/or spleens were collected immediately thereafter. The lymph nodes were crushed between frosted slides into a single-cell suspension with HBSS. The spleens were crushed through a 40-μm filter using a 3-mL syringe plunger with HBSS. Cell suspensions were passed through a 40-μm filter. Red blood cells in splenocyte suspensions were removed with ammonium-chloride-potassium lysis buffer.

Splenocyte culture

Three days after systemic allergen exposure, the spleens were collected and processed under sterile conditions for culture. Cells were resuspended in complete RPMI medium (10% FBS, 1% penicillin-streptomycin, 1% l-glutamine, and 55 μM 2-mercaptoethanol) and plated in triplicate at 8×10^6 cells/mL. Cells were left unstimulated or were stimulated with 250 μg/mL CPE. Cell-free supernatants were collected on day 5 and stored at –80°C until use.

PBMC isolation and storage

Up to 80 mL of peripheral blood was collected into heparinized tubes (BD, San Jose, Calif). PBMCs were isolated via Ficoll-Paque (GE Healthcare) density gradient centrifugation, filtered, and resuspended in either enriched RPMI medium for culture or 10% dimethyl sulfoxide–supplemented FBS to be frozen at –80°C overnight in appropriate freezing containers and subsequently in liquid nitrogen for long-term storage. The PBMCs used in this study were frozen for 0 to 6 months before use.

PBMC culture

Before culture, PBMC viability was assessed by using automated viable cell counting (ADAM MC, Bulldog Bio, Portsmouth, NH). All fresh PBMCs were characterized by 95% viability, and freeze-thawed PBMCs were determined to have 90% viability. Cells were resuspended in enriched RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated human AB serum (Corning, Corning, NY), 10 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 55 μM 2-mercaptoethanol, 1% l-

glutamine, and 1% penicillin-streptomycin. Cells were plated at least in duplicate at a density of 1.5×10^6 per mL in 24-well plates for up to 12 days. On days 4 and 8, 1 mL of cell-free supernatant was collected, stored at –80°C, and replaced with 1 mL of enriched medium. As a positive control for IgE production, cells were stimulated with 68.7 ng/mL (8000 IU) of rhIL-4 (Millipore Sigma) and 5 μg/mL of anti-CD40 (Bio X Cell) at the start of culture. As a negative control, cells were incubated in medium alone. Allergen-stimulated cells were cultured with 2.5 ng/mL of CPE. Cells incubated with neutralizing antibodies against IL-4Rα and keyhole limpet antigen (KLH [IgG2a IC for IL-4Rα antibody]) received 50 μg/mL of antibody in the presence or absence of CPE.

ELISA and ELISpots

Peanut-specific mouse IgG1, IgE, and total human IgE ELISAs were performed as previously described.[7,28] For the detection of human peanut-specific IgE antibodies, MaxiSorp plates (ThermoFisher Scientific, Waltham, Mass) were coated with 4 μg/mL of CPE in carbonate-bicarbonate buffer and kept overnight at 4°C. The wells were blocked with 5% skim milk powder in PBS for 2 hours followed by 3 washes with 0.05% Tween-20. Samples were plated and incubated overnight at 4°C. Supernatant samples were lyophilized (Modulyo Freeze Dryer, ThermoFisher Scientific) overnight and reconstituted (10×-concentrated) in 5% skim milk. After washing, biotin-conjugated goat anti-human IgE, cross-adsorbed (polyclonal; Invitrogen, Carlsbad, Calif) was added at a rate of 0.25 μg/mL. Following a 2-hour incubation, the wells were washed, and streptavidin–horseradish peroxidase (BD) was added for 1 hour. The ELISAs were developed with

3,3',5,5'-tetramethylbenzidine (Millipore Sigma), and ODs were acquired with a Multiskan FC photometer (ThermoFisher Scientific). Rat IgG2a ELISA (Invitrogen) and total IgE ELISpots (Mabtech, Nacka, Sweden) were performed with commercially available kits, as per the manufacturers' recommendations.

Cytokine analysis

Cytokines in PBMC culture supernatant were assayed by using MILLIPLEX Immunology Multiplex Assays (HCYTOMAG-60K, Millipore Sigma) and analyzed by using MAGPIX XMAP Technology (Luminex, Austin, Tex). Supernatants from day 4 of culture were assayed for either IL-4, IL-5, and IL-13 or a discovery panel that included sCD40L, epidermal growth factor, eotaxin/CCL11, fibroblast growth factor-2, Fit-3 ligand, fractalkine, G-CSF, GM-CSF, GRO, IFN-α2, IFN-γ, IL-1α, IL-1β, IL-1Rα, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p40), IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, MCP-1, MCP-3, macrophage-derived chemokine (CCL22), macrophage inflammatory protein-1α (MIP-1α), MIP-1β, TGF-α, TNF-α, TNF-β, and vascular endothelial growth factor. Cytokines detected in levels above background and falling within the detectable range (3.2-10000 pg/mL) were included in the statistical analyses.

Cytokines in mouse splenocyte culture supernatant were assayed by using MILLIPLEX Immunology Multiplex Assays (MCYTOMAG-70K, Millipore Sigma) and analyzed by using MAGPIX XMAP Technology (Luminex). Supernatants from day 5 of culture were assayed for IL-4, IL-13, and IFN-γ. Cytokines detected in levels above background and

falling within the detectable range (3.2-10000 pg/mL) were included in the statistical analyses.

Tetramer staining and enrichment

Phycoerythrin (PE)-conjugated peptide:MHC class II tetramers were prepared as previously described[29] and generously provided to us by Dr Marc Jenkins (University of Minnesota). Inguinal lymph node single-cell suspensions were pooled from 4 or 5 mice and incubated (for 1 hour at room temperature) in 100 μL of fluorescence-activated cell sorting (FACS) buffer (0.5% BSA and 2% 0.5 mM EDTA in PBS) with 1 μL each of 2 peptide:MHC class II tetramers containing the immunodominant OVA epitopes OVA2C ($OVA_{325-335}$) and OVA3C ($OVA_{327-338}$).[30] The cells were washed with 10 mL of FACS buffer to remove unbound tetramer. The pellets were resuspended and incubated (for 30 minutes on ice) with 50 μL of anti-PE microbeads (Miltenyi Biotec, Cologne, Germany). Next, 5 mL of FACS buffer was added to cells and the cell suspension was run through an LS Column (Miltenyi Biotec) on a magnetic stand. Tetramer-unbound cells (the flowthrough fraction) were collected. Columns were removed from the magnetic stand and 5 mL of FACS buffer was plunged through the column to collect the tetramer-bound cells (the enriched fraction). The flowthrough and enriched fractions were then counted, pelleted, and stained with fluorochrome-conjugated antibodies for flow cytometry.

Flow cytometry and bulk sorting

Cells (cultured human PBMCs or mouse inguinal lymph nodes) were plated in 96-well plates (<5×10^6 cells/well) in FACS buffer before extracellular staining. The cells were incubated with an antibody cocktail and Fixable Viability Dye eFluor780 (eBioscience, San Diego, Calif) for 30 minutes on ice. The fluorochrome-conjugated antibodies used in the human flow cytometric analyses include CD3– fluorescein isothiocyanate (HIT3a), CD4-BV510 (OKT4), CD19-BV421 (SJ25C1), and CD45-AF700 (HI30). For apoptosis analysis, cells were stained with annexin V–PE in Annexin V Binding Buffer (BioLegend, San Diego, Calif). For the mouse flow cytometric analyses, the following fluorochrome-conjugated antibodies were used: B220-BV711 (RA3-6B2), F4/80-BV711 (BM8), CD3-BV510 (17A2), CD8a-AF700 (53-6.7), CD4-PerCP-Cy5.5 (RM4-4), CD62L-BV605 (MEL-14), CD44-BV650 (IM7), CXCR5-BV421 (L138D7), PD-1–PE/Cy7 (RMP1-30), and human CD2-APC (RPA-2.10, BD). All antibodies were purchased from BioLegend unless otherwise specified. Data were acquired with an LSRFortessa flow cytometer (BD). Bulk cell sorting was performed on a MoFlo XDP cell sorter (Beckman Coulter, Brea, Calif) before single-cell sorting. All data were analyzed with FlowJo, version 10, software (BD).

scRNA-seq

Single-cell 3' RNA sequencing libraries were prepared by using Chromium Single-Cell V3 Reagent Kit and Controller (10X Genomics, Pleasanton, Calif). Libraries were sequenced on HiSeq 4000 sequencers (Illumina, San Diego, Calif). The sequenced data were processed by using Cell Ranger pipeline (version 3.0.1) (10X Genomics

[https://www.10xgenomics.com]). Sequencing reads were aligned to the human transcriptome (GRCh38), followed by filtering and correction of cell barcodes and Unique Molecular Identifiers. Reads associated with retained barcodes were quantified and used to build expression matrices.

The single-cell RNA-sequencing (scRNA-seq) R package Seurat (version 3.1.1) was used for preprocessing, quality control, and downstream analyses.[31] Genes not detected in at least 3 cells were filtered out. Low-quality cells expressing very few genes and potential doublets or multiplets expressing aberrantly high number of genes (>5000) were excluded. Dying or stressed cells with a high percentage of transcripts (>20%) mapping to mitochondrial genes were removed. Samples from the 4 libraries were merged for subsequent clustering and visualization. The data were normalized by using the log normalization method with a scaling factor of 10,000. Highly variable genes were selected by using the mean-variance method, and the data were scaled to regress out the effect of number of unique molecular identifiers and percentage of mitochondrial genes. Dimensionality reduction was performed by using principal component analysis, and the most significant clusters were chosen for subsequent clustering. Graph-based clustering was implemented by calculating the k-nearest neighbors, followed by modularity optimization to clusters cells (FindClusters function). Nonlinear dimensionality reduction and visualization was performed by using Uniform Manifold Approximation and Projection. Clusters were annotated on the basis of canonic markers, and differential gene expression testing was used to determine a gene set signature for each cluster by using the Wilcoxon Rank Sum test.

Quantitative RT-PCR

RNA was isolated from cultured PBMCs with an RNeasy Mini Kit (QIAGEN, Hilden, Germany) and quantified with NanoVue Plus (GE Healthcare). cDNA was synthesized with a Maxima First Strand cDNA Synthesis Kit (ThermoFisher Scientific), as per the manufacturer's recommendations. Immunoglobulin epsilon heavy chain transcripts were amplified (StepOnePlus Real-Time PCR System, Applied Biosystems, Foster City, Calif) by using custom TaqMan gene expression kits (Applied Biosystems) with previously developed primer pairs and probes.[32] Data were analyzed by the ΔΔCT method to represent data as fold change relative to the 18S housekeeping gene (Applied Biosystems) and unstimulated (media-only) control.

Statistical analysis

Data were analyzed with Prism 8 software (GraphPad Software, San Diego, Calif). Comparisons were drawn by using 1-way or 2-way ANOVA, the Student *t*-test, or Pearson *r*. The Benjamini-Hochberg correction for multiple testing was used in analysis of the cytokine discovery panel. Nonlinear regression was interpolated for IgG1 dilution plots, and median effective concentration values were calculated when possible. Data were considered significant with a *P* value less than .05.

RESULTS

Induction of an IgE recall response in human PBMCs

Th2 cell responses to allergens follow a classical adaptive response involving expansion of effector cell populations, a subsequent contraction phase, and memory persistence. However, a comprehensive understanding of the cellular and molecular interplay for recall responses remains to be fully elucidated. To this end, we developed an *in vitro* platform to interrogate allergen-dependent T-cell and B-cell responses concomitantly (Fig 1, *A*). Human PBMC cultures are widely used to assess cytokine production following allergen stimulation; however, we found that these platforms did not support the generation of IgE responses in the absence of a polyclonal stimulus (eg, IL-4 plus anti-CD40).[33-36] To overcome this limitation, we determined that the optimal dose of allergen facilitating IgE^+ B-cell responses is 5 logs less than what has traditionally been used for the assessment of T-cell responses.[34-36] Thus, we proceeded under these modified culture conditions to validate the model with donors without allergy (n = 5) and donors with peanut allergy (n = 12) (Table E1).

To evaluate T-cell responses, we performed a targeted proteomic analysis that included 38 analytes from the 7 donors with peanut allergy with the highest serum peanut-specific IgE levels after either control (nonstimulated) or peanut exposure. In all, 24 analytes were detected above background, fell within the detectable range (3.2-10000 pg/mL [Fig E1]), and were included in subsequent analyses. Of these, IL-5, IL-13, IL-9, and IL-17A exhibited the highest upregulation in response to peanut (Fig 1, *B*). Significant increases were also observed for GM-CSF, IL-2, IFN-γ, TNF-α, IP-10, and IL-10 (Fig 1, *B*). These data demonstrate that the recall response to peanut in this human platform involves a heterogenous cytokine response with potent production of Th2 cytokines.

We next assessed the IgE memory response in culture. Treatment with recombinant IL-4 and anti-CD40, which induces IgE in human PBMCs,[37] resulted in IgE responses detectable at the cellular, molecular, and transcriptional level irrespective of allergy status (Fig E2, *A-C*). Peanut stimulation increased IgE-secreting cells, peanut-specific IgE, and *IGHE* transcripts as compared with nonstimulated cells only in donors with allergy, with no change in allergy-free donors (Fig 1, *C-E*). The intensity of IgE readouts displayed a moderate (IgE-secreting cells [r^2= 0.553]) to strong (peanut-specific IgE [r^2= 0.785]) correlation with serum peanut-specific IgE levels used to ascertain allergy status (Fig E2, *D* and *E*). Production of *IGHE* transcripts did not correlate with serum peanut IgE (r^2 = 0.275 [Fig E2, *F*]).

To investigate the identity of the B cells and T cells involved in peanut recall responses in greater depth, we performed scRNA-seq on PBMCs isolated at day 8 from nonstimulated (n = 3272 cells) and peanut-stimulated (n = 3787 cells) cultures from a representative donor with peanut allergy (P027 [Fig E3, *A*]). After quality control and removal of low-quality cells and doublets, Uniform Manifold Approximation and Projection was used to reduce the number of dimensions and visualize cell clustering. In all, 15 discrete clusters were resolved, assigning nomenclature derived from recognized gene signatures (Fig E3, *B* and *C*), including populations of CD4 T cells, B cells, natural killer (NK) cells, monocytes, and basophils (Fig 1, *F* and *G*). To assess heterogeneity between T-cell populations, cells with high expression of *GATA3, FOXP3, IL7R,* and *MAL* were subclustered (Fig 1, *H* and Fig E4, *A*). These genes were selected to better resolve populations of Th2, Treg, and memory T cells, respectively, with the additional inclusion

of *MAL*-expressing T cells owing to changes in frequency observed in total cell clusters (C0 in Fig 1, *G*). A 2.3-fold increase in the proportion of *MAL*-expressing CD4 T cells (C0) was observed with peanut stimulation (21.8% nonstimulated vs 50.9% peanut-stimulated [Fig 1, *I*]). In this cluster, 43 of 94 differentially expressed genes were ribosomal protein–coding genes, suggesting a high degree of protein synthesis (Table E2). A pathway analysis (G:Profiler) of nonribosomal genes displayed an enrichment in genes relating to lymphocyte differentiation, T-cell activation, and response to IL-4 stimulus (Fig E4, *B*). Notably, *IL4R* was detected almost exclusively in the MAL^{high} cluster, with *MAL* having been suggested to be induced by IL-4 signaling (Fig E4, *C*).[38] Because of the high frequency of these cells at baseline (no stimulus), they are unlikely to be peanut specific; rather, their frequency is likely to expand as a result of bystander proliferation.[39] In contrast, cluster 2 (C2) was uniquely identified in peanut-stimulated PBMCs (0.8% nonstimulated vs 10.6% peanut-stimulated [Fig 1, *I*]). Differentially expressed genes resembled a canonic activated Th2 cell phenotype, including *GATA3, IL17RB, KLRB1, IL2RA,* and *CTLA4* (Fig 1, *J* and Table E3). Additionally, C2 expressed genes encoding prostaglandin D synthase (*HPGDS*) and the prostaglandin D2 receptor CRTH2 (*PTGDR2*), which is consistent with a distinct population of Th2 cells specific to allergic disease, previously coined *Th2A cells*.[40] C3 cells (Fig 1, *H*) expressed both regulatory T cell- and Th2 cell–related genes, including *FOXP3, CTLA4, IL2RA, LGALS3,* and *GATA3,* and they are thus congruous with a T_H2 cell–like Treg phenotype (Fig 1, *J* and Table E3).[41]

Subclustering MBCs, which were identified by expression of the memory markers *CD27* and *AIM2* and low expression of *IGHM, IGHD,* and *TCL1A*[42] (Fig E5, *A*

and *B*), revealed 3 distinct clusters (Fig 1, *K*). C0 and C1 contained heterogeneous IgH expression but did not express *IGHG4* and *IGHE* (highly expressed in C2 cells) (Fig E5, *C*). The number of *IGHE*-expressing cells (C2) expanded with peanut stimulation (Fig 1, *L*). As IgE^+MBCs are extremely rare in circulation (as measured by flow cytometry) and acquisition of tissue samples that may contain IgE^+ MBCs is challenging,[8] our detection of these cells at the single-cell level provided a valuable opportunity to elucidate their transcriptional profile. *IGHE*$^+$ cells were transcriptionally unique (Fig 1, *M* and Table E3), expressing genes associated with costimulation (eg, *CD40* and *CD83*) and IL-4 signaling[43,44] *(*eg, *IL4I1* and *GCSAM),* and high expression of *IL4R* (Fig E5, *D*). In sum, we have developed an *in vitro* platform that yields robust Th2 cell humoral responses with peanut stimulation, positioning it as a novel system with which to study the human allergic recall response. Application of scRNA-seq to *in vitro*–stimulated PBMCs from individuals with peanut allergy revealed the transcriptomic profile of peanut-reactive B-cell and T-cell subsets. Cell populations that expanded with peanut stimulation exhibited an upregulation of genes related to IL-4 signaling.

Of note, the scRNA-seq analysis also identified a population of *bona fide* basophils, which is a known contaminant to the PBMC fraction in density gradient–based isolation strategies. Although basophils encompass just a small percentage (<0.5%) of circulating leukocytes, basophil-derived IL-4 may contribute to the recall response *in vitro*. Differences in basophil frequency between donors may therefore provide an alternative explanation as to the intensity of IgE readouts. Too few basophils were detected by scRNA-seq (5

nonstimulated cells and 7 peanut-stimulated cells) to make meaningful conclusions regarding activation status.

IL-4/IL-13 signaling blockade aborts IgE production and skews the type 2 cytokine response in human PBMCs

Recent work using flow cytometry and single-cell sequencing methods have informed about the frequency and phenotype of human IgE-expressing cells in circulation. In individuals with food allergy, IgE-expressing cells are almost exclusively of a plasmablast phenotype,[9] with rare or absent detection of IgE^+ MBCs.[8,9] This suggests that similar to observations made in mice, IgG^+ MBCs are the reservoir for IgE recall responses in humans and that IL-4/IL-13 signaling may be required to coordinate IgE class switching. Moreover, the unique transcriptional signature that we described in *IGHE*-expressing MBCs further suggests that the cells are responsive to IL-4. To assess the requirement of IL-4/IL-13 signaling in human IgE recall responses, we used the aforementioned *in vitro* platform with the addition of an IL-4Rα–blocking antibody or an IC in 7 donors with peanut allergy and a serum peanut IgE level greater than 40 kU/L.

Remarkably, anti–IL-4Rα resulted in a near-complete inhibition of IgE recall responses, including IgE-secreting cells, peanut-specific IgE, and *IGHE* transcripts (Fig 2, *A*-*C*). Suppression of IgE responses did not appear to result from apoptosisthrough IL-4 deprivation (Fig E6). We applied the 38-plex cytokine array to investigate the implications of IL-4Rα blockade on cytokine production (Fig E7, *A*). IL-5, IL-9, MDC, and IL-17A were significantly downregulated with anti–IL-4Rα; conversely, IL-10, IL-1α, IL-1β, MIP-1α,

G-CSF, and IFN-γ secretion was upregulated (Fig 2, *D*). The Th2 cell–to–Th1 cell and Th2 cell–to–IL-10 ratios were significantly reduced with anti–IL-4Rα (Fig 2, *E*). Of note, IL-4 was excluded from these analyses, as IL-4Rα blockade results in accumulation of secreted IL-4.[45] Without addition of anti–IL-4Rα, no appreciable differences were detected in IL-4 secretion between nonstimulated and peanut-stimulated PBMCs (Fig E7, *B*); however, IL-4Rα blockade resulted in a 2.3-fold change increase over IC-treated peanut-stimulated PBMCs (IC = 80 pg/mL; anti–IL-4Rα = 184 pg/mL [Fig E7, *B*]). This indicates that IL-4 is produced in response to peanut stimulation; however, rapid consumption of the cytokine likely impairs its detection.

We used scRNA-seq to compare the profiles of more than 6000 human peanut-stimulated PBMCs with or without anti–IL-4Rα treatment (3427 peanut + IC and 2718 peanut + anti–IL-4Rα). The peanut-induced expansion of the MAL^{high} population was completely inhibited by IL-4Rα blockade (Fig 2, *F* and *G*). This was consistent with their high level of expression of *IL4R* and suggests that IL-4/IL-13 signaling is critical for their expansion. In contrast, IL-4/IL-13 signaling was dispensable for maintaining terminally differentiated T_H2A cells (C2). With regard to MBCs, the expansion of $IGHE^{+}$ cells was impaired by IL-4Rα blockade, which coincides with upregulation of IL-4-signaling genes in this cluster (Fig E8). These data establish the critical requirement of IL-4/IL-13 signaling in human IgE recall responses to peanut.

CD4 T cells drive the recall response in vivo

Given the critical requirement of IL-4/IL-13 signaling in the *in vitro* recall response, we used a mouse model of food allergy to validate and expand on these findings *in vivo*. Here, we used a previously described model of epicutaneous sensitization.[16] This approach enables temporal control over the IgE response in which sensitization initially produces allergen-specific IgG1-mediated anaphylaxis, which can be blocked with anti-CD16/32 treatment. Following contraction of the primary immune response, subclinical, nonsensitizing subcutaneous reexposures activate a memory response and the conversion of IgG1 responses to IgE.[16]

First, we assessed the cellular sources of IL-4 in a recall response to peanut in 4get/KN2 mice. In these mice, green fluorescent protein (GFP) and human CD2(hCD2) expression report *IL4* gene transcription (GFP^+hCD2^-) and IL-4 secretion (GFP^+hCD2^+), respectively.[39] Whereas the source of IL-4 following Th2 cell sensitization has been studied extensively, the source of IL-4 in the recall response to food allergens remains unknown. In the inguinal lymph nodes, GFP^+hCD2^+ and GFP^+hCD2^- cells peaked at 3 and 7 days after reexposure, respectively, and contracted by day 14 (Fig 3, *A* and *B*). For up to and inclusive of 7 days after reexposure, CD4 T cells accounted for more than 92% of the IL-4–secreting and transcribing populations (Fig 3, *A* and *B* [*shaded regions*]). Expectedly, almost all GFP^+hCD2^+ CD4 T cells expressed the activation marker CD44 (>95% [Fig 3, *C*]) and 25% were follicular helper T (TFh) cells (Fig 3, *D*). Thus, in the draining lymph nodes CD4 T cells provide the overwhelming majority of IL-4, with contributions from both Th2 cells and TFh cells.

The requirement of CD4 T cells in IgE recall responses has been evaluated in the context of helminth infections and/or utilization of highly selective adoptive transfer models, but not in intact food allergy models. Thus, we evaluated the contribution of CD4 T cells in the recall response to peanut by using an anti-CD4 antibody to deplete CD4 T cells before subcutaneous reexposures. As CD4-targeted depletions also eliminate CD4-expressing NK T (NKT) cells, a separate group that received anti-NK1.1 was included to discriminate the contribution of conventional CD4 T cells and CD4-expressing NKT cells. After induction of the recall response, peanut IgG1 production was similar between untreated and anti-NK1.1–treated mice, although depletion of CD4 T cells significantly impaired this response (Fig 3, *E*). Similarly, depletion of CD4 T cells, but not $NK1.1^+$ cells, prevented the emergence of peanut IgE (Fig 3, *F*). Accordingly, anti-CD4–treated mice were protected from IgE-mediated anaphylaxis (Fig 3, *G-I*). Thus, in the absence of conventional CD4 T cells, other cells (eg, NKT cells, basophils, etc) are insufficient at mounting an IgE recall response. Taken together, these data highlight a critical role of CD4 T cells for the IgE recall response *in vivo* and positions these cells as key therapeutic targets.

Th2 cell polarization is maintained in allergen-specific CD4 T cells

Next, to elucidate the impact of anti–IL-4α on T-cell phenotype *in vivo* during recall and validate findings revealed by scRNA-seq *in vitro*, we used the same epicutaneous sensitization model in 4get/KN2 mice. For these experiments, mice were sensitized to OVA, an allergenic protein in egg. Epicutaneous sensitization to OVA yields the same allergic phenotype as peanut sensitization[15] and enables the use of peptide:MHC class

II tetramers for detection of OVA-specific CD4 T cells. Draining (inguinal) lymph nodes were collected 3 days after the final subcutaneous injection, at the peak of IL-4 production from CD4 T cells. Cells were stained with 2 peptide:MHC class II PE-conjugated tetramers loaded with the immunodominant OVA peptides OVA2C ($OVA_{325-335}$) and OVA3C ($OVA_{327-338}$).[40] Tetramer-positive (OVA^+) cells were enriched with anti-PE magnetic microbeads and then stained with antibodies for flow cytometry analysis.

The impact of anti–IL-4Rα on IL-4 transcript and protein expression was assessed in $B220^-$ $F4/80^-CD3^+CD8^-CD4^+$ cells. An average of 61 and 33 OVA^+ CD4 T cells were detected in IC- and anti–IL-4Rα–treated mice, respectively, which was not significantly different ($P = .2998$ [Fig 4, *A*]). As TFh cells produce IL-4 independent of the type of immune response (eg, Th1 and Th2 cells), we separately analyzed TFh cells ($CD44^{high}CD62L^-PD\text{-}1^+CXCR5^+$) and activated non-TFh CD4 T cells ($CD44^{high}CD62L^-CXCR5^-$) (Fig 4, *B*); previous literature has described the GFP^+ non-T_{FH} cells to be the Th2 cell population.[39,41] In both the OVA^+ TFh and OVA^+ Th2 cell populations, *IL4* transcription and IL-4 secretion were unchanged by IL-4Rα blockade (Fig 4, C-*F*). This is in agreement with the observation *in vitro*, where human T_H2A cells maintained their phenotype in the presence of anti–IL-4Rα blockade. Overall, these data demonstrate that although anti–IL-4Rα abrogates IgE production and anaphylaxis in a recall response, Th2 cell–polarized allergen-specific cells maintain their pathogenic phenotype in the absence of key polarizing signals via IL-4 and IL-13.

IL-4Rα blockade inhibits IgE recall response and anaphylaxis in epicutaneously sensitized mice

Lastly, we evaluated the functional impact of IL-4Rα blockade *in vivo* by challenging mice after anti–IL-4Rα (or IC) treatment and subcutaneous reexposures. All groups of mice that underwent epicutaneous sensitization presented with similar levels of peanut-specific IgG1 and background levels of peanut-specific IgE (Fig 5, *A*). Following subcutaneous peanut reexposures, peanut IgG1 levels remained similar and an IgE response was generated, which was fully abrogated by anti–IL-4Rα treatment (Fig 5, *B*). To assess the functional impact of IL-4Rα blockade, mice were challenged intraperitoneally with peanut. The mice receiving anti–IL-4Rα exhibited clinical reactivity after challenge, although to a lesser extent than untreated mice did (Fig 5, *C*-*E*). We reasoned that this halfway response could be a consequence of IgG-mediated alternative anaphylactic pathways, as anti–IL-4Rα–treated mice develop normal IgG1 responses. To this end, groups of mice were treated with anti-CD16/32 before challenge to block IgG binding to Fcγ receptors.[27] Mice cotreated with anti–IL-4Rα and anti-CD16/32 displayed no clinical reactivity following challenge, as evidenced by a maintenance of homeostatic core body temperature and hemoconcentration (Fig 5, *C*-*E*). To confirm that anaphylaxis was inhibited due to anti–IL-4Rα–mediated events occurring before challenge (eg, preventing IgE production) and was not due to the blockade of IL-4Rα during challenge, we assessed the impact of anti–IL-4Rα administered the day before challenge. Although IL-4 has been shown to exacerbate anaphylaxis,[47] we found that blockade of IL-4Rα before challenge did not affect

clinical reactivity (Fig E9). Thus, IL-4Rα blockade specifically affects the recall response to allergens and, as a consequence, prevents anaphylaxis.

To establish whether IL-4Rα blockade exhibited long-lasting impact on the allergic response, we conducted experiments involving tertiary reexposures following anti–IL-4Rα clearance. Clearance of anti–IL-4Rα was ascertained by 2 independent approaches. First, IL-4 is known to be critically required for allergic sensitization.[28,48] To this end, naive mice were treated with anti–IL-4Rα 1 day, 2 weeks, or 6 weeks before sensitization. We reasoned that any residual antibody would, expectedly, prevent or stunt allergic sensitization. By 6 weeks after anti–IL-4Rα treatment, the clinical reactivity of anti–IL-4Rα mice was no different from that of untreated mice (Fig E10, *A* and *B*). Second, we measured rat IgG2a from the serum of antibody-treated mice and found that it was undetectable after 6 weeks (Fig E10, *C*). Thus, to establish the lasting impact of IL-4Rα blockade, mice with allergy were treated with anti–IL-4Rα and subjected to subcutaneous reexposures (as per the experiments in Fig 5, *A*-*E*), followed by a 6-week wait for antibody clearance and an additional round of subcutaneous reexposures (tertiary exposure). A sustained and complete inhibition of IgE production in mice previously receiving anti–IL-4Rα was observed (Fig 5, *F*). Moreover, at this time point, anti–IL-4Rα–treated mice exhibited a significantly dampened production of IL-4, but not IL-13, in *in vitro*–stimulated splenocytes (Fig 5, *G*). The reduction in IL-4 was paired with a significant increase in IFN-γ production. Thus, anti–IL-4Rα appears to transform IL-4/IL-13 signaling where over time, IL-4 production is lessened in conjunction with an increase in the Th1 cytokine IFN-γ.

DISCUSSION

Onset of food allergies, such as those to peanut, routinely occurs before the age of 2 years and persists for a lifetime in most patients. The current standard of care is strict allergen avoidance; however, accidental exposures, which are estimated to occur in 14% of people with peanut allergy each year, pose a continual risk of life-threatening reactions.[5] The lifelong nature of some food allergies is thought to result from repeated activation, through clinical or subclinical allergen exposures, of lifelong allergen-specific memory cells that regenerate transient IgE-secreting cells. It is thus necessary to elucidate critical requirements for memory cells to generate IgE recall responses against allergens. This has remained elusive in humans, in part owing to a lack of platforms that facilitate the *in vitro* regeneration of IgE and IgE-secreting cells. To this end, we established a novel human PBMC culture that yields both T-cell and B-cell readouts following allergen stimulation exclusively in patients with allergy. We surmise that this model is the first of its kind to enable rapid screening of human biologics with the potential to inhibit recall responses in food allergy and other allergic diseases.

As IL-4/IL-13 signaling is central to the initiation of food allergy[28] and other Th2 cell–mediated disease (eg, helminth infection),[48] we probed the requirement of this signaling pathway in the context of a recall response. Application of an IL-4Rα–blocking antibody entirely abrogated IgE transcription and production following peanut stimulation of human PBMCs from donors with peanut allergy. Moreover, Th2 cytokine production was broadly suppressed with a remarkable inhibition of IL-9 secretion, which

has been demonstrated to be critically required for secondary antibody responses.[49] The advent of single-cell technology has provided an unparalleled depth of understanding of biologic processes. Here, we applied scRNA-seq to generate novel insights into the transcriptomic profile of different populations of peanut-reactive CD4 T cells and B cells during a recall response in the presence or absence of IL-4/IL-13 blockade. Moreover, despite the extreme rarity of IgE^+ MBCs in circulation as measured by flow cytometry, we were able to detect these cells at the single-cell level following culture with peanut to elucidate their transcriptional profile. In concordance with Wambre et al,[40] a unique population of Th2 cells thought to be pathogenic in allergy, termed *Th2A cells*, expanded following peanut stimulation compared with the nonstimulated control. We have extended on this work by demonstrating that proliferation of Th2A cells, as well as maintenance of their phenotype during allergen exposure, occurs independently of IL-4/IL-13 signaling.

A number of studies have described a role of IL-4/IL-13–secreting cells in the allergic cascade. For example, basophils have been shown to supply IL-4 and IL-13 in an IgD-dependent manner[50] and ILC2-derived IL-4 constrains the production of regulatory T cells,[51] both of which amplify the IgE response. Thus, there appears to be a number of IL-4/IL-13–secreting cells that have seemingly relevant effects on the IgE response. In a recall response, however, the essential source of IL-4 remained unknown. By using IL-4 dual-reporter mice, we have demonstrated that Th2 and TFh cells provide the vast majority of IL-4 locally. Through antibody-mediated depletion of $CD4^+$ cells, which unlike adoptive transfer of B cells and T cells into immunodeficient mice, leaves $CD4^-$ cells (eg, basophils and ILC2s) intact, we have demonstrated that CD4 T cells are critically required for the

differentiation of $IgG1^+$ MBCs to IgE-secreting cells. Moreover, NKT cells, which also secrete IL-4 and may be critical for the development of allergen-induced airway hyperreactivity,[52] were not critically required, nor did they amplify the recall response.

We next sought to determine whether blockade of IL-4/IL-13 signaling impaired the pathogenic T_H2 cell response *in vivo* by altering IL-4 production and preventing IgE recall responses. We administered an IL-4Rα–blocking antibody to mice with peanut allergy following contraction of the primary immune response and immediately preceding the induction of the recall response via subcutaneous injections. Production of IL-4 from T_H2 and T_{FH} cells was unchanged during IL-4Rα blockade, although IgE production was abrogated, resulting in complete protection from anaphylaxis following allergen challenge. Therefore, it is likely that the immediate effects of anti–IL-4Rα are a result of IL-4Rα blockade on $IgG1^+$MBCs preventing IgE class switch recombination and are not due to interruption of IL-4/IL-13 signaling on T cells. Importantly, however, the inhibition of IgE regeneration was sustained following clearance of anti–IL-4Rα, demonstrating that the immunologic changes imparted by anti–IL-4Rα persist beyond the duration of therapy. We posit that this sustained effect could be due, at least in part, to the decrease in IL-4 and substantial increase in IFN-γ seen *in vitro*, given that IFN-γ has been shown to potently suppress primary IgE responses both *in vitro* and *in vivo*.[53,54] Recently, IL-21 was demonstrated to negatively regulate IgE responses more so than IFN-γ in an alum-adjuvanted model.[55] In our experiments, IL-21 was undetectable in *in vitro*–stimulated splenocytes, although this does not negate the potential for IL-21–mediated

effects *in vivo.* Ultimately, the precise mechanism by which anti–IL-4Rα confers sustained protection from IgE regeneration is complex and remains an active area of investigation.

The human mAb targeting IL-4Rα is licensed for the treatment of moderate-to-severe atopic dermatitis, eosinophilic asthma, and chronic rhinosinusitis with nasal polyposis, and it remains in clinical trials for other atopic diseases. Anti–IL-4Rα treatment in humans comprehensively improves disease scoring (eg, FEV_1 for asthma) and decreases total IgE levels by 35% to 38% after 12 weeks of treatment[56,57]; however, its therapeutic potential in food allergy remains largely unexplored. Herein, we demonstrated that signaling through IL-4Rα is critically required for the regeneration of IgE in both mice and humans with allergy. Importantly, this resulted in protection from anaphylaxis on allergen challenge. Moreover, blockade of IL-4/IL-13 signaling skews the Th2 cell–dominant cytokine response and imparts lasting protection against the regeneration of IgE following allergen reexposure beyond the duration of therapy. These results spotlight IL-4Rα blockade as a therapy with disease-transformative potential for the treatment of food allergy.

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TABLES & FIGURES

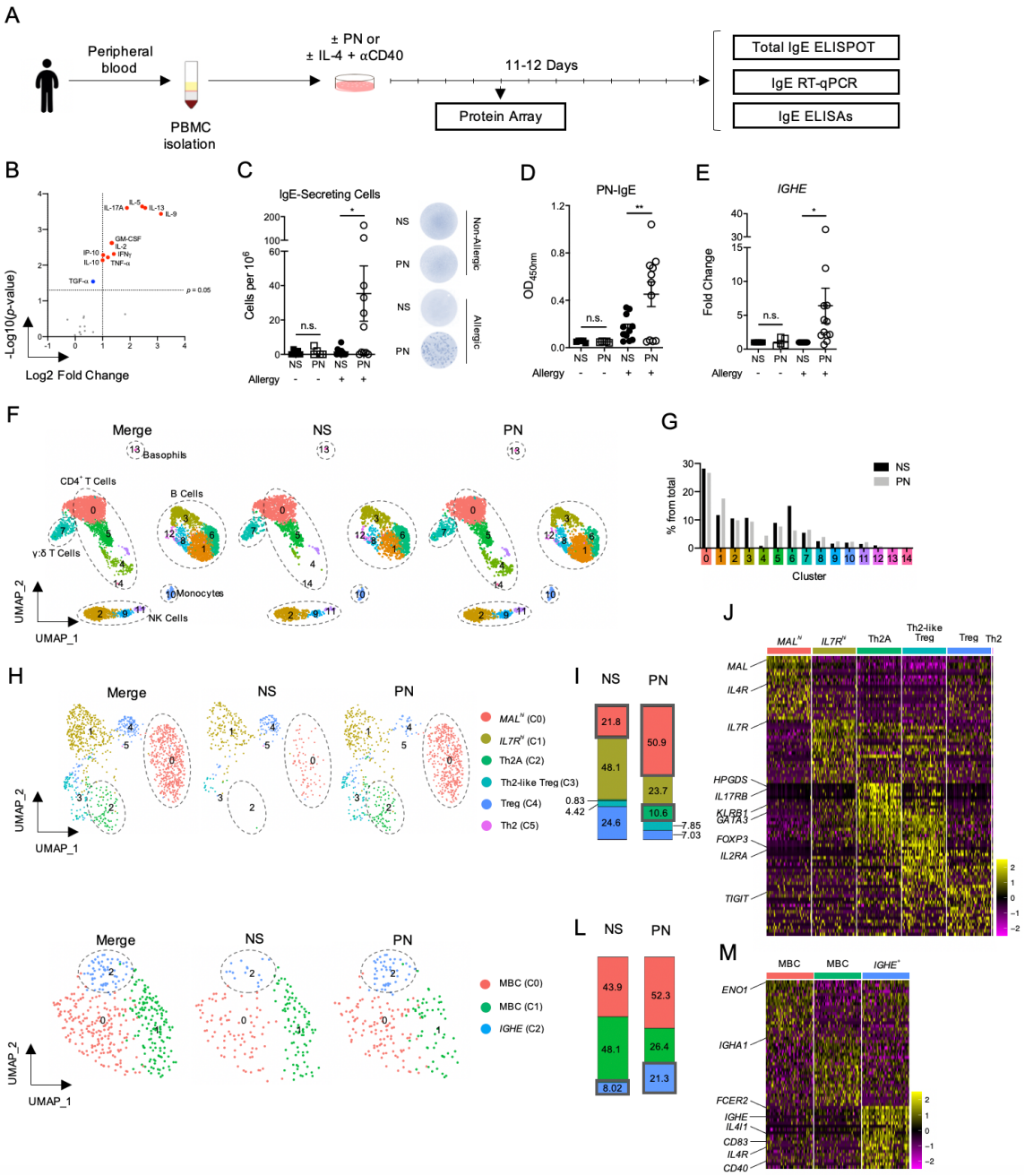

Figure 1. Induction of an IgE recall response to PN in human PBMCs. (A) Schematic of culture with PN-allergic or non-allergic donor PBMCs. **(B)** Volcano plot of cytokines detected with 38-analyte array in PN-stimulated PBMCs from PN-allergic donors. Benjamini-Hochberg correction was applied to correct for multiple comparisons. Data are plotted as upregulated cytokines (red), downregulated cytokines (blue), and unchanged (grey). **(C)** Enumerated IgE-secreting cells detected at end of culture with representative images. **(D)** PN-specific IgE measured from culture supernatant. **(E)** Fold change of *IGHE* mRNA expression relative to housekeeping gene (*18S*) and non-stimulated control. Data are representative of 5 non-allergic donors **(C-E)** and 7-11 PN-allergic donors **(B-E)** and are plotted as mean ± SEM **(C-E)**. *$P < 0.05$, **$P<0.01$. **(F)** UMAP identifying distinct leukocyte populations from a PN-allergic donor. **(G)** Frequency of cells in each cluster. **(H)** UMAP of subclustered T cells. **(I)** Stacked bars of subcluster frequency (%) in H. **(J)** Heatmap of top 20 differentially expressed genes in T cell subclusters. **(K)** UMAP of subclustered MBCs. **(L)** Stacked bars of subcluster frequency (%) in K. **(M)** Heatmap of top 20 differentially expressed genes in MBC subclusters.

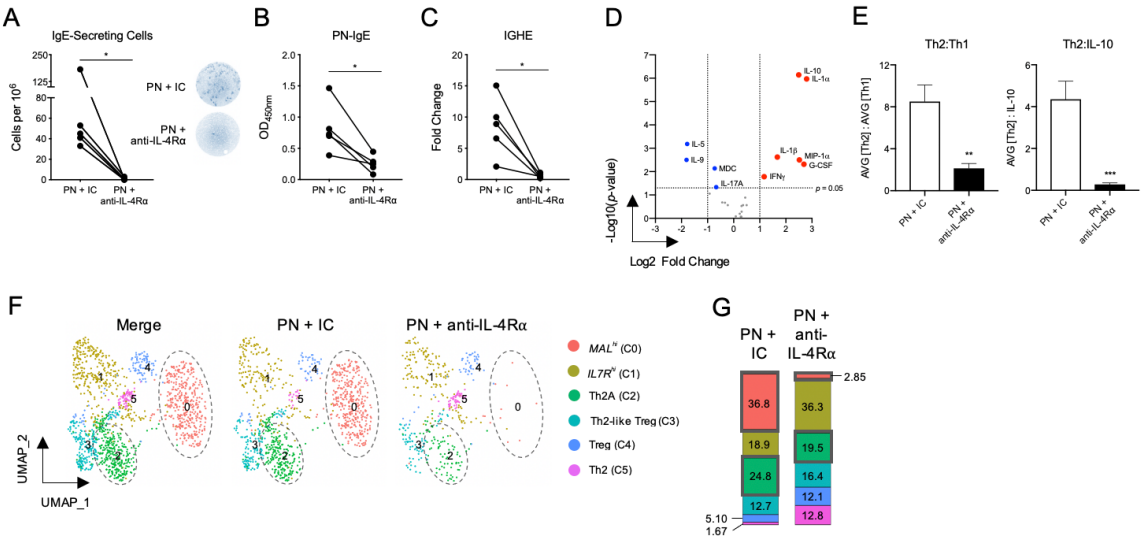

Figure 2. IL-4/IL-13 signaling blockade aborts IgE recall response and skews cytokine response in human PBMCs. (A) Enumerated IgE-secreting cells detected at the end of culture with representative images. **(B)** PN-specific IgE measured from culture supernatant. **(C)** Fold change of *IGHE* mRNA expression relative to housekeeping gene (*18S*) and non-stimulated control. **(D)** Volcano plot of cytokines detected with 38-analyte array in IC or anti-IL-4Rα-treated PN-stimulated PBMCs from PN-allergic donors. Benjamini-Hochberg correction was applied to correct for multiple comparisons. Data are plotted as upregulated cytokines (red), downregulated cytokines (blue), and unchanged (grey). **(E)** Ratio of type 2 cytokine (IL-5, IL-9, IL-13) to type 1 cytokines (IL-2, IFNγ, IL-12(p40)) or IL-10 concentration. Data are plotted as mean ± SEM **(E)** or individual donors **(A-C).** **P* < 0.05, ***P*<0.01, ****P*<0.001. **(F)** UMAP of subclustered T cells. **(G)** Stacked bars of subcluster frequency (%) in F.

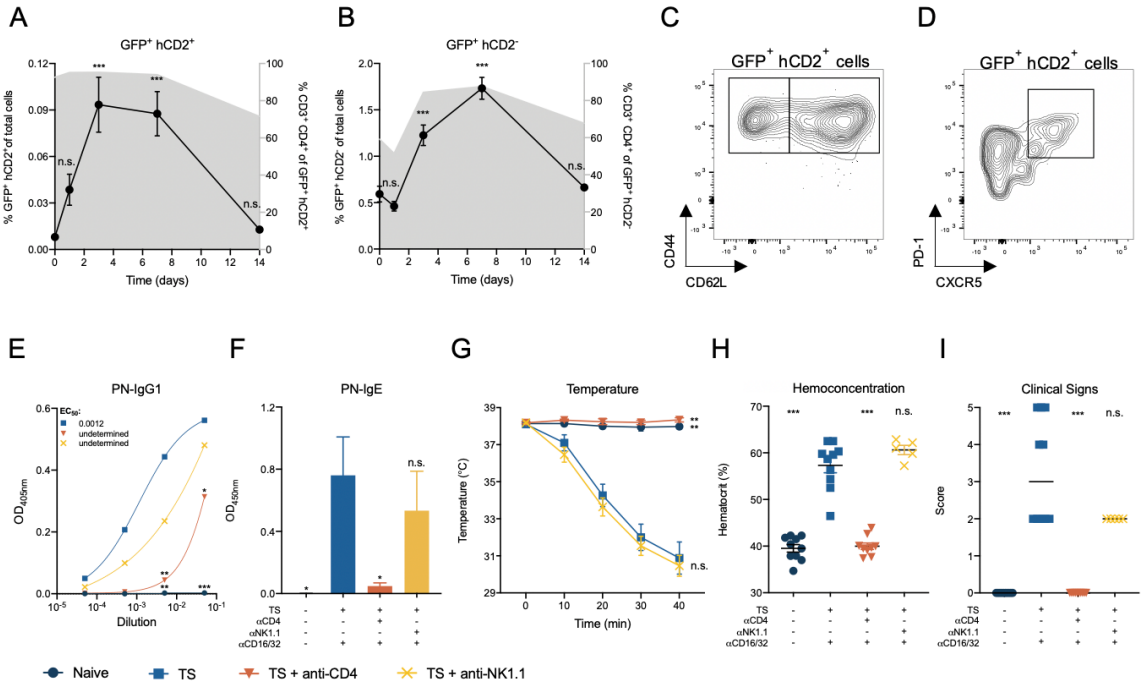

Figure 3. CD4 T cells are critically required for IgE recall response. **(A-B)** Frequency of *Il4* transcribing cells **(A)** and IL-4-secreting cells **(B)** in draining lymph nodes of 4get/KN2 mice after *s.c.* re-exposures. Shaded region is percent of CD4 T cells from GFP$^+$ hCD2$^+$ (A) and GFP$^+$ hCD2$^-$ (B) cells. (**C-D**) Representative contour plots of CD44 and CD62L expression (C) and T follicular helper cells (D) from GFP$^+$ hCD2$^+$ cells at 3 days post-re-exposure. **(E-F)** Serum PN-specific IgG1 (**E**) and IgE (**F**) following sensitization. **(G)** Core body temperature at 10-minute intervals during systemic challenge. **(H)** Hemoconcentration at 40-minutes post-challenge. **(I)** Scoring of clinical signs during systemic challenge. Data are representative of 2 experiments with 4-5 mice per group, plotted as mean ± SEM or median (I). Anti-NK1.1 group is from one experiment only. **P*<0.05, ***P*<0.01, ****P*<0.001. IC, isotype control.

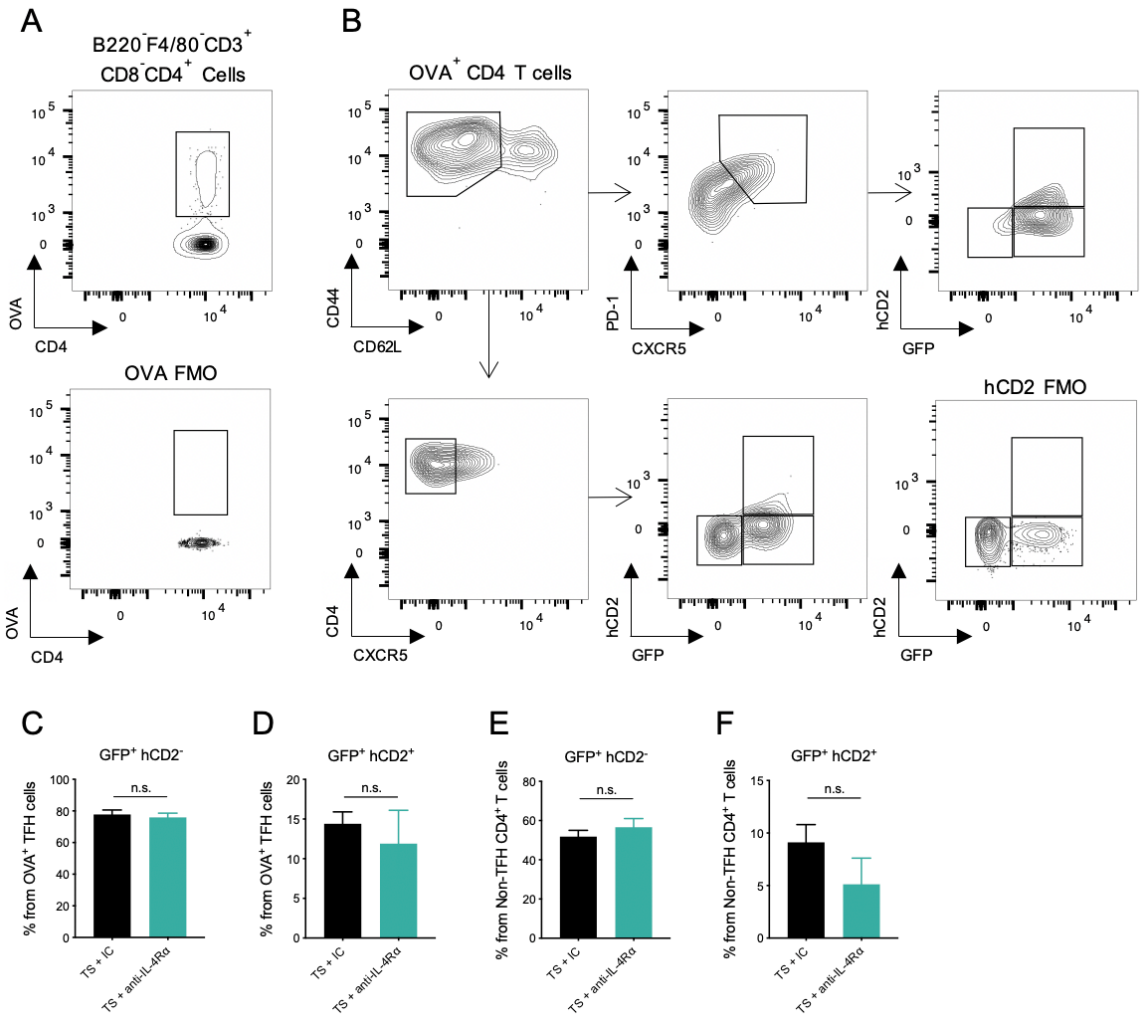

Figure 4. Th2 phenotype persists with IL-4Rα blockade. (A) Identification of OVA-specific CD4 T cells in OVA-allergic mice at 3 days post-subcutaneous re-exposure. **(B)** Gating strategy for detection of IL-4 production in OVA-specific TFh cells and non-TFh cells from pooled inguinal lymph nodes. **(C, D)** Frequency of GFP^+ $hCD2^-$ cells **(C)** and GFP^+ $hCD2^+$ cells **(D)** from OVA-specific TFh cells. **(E, F)** Frequency of GFP^+ $hCD2^-$ cells **(E)** and GFP^+ $hCD2^+$ cells **(F)** from OVA-specific non-TFh cells. Data are representative of 2 experiments with 4-5 mice per experimental group and are plotted as mean ± SEM. $^{**}P<0.01$, $^{***}P<0.001$. FMO, fluorescence minus one (flow cytometry control); TS, tape strip; IC, isotype control.

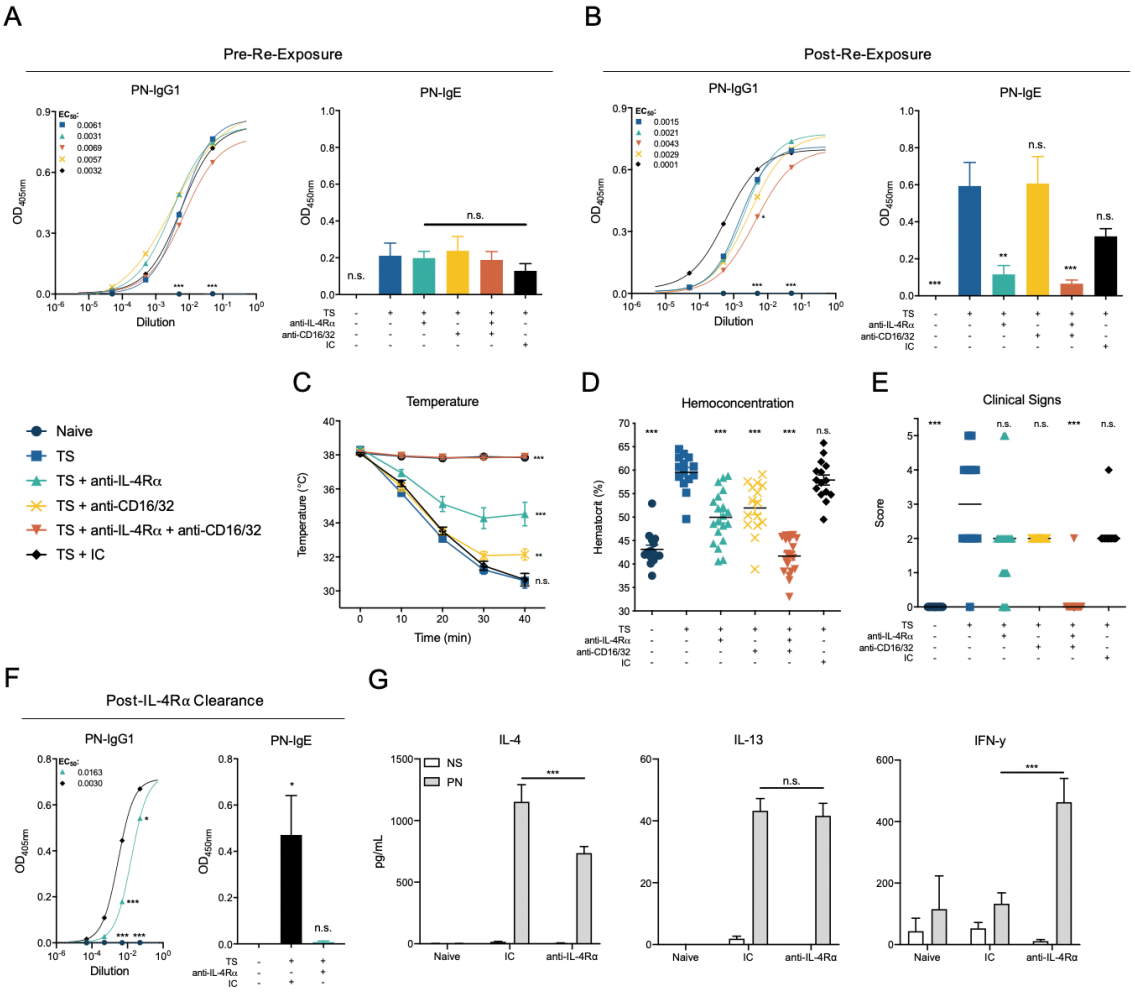

Figure 5. IL-4Rα blockade inhibits IgE recall response and anaphylaxis in epicutaneously-sensitized mice. (A) Serum PN-specific IgG1 and IgE following sensitization. **(B)** Serum PN-specific IgG1 and IgE following PN re-exposure. **(C)** Core body temperature at 10-minute intervals during systemic challenge. **(D)** Hemoconcentration at 40-minutes post-challenge. **(E)** Scoring of clinical signs during systemic challenge. **(F)** Serum PN-specific IgG1 and IgE following anti-IL-4Rα clearance and *s.c.* re-exposures. **(G)** Cytokines measured from splenocyte culture after systemic challenge. Data are representative of 2-3 experiments with 3-5 mice per experimental group, plotted as mean ± SEM or median (E). **P*<0.05, ***P*<0.01, ****P*<0.001. TS, tape strip; IC, isotype control.

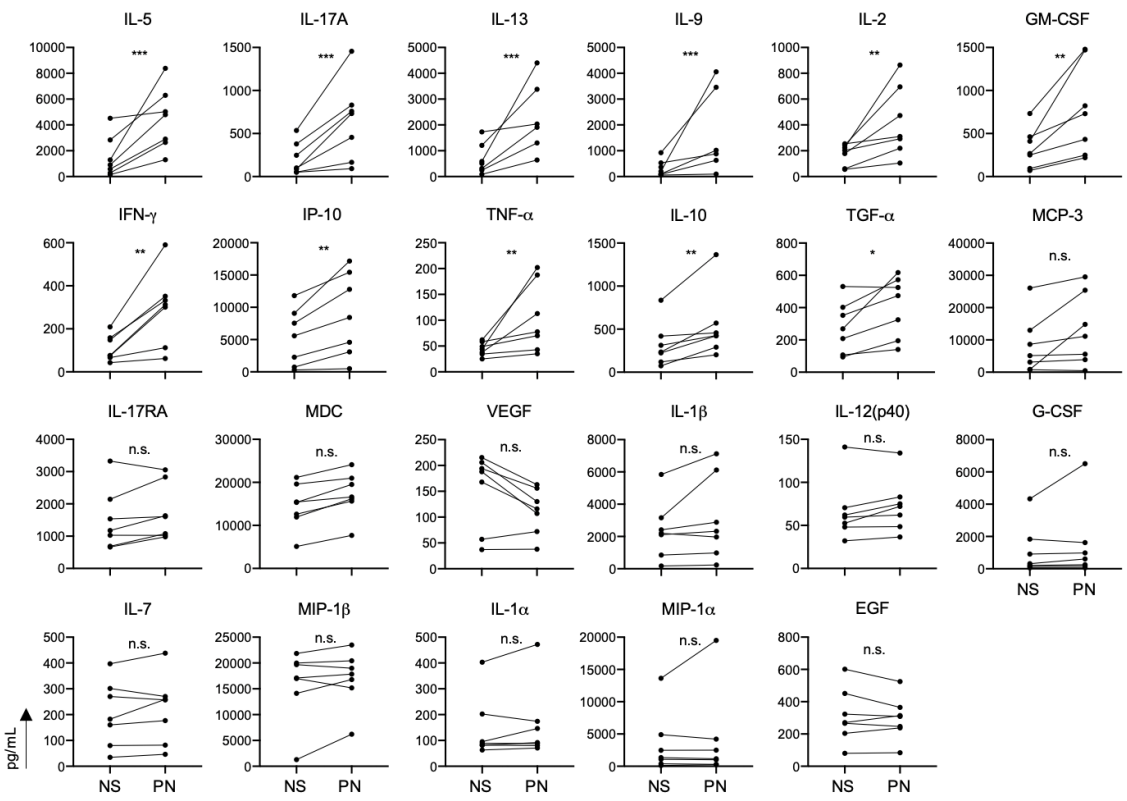

Figure S1. Cytokine response in culture. Cytokines detected with 38-analyte array in NS or PN-stimulated PBMCs from PN-allergic donors (n=7). Each line represents an individual donor and plots are ordered (left to right) by ascending *p* value. **P*<0.05, ***P*<0.01, ****P*<0.001.

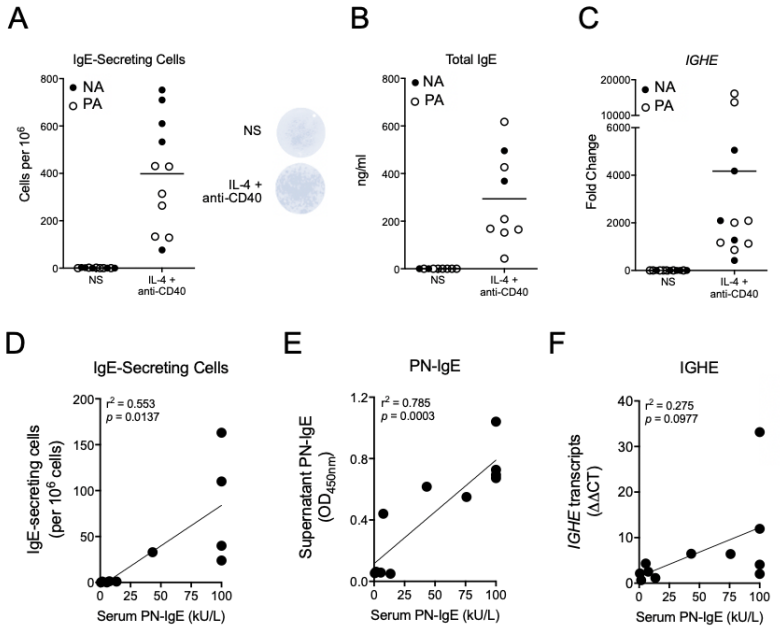

Figure S2. ***In vitro*** **IgE response. (A)** Enumerated IgE-secreting cells detected at end of culture with representative images. **(B)** Total IgE measured from culture supernatant. **(C)** Fold change of *IGHE* mRNA expression relative to housekeeping gene (*18S*) and non-stimulated control. Data are representative of 2-5 non-allergic donors (NA; filled circle) and 6-7 PN-allergic donors (PA; open circle) and plotted as individual data points with mean (horizontal bar). (**D-F)** Relationship between serum PN-specific IgE levels (kU/L) and IgE readouts in PN-allergic donors.

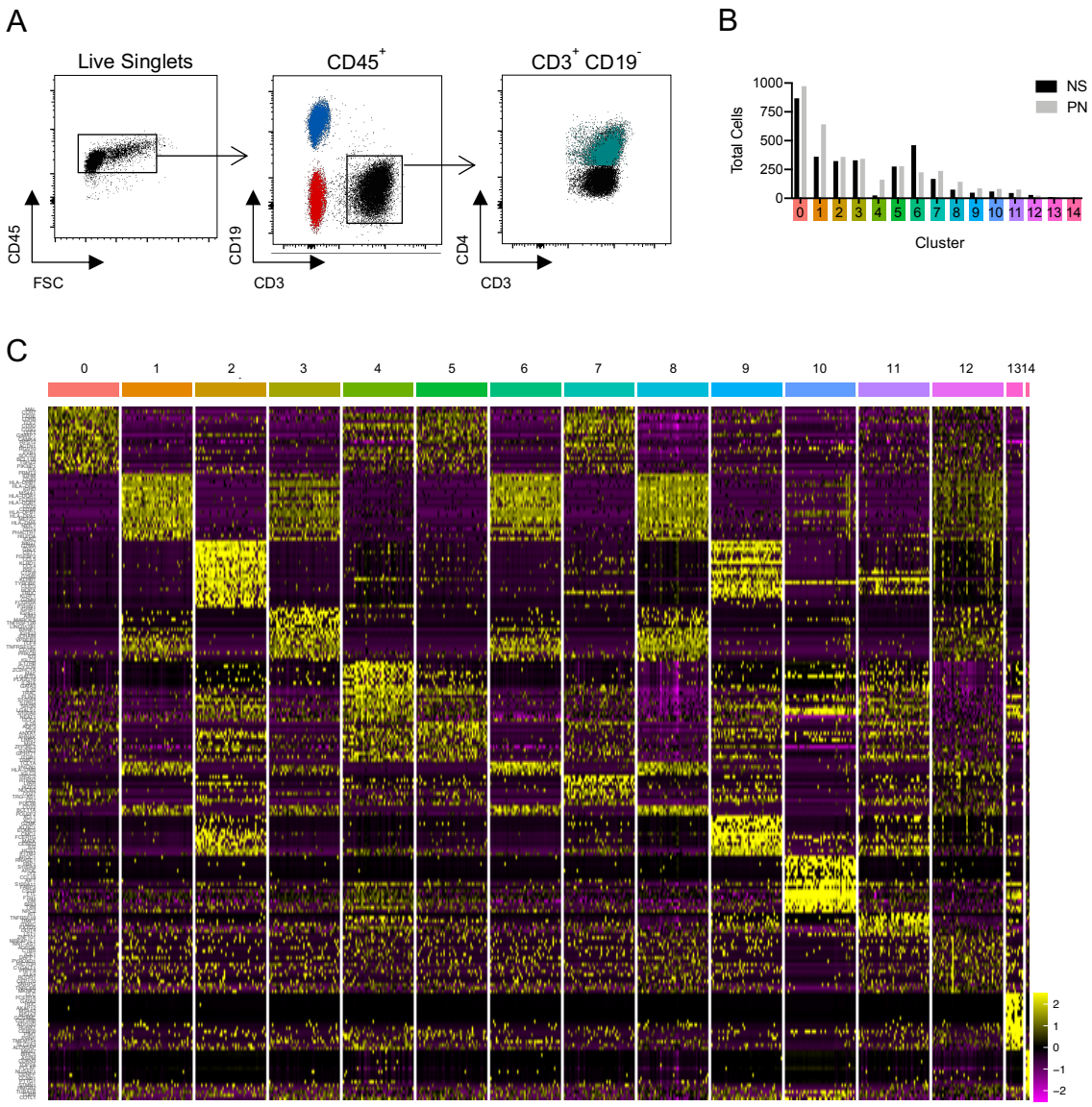

Figure S3. **(A)** Gating strategy for sorting of B cells, CD4 T cells, and non-T/B cells for single-cell RNA-sequencing. Sorted populations were pooled at the approximate frequencies: 40% CD4 T cells, 40% B cells, 20% non-T/B cells. **(B)** Total cells in each cluster of Fig. 1F. **(C)** Heatmap of top 20 differentially expressed genes in leukocyte clusters from Fig. 1F.

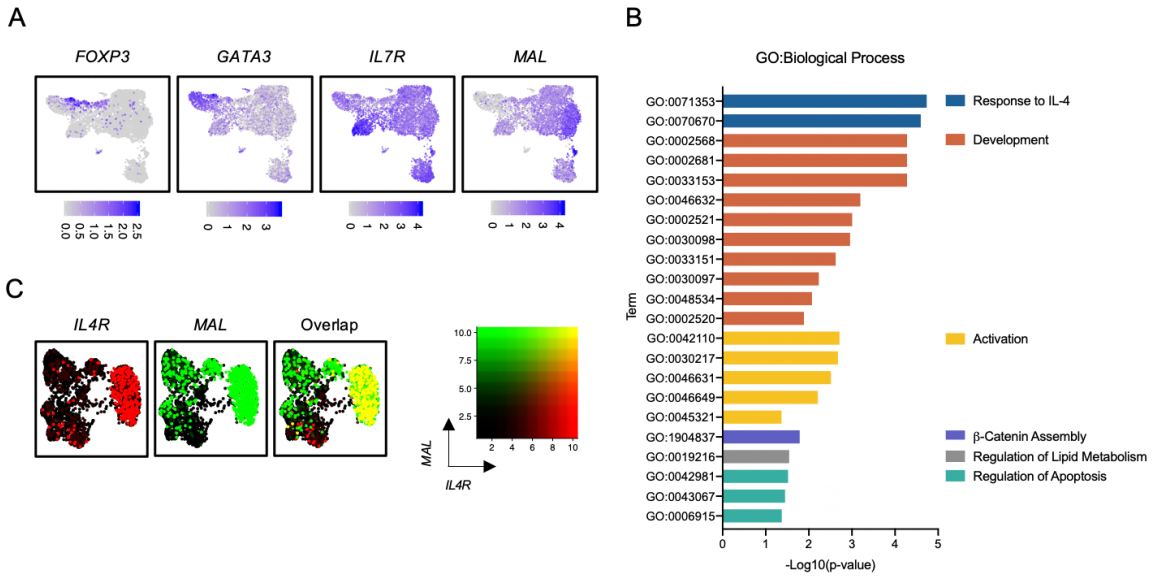

Figure S4. Gene expression in T cell subclusters. (**A**) UMAP visualization of all T cell clusters with overlay of gene expression used for T cell subclustering in Fig. 1H. (**B**) Pathway analysis (G:Profiler) of differentially expressed genes (excluding ribosomal genes) in *MAL*^{hi} cluster. (**C**) Feature plots depicting *IL4R* and *MAL* expression and co-expression in T cell subclusters.

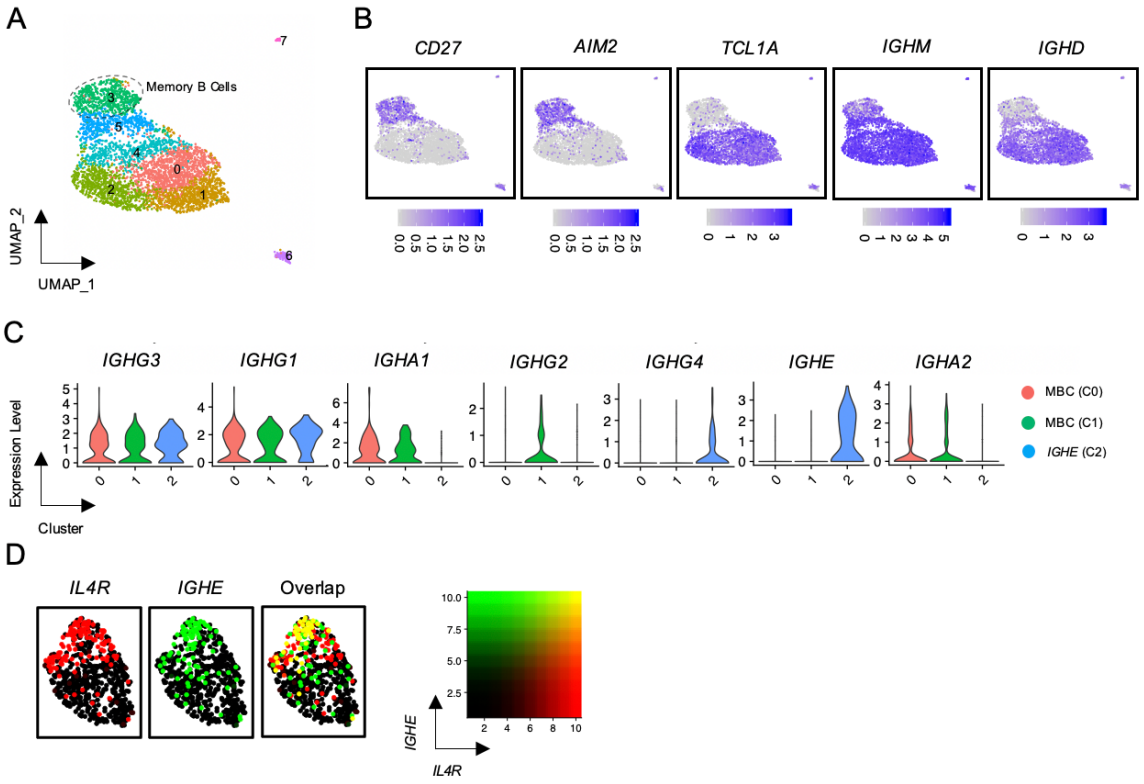

Figure S5. Gene expression in MBC subclusters. (A) UMAP of total B cells. **(B)** Feature plots of key genes used to subcluster MBCs in Fig. 1K. **(C)** Violin plots of IgH gene expression in MBC subclusters. **(D)** Feature plots depicting *IL4R* and *IGHE* expression and co-expression in MBC subclusters.

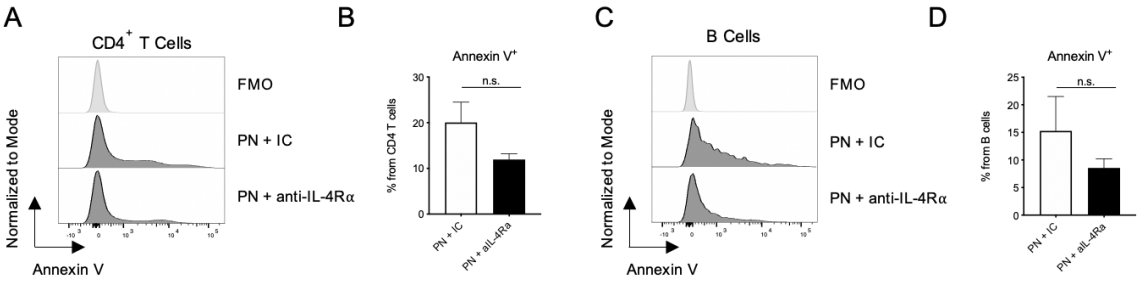

Figure S6. IL-4Rα blockade does not induce apoptosis. Representative histograms of Annexin V staining in CD4 T cells **(A)** and B cells **(C)** assessed by flow cytometry at end of culture. Frequency of Annexin V^+ CD4 T cells **(B)** and B cells **(D)**. Data are representative of two PN-allergic donors, plotted as mean ± SEM.

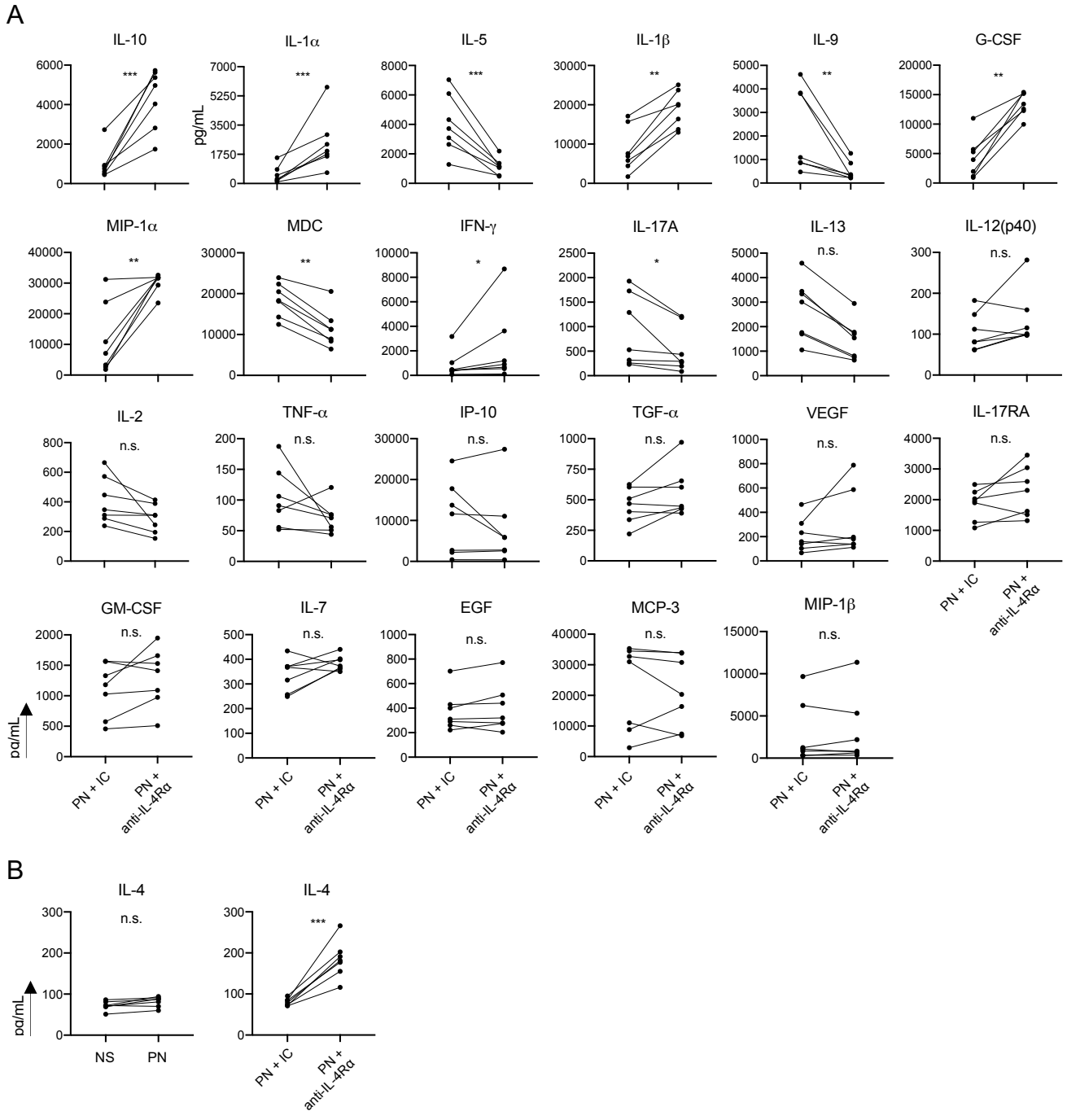

Figure S7. Cytokine response in anti-IL-4Rα-treated PBMCs and detection of IL-4. **(A)** Cytokines detected with 38-analyte array in anti-IL-4Rα- or IC-treated PN-stimulated PBMCs from PN-allergic donors (n=7). Plots are ordered (left to right) by ascending *p* value. **(B)** Detection of IL-4 in all treatment conditions. Each line represents an individual donor. **P*<0.05, ***P*<0.01, ****P*<0.001.

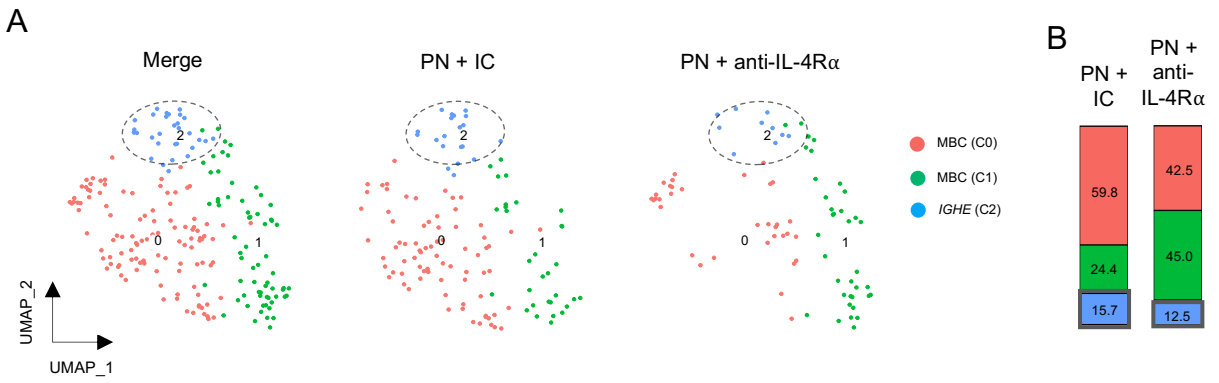

Figure S8. Effect of IL-4Rα on MBC clusters. (A) UMAP of subclustered memory B cells. **(B)** Stacked bars subcluster frequency (%) in A.

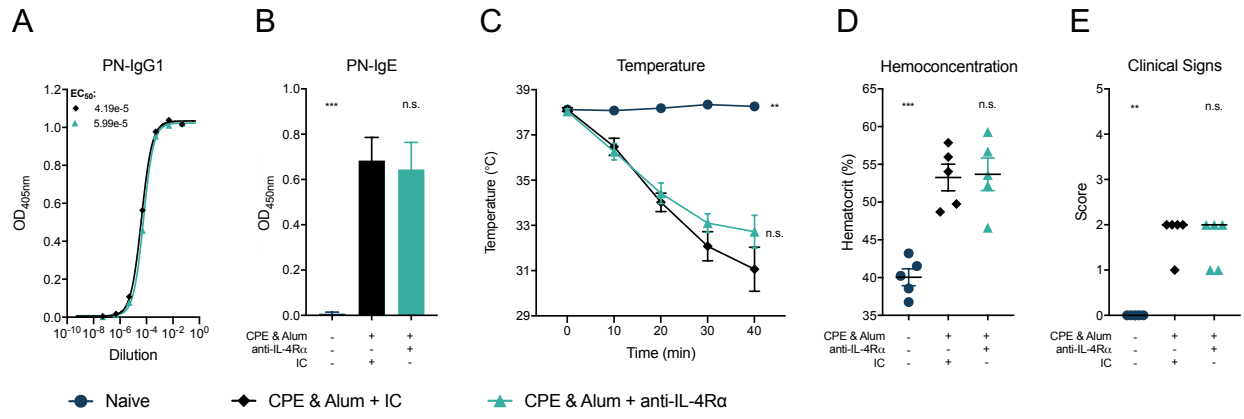

Figure S9. IL-4/IL-13 signaling is dispensable during allergen challenge. Tape-stripped mice were treated with anti-IL-4Rα or an isotype control the day before allergen challenge. Serum PN-specific IgG1 (**A**) and IgE (**B**) following sensitization. **(C)** Core body temperature at 10-minute intervals during systemic challenge. **(D)** Hemoconcentration at 40-minutes post-challenge. **(E)** Scoring of clinical signs during systemic challenge. Data are from a single experiment with 5 mice per experimental group, plotted as mean ± SEM or median (E). ***P*<0.01, ****P*<0.001. IC, isotype control.

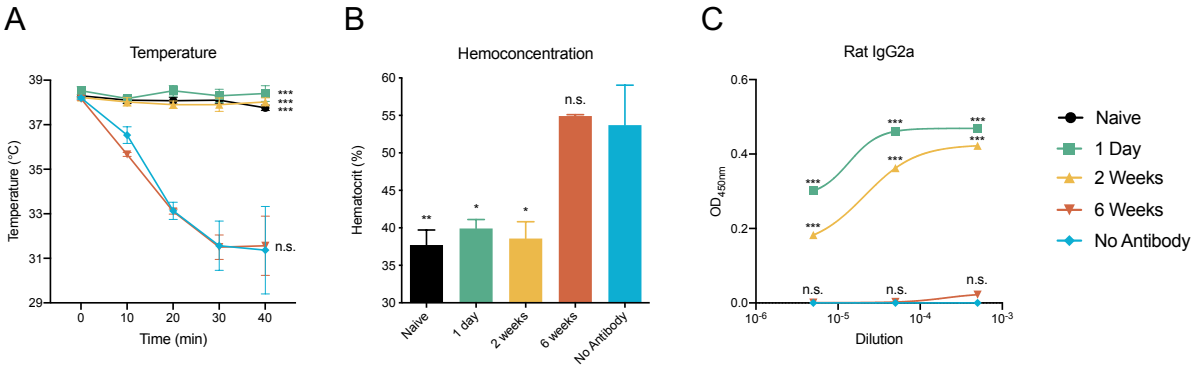

Figure S10. Clearance of anti-IL-4Rα *in vivo*. To demonstrate functional clearance of anti-IL-4Rα, mice were treated with anti-IL-4Rα 1 day, 2 weeks, or 6 weeks *before* epicutaneous sensitization, as IL-4 has previously been demonstrated to be critically required for allergic sensitization. Two weeks following epicutaneous sensitization, mice were challenged intraperitoneally with PN. **(A)** Core body temperature at 10-minute intervals during systemic challenge. **(B)** Hemoconcentration at 40-minutes post-challenge. **(C)** Serum rat IgG2a titers in mice injected with anti-IL-4Rα to demonstrate clearance of circulating antibody. Data are from 1 experiment with 3-5 mice per experimental group, plotted as mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001.

Table S1. Patient demographics.

Donor ID	Sex	Age	Serum PN-IgE (kU/L)	SPT (S-H-PN; mm)	Clinical history of PN allergy
P001	M	21	<0.1	2-5-2	No
P007	M	23	<0.1	1-4-1	No
P009	F	24	<0.1	1-4-1	No
P026	M	20	<0.1	1-5-1	No
P021	M	21	<0.1	1-4-1	No
P011	M	19	0.66	1-5-7	Yes
P016	M	21	1.66	1-4-8	Yes
P024	M	61	5.54	1-4-10	Yes
P020	M	26	7.56	1-4-12	Yes
P006	F	23	13.48	1-4-5	Yes
P017	M	22	43.28	1-4-10	Yes
P034	M	22	75.84	1-4-7	Yes
P040	F	12	>100	0-4-10	Yes
P041	F	13	>100	1-4-9	Yes
P042	F	14	>100	0-3-8	Yes
P027	M	13	>100	1-4-5	Yes
P043	M	10	>100	0-4-10	Yes

Chapter 4: Reprogramming of pathogenic IgE memory with IL-4Rα blockade

Kelly Bruton[1,2], Sharon Khavkine-Binstock[1,2], Allyssa Phelps[1,2], Jake Colautti[1,2], Tina Walker[1,2], Jianping Wen[1,2], Atai Ariaz[1,2], Olivia Mann-Delany[1,2], Siyon Gadkar[1,2], Emily Grydziuszko[1,2], Joshua Koenig[1,2], Susan Waserman[2,3,4], Manel Jordana[1,2]

[1] McMaster Immunology Research Centre, Department of Medicine, McMaster University, Hamilton, Ontario, Canada
[2] Schroeder Allergy and Immunology Research Institute, McMaster University, Hamilton, Ontario, Canada
[3] Department of Medicine, Division of Clinical Immunology & Allergy, McMaster University, Hamilton, Ontario, Canada
[4] The Research Institute of St. Joe's, Hamilton, Ontario, Canada

Corresponding Author: Manel Jordana (jordanam@mcmaster.ca)

ABSTRACT

Lifelong allergies are perpetuated by allergen-specific memory B cells (MBCs), primarily of an IgG1 isotype. Upon allergen re-exposure, IL-4 is critically required to replenish the transient pool of IgE^+ plasmablasts. Whether therapeutic intervention can deviate existing MBCs away from an IgE fate upon a recall response remains unknown. Here we investigated the plasticity of allergen- specific MBCs when deprived of IL-4 during a recall response. Our data demonstrate that antibody-mediated IL-4Rα blockade facilitates skewing of B cell responses away from a Th2 phenotype. This included the induction of serum allergen-specific IgG2b and IgG2c, a long-lived OVA^+ $IgG2c^+$ MBC compartment,

and loss of MBC Th2-like polarization. IgG2b and IgG2c isotypes were, at least in part, derived from existing class-switched MBCs, suggesting that pathogenic MBCs can be functionally reprogrammed. Altering the type of T cell help provided in recall responses might thus provide an efficacious approach to mitigate lifelong allergies and, perhaps, promote allergen tolerance.

INTRODUCTION

Type I hypersensitivities are mediated by pathogenic IgE responses against innocuous antigens. Studies from mice and humans indicate that IgE responses are relatively short-lasting, yet allergies to certain foods (*e.g.* peanuts) can persist for a lifetime.[1–4] Central to the persistence of lifelong allergies are IgG^+ memory B cells (MBCs). Upon allergen re-exposure, IgG^+ MBCs undergo sequential class-switching to replenish the transient IgE plasma cell pool.[4–6] Accordingly, methods to disarm or disable such memory must be established to transform disease.

Seminal work on IgE established IL-4 as a critical molecule for IgE class switch recombination in primary immunizations.[7] We and others have since established that the requirement of IL-4 persists beyond the initiation of IgE responses as it is necessary for the generation of secondary IgE responses in allergy and helminth infection.[8–10] Beyond IgE class-switching, the roles of IL-4 are pervasive within B cell immunity, including B cell survival and proliferation *in vitro*, upregulation of CD23, and augmenting the development of germinal center (GC) light zones.[11–13] Recently, single cell transcriptomics have revealed an IL-4-responsive phenotype unique to allergen-reactive/specific MBCs, including

heightened CD23, IL-4Rα, and ε germline transcript expression.[8,14] Thus, it appears that these cells are fated to become IgE-expressing in a recall response, though whether this commitment is irreversible remains to be elucidated.

Monoclonal antibody blockade of IL-4Rα (dupilumab) is an emerging therapeutic for the treatment of certain atopic diseases, including atopic dermatitis, moderate-to-severe asthma, and chronic rhinosinusitis. In all instances, dupilumab treatment was found to effectively decrease pathogenic Th2 biomarkers, including serum IgE.[15–17] Clinical trials assessing the efficacy of dupilumab during oral immunotherapy for desensitization to food allergens are currently underway. Given the success in other atopic diseases and preclinical evidence generated with human peripheral blood mononuclear cells *in vitro*, it is expected that dupilumab will be effective in ameliorating food-specific IgE responses by preventing IgE class-switching in non-IgE$^+$ MBCs.[8] This does not, however, address whether IL-4Rα blockade exerts more profound effects on B cell immunity and, in particular, whether the memory response is fundamentally reprogrammed away from an IgE fate.

Using a murine model of IgE-mediated food allergy, we found that while IL-4 supported allergen-specific B cell reactivation and germinal center (GC) formation, it was not critically required to establish secondary GCs. Recall responses with IL-4Rα blockade overcame the IgE-biased fate by promoting IgG2 production and diminishing pathogenic Th2 skewing in allergen-specific MBCs in an IFN-γ-dependent manner. Allergen-specific IgG2 responses were, at least in part, derived from existing class-switched MBCs.

Collectively, our findings elucidate a malleable fate of pathogenic allergen-specific MBCs and highlights the potential to therapeutically reprogram refractory lifelong allergies.

METHODS

Mice

Female C57BL/6 mice (6-8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). Female B6.129S7-*Ifng*^{tm1Ts}, B6.129S2-*Ighm*^{tmCgn}, and B6.SJL-*Ptprc*^{a} *Pepc*^{b}/Boy mice (6-8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). For IFN-γ KO experiments, control C57BL/6 mice were purchased from Jackson Laboratories. 4get and KN2 breeders were a generous gift from Dr. Irah King (McGill University). Homozygous 4get (*Il4*^{tm1Lky}) and KN2 (*Il4*$^{tm1(CD2)Mmrs}$) mice on C57BL/6 backgrounds were crossbred to generate 4get/KN2 mice, described previously.[8] All experimentation was in compliance with the McMaster University Research Ethics Board.

Immunizations

Epicutaneous sensitization: Epicutaneous sensitization to ovalbumin (Sigma-Aldrich, St. Louis, MO) was conducted as previously described.[8] For recall responses, three low-back subcutaneous injections of 100 μg OVA were given over the course of a week. *Gastric sensitization:* Mice received one intragastric gavage of 1 mg OVA with 5 μg cholera toxin in PBS. Four weeks later, mice received 1 mg OVA in PBS weekly for three weeks via gavage. Stool pellets were collected two weeks later for immunoglobulin analysis. *Intraperitoneal sensitization:* Mice were immunized intraperitoneally with 225 μL

Alhydrogel 2% (Invivogen, San Diego, CA) with 250 μg OVA. *Th1 immunization:* Mice were immunized intraperitoneally with 40 μg CpG ODN 1826 (Invivogen, San Diego, CA) and 250 μg OVA.

Serum for analysis of OVA-specific antibodies was collected through retro-orbital bleeding. For allergen challenges, 5 mg OVA was intraperitoneally injected. Core body temperature and clinical signs were recorded, as previously described, and hematocrit was measured at 40 minutes.[18]

Antibody Administration

Anti-CD40L (MR-1) and anti-trinitrophenol IgG2a isotype control (2A3) were purchased from Bio X Cell (Lebanon, NH). Anti-IL-4Rα (M1) was produced in-house, as previously described.[8] Original hybridomas were generated by Immunex Corporation (Amgen, Thousand Oaks, CA) and were generously provided by Dr. Fred Finkelman (University of Cincinnati).

Anti-IL-4Rα (1 mg) or isotype control was intraperitoneally injected the day prior to s.c. re-exposures. Anti-CD40L (0.35 mg) or isotype control was intraperitoneally injected on days -1, 0, and 2 of s.c. re-exposures.

Tetramer Staining and Enrichment

Purified OVA (Sigma-Aldrich, St. Louis, MO) was biotinylated (ThermoFisher Scientific, Waltham, MA) and tetramerized with streptavidin-PE or streptavidin-APC (Agilent, Santa

Clara, CA), as previously described.[24] For decoy tetramer, β-lactoglobulin (Sigma-Aldrich, St. Louis, MO) was biotinylated. Streptavidin-PE or -APC was conjugated with DyLight 594 or DyLight 755 esters (ThermoFisher Scientific) and was used to tetramerize the biotinylated- β-lactoglobulin.

Cell pellets were resuspended and stained with 5 nM decoy tetramer in 20 µL FACS buffer (10% FBS and 1 µM EDTA in PBS) and 2% rat serum for five minutes on ice. Five nM OVA tetramer was added and incubation continued for 30 minutes. Cells were washed to remove unbound tetramer and were incubated with anti-PE or -APC microbeads (Miltenyi Biotec, Germany) for 15 minutes on ice. Cells were passed through LS columns affixed to MACS magnetic separator (Miltenyi Biotec). Bound fractions were eluted with FACS buffer.

For CD45.1 enrichments, cell pellets were resuspended in 20 µL FACS buffer with 0.25 µg FcBlock (BioLegend) for five minutes on ice, then stained with anti-CD45.1-APC (A20; BioLegend) for 30 minutes. Enrichment with anti-APC microbeads proceeded as described above.

Flow Cytometry and Cell Sorting

Single-cell suspensions were plated in U-bottom 96-well plates and incubated with 0.25 µg FcBlock for 10 minutes on ice. Cells were stained extracellularly for 30 minutes on ice with antibody cocktails prepared in FACS buffer. For intracellular staining, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), as per manufacturer's

instructions. Intracellular antibody cocktails were prepared in 1x perm wash and cells were stained for 30 minutes on ice. The following anti-mouse fluorochrome-conjugated antibodies were used: B220-AF700 (RA3-6B2), CD138-PE/Dazzle 594 (281-2), CD3-BV711 (17A2), CD3-BV510 (17A2), F4/80-BV711 (BM8), F4/80-BV510 (BM8), CD38-PE/Cy7 (90), GL7-PerCP.Cy5.5 (GL7), GL7-BV421 (GL7; BD, Franklin Lakes, NJ), IgD-BV605 (11-26c.2a), IgM-BV786 (II/41; BD), IgM-FITC (II/41), IgG1-BV650 (RMG1-1), IgG2c-FITC (polyclonal; SouthernBiotech, Birmingham, AL), IL-4Rα-PE (REA235; Miltenyi Biotec), and CD23-BV421 (B3B4). All antibodies were purchased from BioLegend (San Diego, CA), unless otherwise noted. Viability staining was executed with Fixable Viability Dye eFluor780 (eBioscience, San Diego, CA) or Fixable Viability Stain 510 (BD Biosciences).

Data were acquired with an LSRFortessa flow cytometer (BD). Bulk cell sorting was performed on a MoFlo XDP cell sorter (Beckman Coulter, Brea, CA). All data were analyzed with FlowJo, version 10 software (Tree Star, Ashland, OR).

Adoptive Transfers

Class-switched memory B cells ($B220^+$ $CD3^-$ $F4/80^-$ $CD38^+$ $GL7^-$ IgD^- IgM^-) from tetramer enriched fractions were isolated by flow sorting four weeks after donor immunization. 10^4 cells were intravenously injected in the tail-vein of naïve recipient (muMT) mice. The day after transfer recipients were intraperitoneally injected with 200 μg OVA with either 40 μg CpG or 5 μg CT. One week later, spleens and mesenteric lymph nodes were collected for

flow cytometric analysis of donor B cells, and serum was collected for OVA-specific antibody analysis by ELISA.

ELISAs

OVA-specific immunoglobulins were measured by sandwich ELISA, described previously, with some modifications.[19] Briefly, MaxiSorp plates (ThermoFisher Scientifc) were coated with 100 ng anti-IgG2b, -IgG2c, -IgE, or -IgA overnight at 4°C. Plates were blocked in 5% skim milk at 37°C for one hour, then diluted samples were added and incubated overnight at 4°C. Digoxigenated OVA (15 ng) prepared in-house was added for two hours at room temperature, followed by anti-digoxigenin Fab fragments (Roche, Basel, Switzerland) for one hour. Plates were developed with 3,3',5,5'-tetramethylbenzidinie (Sigma-Aldrich) and stopped with sulfuric acid. Optical densities were acquired with a Multiskan FC photometer (ThermoFisher Scientific).

For OVA-IgG1, plates were coated with 400 ng OVA in carbonate-bicarbonate buffer overnight at 4°C. Plates were blocked in 5% skim milk at 37°C for two hours, then diluted samples were added and incubated overnight at 4°C. Biotinylated anti-mouse IgG1 (SouthernBiotech;1.25 ng) was added for two hours at room temperature, followed by alkaline-phosphatase streptavidin (ThermoFisher Scientific) for one hour. Plates were developed with 4-nitrophenyl phosphate (Sigma-Aldrich) and stopped with sodium hydroxide. Optical densities were acquired as above.

Immunofluorescence

Lymph nodes were fixed in 4% paraformaldehyde for two hours, rinsed with PBS, then incubated overnight in 30% sucrose. Fixed tissues were snap frozen in OCT compound (Sakura Finetek, Torrance, CA) and stored at −80°C until sectioning. Tissues were cut into 20 μm sections and mounted onto gelatin-coated slides. Following a one-hour block with 5% rat serum, tissues were stained overnight at 4°C with the following antibodies: anti-GFP-AF488 (polyclonal; ThermoFisher Scientific), CD3-AF700 (17A2), GL7-AF647 (GL7), IgD-APC/Cy7 (11-26c.2a), and CD35-PerCP-Cy5.5 (7E9). All antibodies were purchased from BioLegend, unless otherwise noted. Images were acquired with a Stellaris 5 confocal microscope (20x Oil; Leica, Wetzlar, Germany). ImageJ (Bethesda, MD) was used for image analysis.

Statistical Analysis

Data were analyzed with Prism 9 software (GraphPad, San Diego, CA). Student's t-test, ANOVA, or Kruskal-Wallis tests (for nonparametric data) were performed. $P < 0.05$ was considered statistically significant.

RESULTS

Loss of IL-4/IL-13 signaling stalls secondary GC activity

Beyond IgG1 and IgE class switch recombination, previous reports suggest a critical requirement of IL-4 signaling for primary GC activity in models of Th2 immunity.[20,21] It is not clear whether this requirement continues for recall responses, especially because certain MBC subsets arising from the primary immune response are poised for GC reentry

(CD80$^-$ CD73$^-$/PD-L2$^-$).[5,22] To further elucidate the role of IL-4 in GCs, we employed a well characterized model of food allergy involving epicutaneous sensitization to ovalbumin (OVA).[23] This approach generates CD80$^-$ PD-L2$^-$ IgG1$^+$ MBCs and provides temporal control over the IgE response through non-sensitizing subcutaneous re-exposures (s.c.).[23] Firstly, we assessed whether MBCs generated upon epicutaneous sensitization do, in fact, require secondary GCs to produce IgE and facilitate IgE-mediated clinical reactivity. To this end, anti-CD40L blocking antibodies were administered to sensitized mice before and during *s.c.* re-exposures (Fig. S1 A). Upon systemic challenge, anti-CD40L-treated mice were fully protected from IgE-mediated anaphylaxis, as indicated by a maintenance of homeostatic core body temperature and hemoconcentration, with an absence of clinical signs (Fig. S1 B-D). Likewise, serum OVA-specific IgE was absent and IgG1 titers were blunted with the loss of CD40 engagement (Fig. S1 E).

To test whether IL-4Rα blockade impaired secondary GC activity, we treated epicutaneously-sensitized mice with anti-IL-4Rα (A4RA) or an isotype control (IC) prior to *s.c.* OVA re-exposures. OVA-specific B cells from inguinal lymph nodes were identified using fluorochrome-conjugated OVA tetramers and a magnetic bead-based enrichment strategy (Fig. 1 A).[24] The frequency of OVA$^+$ B cells was 3-fold lower in A4RA-treated mice, with an average of 406 versus 1269 cells per mouse in A4RA and IC groups, respectively (Fig. 1 B). Expression of CD38 and GL7 identifies three populations of B cells differing in their activation states: CD38$^+$ GL7$^-$ resting naïve/memory (black), CD38$^+$ GL7$^+$ transitional cells (blue), and CD38$^-$ GL7$^+$ GC B cells (orange) (Fig. 1 C).[25] At three days post-final re-exposure, the A4RA-treated group had significantly impaired OVA-specific

B cell activation, evidenced by an increased frequency of $CD38^+$ $GL7^-$ OVA-specific B cells and a concomitant decrease in the $CD38^-$ $GL7^+$ population (Fig. 1 D and F). Similarly, GCs appeared underdeveloped with loss of IL-4 signalling as assessed by immunofluorescent imaging (Fig. 1 G). However, 23% of OVA-specific B cells in A4RA-treated mice were in a transitional state compared to 2.6% in IC-treated mice (Fig. 1 E), which suggested that loss of IL-4 signaling altered the kinetics of MBC reactivation. Indeed, assessment of GC activity at 7-, 14-, and 28-days post-re-exposure demonstrated that the magnitude of GC activity is no different between groups and A4RA merely stalls early GC formation (Fig. 1 H).

IL-4Rα blockade induces long-lasting IgG2c responses in allergic mice

Given that A4RA prevents IgE CSR, but B cell reactivation proceeds albeit with delayed kinetics, we sought to define the product of MBC responses by way of Ig isotype. Beyond three days post-re-exposure, the frequency of OVA^+ B cells that were class-switched (IgD^- IgM^-) was no different between treatment groups (Fig. 2 A and B). Since IL-4Rα blockade promotes IFN-γ production, it was of particular interest to determine whether OVA-specific B cells were retained as IgG1-expressing or if there was an induction of IgG2c-expressing cells (Fig. 2 C).[8] IgG1 expression dominated GC B cells, with no change in the frequency of $IgG1^+$ GC B cells between groups (Fig. 2 D); however, a population of $IgG2c^+$ GC B cells emerged in A4RA-treated mice, at seven times the frequency compared to control mice (Fig. 2 D). Within the OVA^+ MBC compartment, both $IgG1^+$ and $IgG2c^+$ cells were present beyond five months post-re-exposures (Fig. 2 E). Similar to isotype expression in

GCs, IL-4Rα blockade significantly increased the frequency of $IgG2c^+$ cells, but also facilitated a decrease in $IgG1^+$ cells within the OVA^+ MBC population (Fig. 2 E). By ELISA, all OVA-specific antibody isotypes inclusive of and downstream to IgG1, including IgG2b, IgG2c, IgE, and IgA were measured. As we previously demonstrated, A4RA-treated mice produced similar levels of OVA-IgG1 and no OVA-IgE (Fig. 2 F).[8] Consistent with the cellular analysis, there were increased serum levels of OVA-IgG2c. Serum IgG2c titers were transient returning to baseline by nine weeks post-re-exposure (Fig. 2 F); however, the persistence of $IgG2c^+$ OVA^+ MBCs beyond five months suggests that the secreted IgG2c response may be renewed upon subsequent allergen exposures. In both A4RA- and IC-treated mice OVA-IgA was undetectable (data not shown).

Classically, gastrointestinal exposures to food allergens in non-allergic individuals yields active tolerance marked by the presence of IL-10- and TGF-β-secreting regulatory T cells. In mice, allergen feeding in the absence of adjuvant elicits production of allergen-specific IgA.[26] Hence, we hypothesized that IL-4Rα blockade during oral re-exposures might restore the homeostatic IgA-dominant response, rather than an emergence of Th1-skewing which occurred in the context of epicutaneous re-exposures. To establish allergic sensitization, mice received one gavage of OVA and CT.[26] One month later, intragastric re-exposures of OVA preceded by A4RA or IC were administered once weekly for three weeks (Fig. S2 A). In contrast with the epicutaneous model, A4RA did not reduce the frequency of OVA^+ B cells (Fig. S2 B). Within the OVA^+ B cell pool, the frequency of IgD^- IgM^- cells persisting one month post-re-exposures was no different between treatment groups (Fig. S2 C); however, comparable to that observed in epicutaneously sensitized

mice, IL-4Rα blockade reduced the size of the $IgG1^+$ MBC pool and similarly induced a population of $IgG2c^+$ OVA-specific MBCs (Fig. S2 D). This was also reflected in the profile of serum immunoglobulins (Fig. S2 E). While OVA-IgA was detectable at low levels in serum and stool samples, it was not different irrespective of IL-4 signaling, though we did observe a significant increase in IgG2b – a TGF-β-induced isotype (Fig. S2 E). Thus, loss of IL-4 signaling during allergen re-exposures preferentially promotes IgG2c responses irrespective of tissue site.

Having demonstrated evidence of Th1-skewing in both T and B cells, we next compared how similar/dissimilar this reprogrammed recall response was to a *bona fide* Th1 response against the same antigen. Herein, wild-type mice were i.p. immunized to OVA with either the Th1 adjuvant, CpG ODN 1826, or Alum as a Th2 control. Two weeks post-sensitization, mice were i.p. challenged with OVA to evaluate clinical reactivity. As anticipated, Alum- but not CpG-immunized mice experienced severe anaphylaxis as assessed by core body temperature, hemoconcentration, and clinical signs (Fig. S3 A-C). Most strikingly, the profile of serum Igs was nearly identical to that of anti-IL-4Rα-treated mice, wherein CpG immunization induced OVA-IgG1, -IgG2b, and -IgG2c, but not OVA-IgE (Fig. S3 D). Taken together, the B cell response in A4RA-treated mice closely mirrors *bona fide* Th1-immunization in both the absence of clinical reactivity upon OVA challenge and the profile of OVA-specific serum Igs.

Anti-IL-4Rα ameliorates pathogenic Th2-skewing in memory B cells

Two recent transcriptomic studies identified a MBC cluster that co-expresses ε germline transcripts (*IGHE*), the low affinity IgE receptor (*FCER2*/CD23), and *IL4R*.[8,27] Likewise, *IGHE*$^+$ *FCER2*$^+$ IgG$^+$ MBCs were found to be clonally related to IgE$^+$ plasmablasts in birch-allergic humans, suggesting that this Th2-polarized MBC cluster is the reservoir for IgE responses.[14] Therefore, we next investigated whether IL-4Rα blockade altered the Th2-polarized phenotype. Co-expression of surface IL-4R and CD23 was assessed four weeks following *s.c.* re-exposures (Fig. 3 A). A4RA-treated mice exhibited a 61% reduction in the frequency of IL-4Rα$^+$ CD23$^+$ cells in the OVA-specific MBC compartment (Fig. 3 B). Loss of IL-4Rα expression was not an artifact of impaired detection due to the *in vivo* blocking antibody as there was no increase in the frequency of CD23 single-positive cells (Fig. 3 B). In both groups, the majority of Th2-polarized OVA-specific MBCs were IgG1$^+$ (Fig. 3 C). Consistent with a role of IL-4 in inducing CD23 expression, A4RA facilitated a decreased surface expression of CD23 (Fig 3 D).[12] CD23 downregulation was not, however, unique to allergen-specific cells, but instead was broadly observed throughout non-specific MBCs (Fig 3 E). As CD23 has been proposed to augment allergen presentation to T cells, its broad downregulation on all B cells may be beneficial in restricting secondary T cell responses.[28]

With the prospect of using A4RA (dupilumab) as a therapeutic for food allergy treatment, we next evaluated whether loss of Th2 MBC skewing also occurred in humans. In this regard, we performed a targeted analysis in our existing single-cell RNA-sequencing dataset of A4RA- or IC-treated human peripheral blood mononuclear cells from a peanut-

allergic donor.[8] Unsupervised clustering of non-naïve B cells resolved five clusters (Fig. 3 F). Cluster 3 (green) was identified as the Th2-polarized MBC population, characterized by distinct expression of *IL4R*, *FCER2*, and *IGHE* (Fig. 3 G). While this cluster was still present in A4RA-treated cells, the expression level of each transcript was markedly reduced compared to the IC group (Fig. 3 H). Taken together, these data indicate that Th2-skewing of allergen-specific MBCs can be impacted; however, in the contexts tested here, we did not observe a complete therapeutic reversal questioning whether resistant cells may still be poised for an IgE fate. Notably, this was the effect of a single administration of A4RA, which questions whether a longer duration of treatment may exert a more profound effect.

Establishing the IgG2c$^+$ MBC compartment is IFN-γ-dependent

Blockade of IL-4Rα during a recall response to allergen induced IFN-γ production in both mice and humans.[8] Given that IFN-γ is a potent inducer of IgG2c class switch recombination in mice, we reasoned that increased IFN-γ production was likely to have facilitated the Th1-skewed B cell responses. To this end, IFN-γ$^{-/-}$ mice were epicutaneously sensitized to OVA, treated with A4RA and IC, and re-exposed to OVA as described earlier. As anticipated, A4RA-treated IFN-γ$^{-/-}$ mice lacked an OVA-specific IgG2c$^+$ MBC population, unlike their WT counterparts, though the reduction in OVA$^+$ IgG1$^+$ MBC frequency was conserved (Fig. 5A and B). The impact of IL-4Rα blockade on OVA-specific IgG1 and IgE serum titers was similar between WT and IFN-γ$^{-/-}$ mice (Fig. 5C). Quite surprisingly, however, IFN-γ$^{-/-}$ mice produced comparable levels of OVA-IgG2c when treated with A4RA, though exhibited increased OVA-IgG2b titers (Fig. 5C). The

divergent findings between the cellular and humoral observations propose a model in which IgG2c class switch recombination can proceed independent of IFN-γ signaling, but IFN-γ facilitates a critical role in the generation of IgG2c$^+$ MBCs. Indeed, in T cell-dependent type 1 responses, others have shown that IgG2a/c responses can occur in the absence of IFN-γ/IFN-γR, though the antigen-specific MBC compartment may be diminished.[29-32]

We also assessed the impact of IFN-γ deficiency on the Th2 phenotype of OVA-specific MBCs (Fig. 5 D). Here, loss of IL-4Rα and CD23 surface expression occurred independently of IFN-γ (Fig. 5E). Taken together, these data demonstrate that loss of IL-4 signaling is adequate to reduce MBC Th2 polarization, though the reprogramming to long-lived IgG2c$^+$ MBCs requires active input from IFN-γ.

IgG2 responses arise from allergic class-switched memory B cells

The isotypic and phenotypic findings described above indicate the potential to reprogram pathogenic IgE responses to allergens; however, it is unclear if these altered responses are derived from naïve B cells or direct reprogramming of existing Th2-skewed MBCs. To this end, we developed an adoptive transfer model involving the transfer of class-switched MBCs from Th2 immunized CD45.1$^+$ mice into naïve B cell-deficient (muMT) mice (Fig. 5 A). Recipient mice were then immunized with OVA+CT or OVA+CpG to promote Th2 or Th1 recall responses, respectively. Thus, all resulting B cell responses in recipient muMT mice are generated from donor cells, enabling the fate mapping of OVA-specific MBCs. CD45.1$^+$ OVA$^+$ B cells were identified in the spleens and mesenteric lymph nodes

of recipient mice (Fig. 5 B). Consistent with the input population, all CD45.1$^+$ OVA$^+$ B cells were class-switched (Fig. 5 C). IgG1$^+$ cells were present in both groups of recipient mice, coinciding with a dominance of IgG1 in the pool of adoptively transferred class-switched MBCs (Fig. 5 D). IgG2c$^+$ OVA-specific B cells were detected only with CpG immunization, establishing the potential for allergic class-switched MBCs to attain a Th1 fate. Likewise, OVA-IgG1, -IgG2c, as well as OVA-IgG2b, but not -IgE was present in the serum of CpG-immunized mice; in contrast, only OVA-IgG1 and -IgE were produced with OVA+CT immunization (Fig. 5 E). Overall, re-exposure with OVA+CpG overcame existing Th2 biases, suggesting that direct reprogramming of allergic MBCs is plausible.

DISCUSSION

The potential to reprogram allergen-specific MBCs away from an IgE fate remains uncertain. Previous work has identified IgG1$^+$ MBCs as the primary reservoir of IgE memory in mice and humans, with an apparent absence of IgE$^+$ MBCs.[4,5,33,34] This point has been further supported through clonal lineage mapping in which shared clones are observed between IgG$^+$ MBCs and IgE$^+$ plasmablasts.[14] Allergen-specific/reactive IgG$^+$ MBCs have been found to express ε germline transcripts, suggestive of their commitment to IgE upon reactivation.[8,14] Likewise, these cells upregulate genes relating to IL-4 signaling, including *IL4RA* and *FCER2,* suggesting a future propensity to respond to IL-4. Indeed, we recently demonstrated that CD4 T cells and IL-4 are critically required to establish secondary IgE responses from non-IgE-expressing MBCs.[8] Beyond prevention of IgE class switching, loss of IL-4 signaling enhanced IFN-γ and IL-10 production,

suggestive of non-Th2 skewed T cell responses. Since these data intimated a reprogrammed T cell response, we sought to investigate whether IL-4Rα blockade deviated allergen-specific MBCs away from a Th2-skewed fate or simply prevented IgE class switch recombination while maintaining the pool of pathogenic $IgG1^+$ MBCs.

It was previously reported that IL-4 was required for GC formation in primary Th2 immunization models[20]; therefore, it was plausible for A4RA-treatment to have blunted GC B cell responses, while maintaining the pool of pathogenic $IgG1^+$ MBCs and perhaps enabling extrafollicular $IgG1^+$ plasmablast differentiation. Instead, we found that GCs could form in the absence of IL-4 signaling albeit with delayed kinetics and, strikingly, the frequency of OVA-specific $IgG1^+$ MBCs declined. The former is at variance with the previous report perhaps due to differences in model and/or use of antibody-mediated blockade instead of knockout mice. Nonetheless, this provided insight that B cell responses in a recall response could proceed without IL-4 and, therefore, questions what the product of this response was if not IgG1 or IgE.

The end goal of a disease-transforming therapeutic is to achieve allergen tolerance. Often, allergies to cow's milk and egg are outgrown presenting a form of naturally acquired tolerance. In instances where food allergies are not outgrown, oral immunotherapy can transiently induce a tolerant state.[35] In humans, both naturally- (outgrowth) and therapeutically-acquired tolerance are associated with increased allergen-specific IgG4 and, less acknowledged, IgG2 titers.[36–39] Elevated allergen-specific IgG can inhibit IgE-mediated mast cell and basophil activation through inhibitory FcγRIIb.[36,40] As circulating

IgG is much more abundant than IgE, allergen-specific IgG may also provide steric blockade of allergen preventing engagement with FcεRI-bound IgE.[36,41] During oral immunotherapy, however, the path to increased inhibitory IgG is accompanied by an initial increase in allergen-specific IgE, which may position patients at an increased risk of adverse events.[42-45] Here we show that the loss of Th2-skewed B cell responses observed upon A4RA treatment was accompanied by an increase in allergen-specific IgG2b and IgG2c while maintaining an absence of IgE. Although the humoral IgG2c responses observed in our experiments were transient, we observed a population of allergen-specific $IgG2c^+$ MBCs arise in an IFN-γ-dependent manner that persisted beyond six months after a single A4RA injection. Thus, IL-4Rα blockade appears to give rise to long-lived $IgG2c^+$ MBCs which may restore secreted IgG2 upon tertiary allergen re-exposure.

Beyond isotypic reprogramming, we evaluated the requirement of IL-4 for the maintenance of the Th2-skewed MBC phenotype. Indeed, a large-scale analysis of B cell surface markers put forth the concept that phenotype, rather than isotype, better predicts B cell function.[46] In humans, expression of IL-4Rα and the low-affinity IgE receptor, CD23, appear on MBCs that are clonally related to IgE^+ plasmablasts.[14] While the mechanism by which these surface markers yield an IgE fate remain to be elucidated, it can be inferred that heightened IL-4Rα expression increases responsiveness to IL-4 necessary for IgE class switch recombination. Regarding CD23, existing evidence indicates a role of CD23 in amplifying presentation of allergen peptides to cognate T cells through IgE-mediated antigen presentation, which is suggested to intensify allergen-specific B cell responses.[47] We found

that recall responses in the absence of IL-4 signaling substantially reduced the frequency of IL-4Rα$^+$ CD23$^+$ OVA-specific MBCs; however, approximately 10% were retained within this double-positive population, almost all of which expressed IgG1. These data were mirrored in a single-cell transcriptomic analysis of peanut-allergic human PBMCs, where expression level of *IL4R* and *FCER2* transcripts were reduced but remained elevated relative to other MBC clusters. Therefore, unless adequately suppressed or outcompeted by other cells, perhaps through repeated drug and allergen administrations, the persistence of few Th2-skewed IgG1$^+$ MBCs may compromise the duration of protection/tolerance, much like the small population of Th2A cells maintained after oral immunotherapy.[48,49]

Skewing existing Th2 responses towards Th1 has previously been achieved using Th1 adjuvants, including CpG ODN. In murine asthma models, both prophylactic and therapeutic use of CpG ODN diminishes Th2 responses (IgE, eosinophilia, etc.) and enhances regulatory and Th1 responses, including IL-10, IFN-γ, and IgG2a/c.[50] In orally sensitized mice, treatment with CpG-coated nanoparticles containing peanut extract impaired anaphylaxis upon challenge, in addition to reducing Th2 cytokines and peanut-IgE titers, and increasing IFN-γ and serum peanut-IgG2a.[51] While these preclinical models demonstrate success in therapeutically diminishing established Th2 responses, it had not been elucidated whether the Th1-skewing arises as a *de novo* response (i.e. from naïve B cells) or if existing MBCs acquire a Th1 fate. Through adoptive transfer of class-switched MBCs, we determined that existing MBCs can be directly reprogrammed away from their

Th2 fate, though this does not negate the possibility of a naïve B cell contribution in immunocompetent systems.

Overall, our study reveals a functional plasticity of allergen-specific MBCs. Despite being phenotypically poised to maintain a Th2 fate, loss of IL-4 signaling during allergen re-exposures was sufficient to provoke IgG2 responses. As the product of B cell responses are directly influenced by the nature of T cell help, a more comprehensive understanding of allergen-specific T cell plasticity is most warranted.

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FIGURES

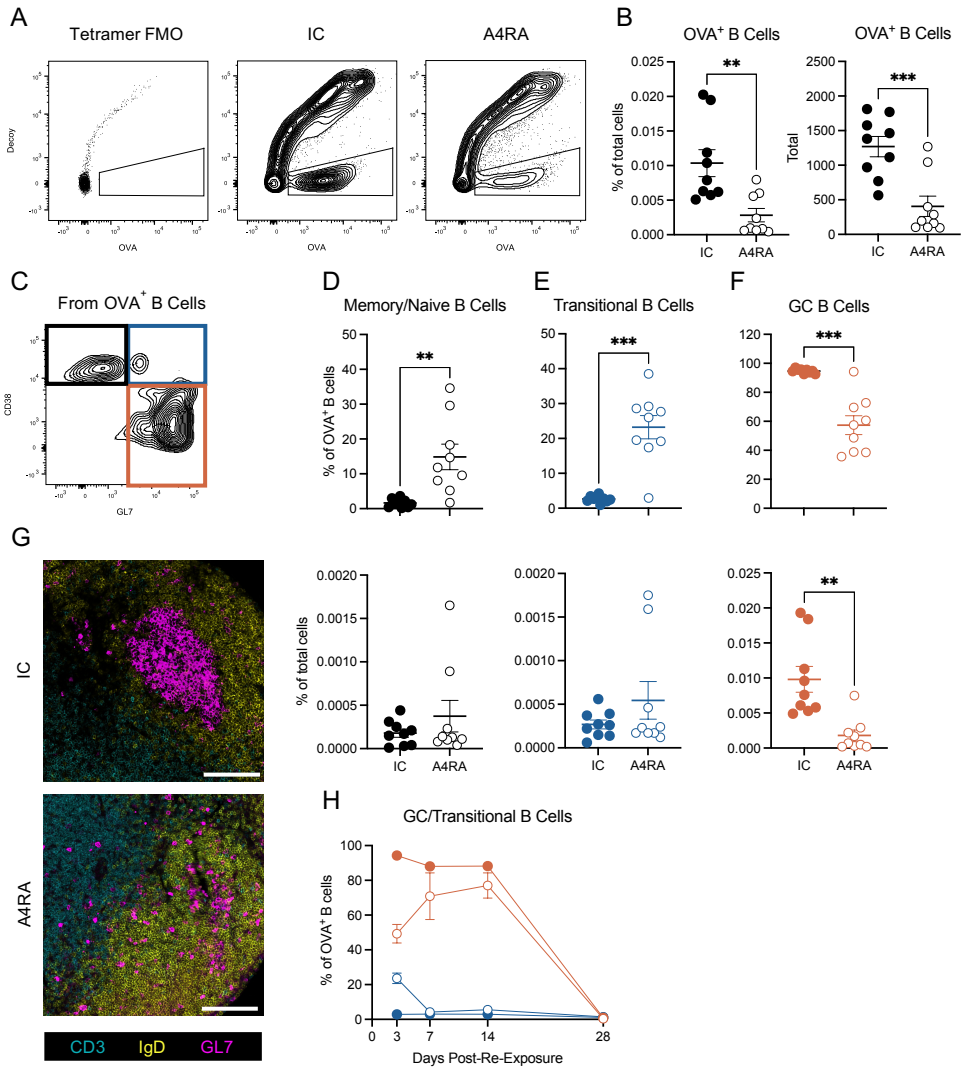


Figure 1. Loss of IL-4/IL-13 signaling stalls secondary germinal center activity.
(A) Representative contour plots of tetramer staining in inguinal lymph nodes of epicutaneously-sensitized mice. Events were pre-gated as live, singlet, $CD3^-$ $F4/80^-$ $B220^+$. FMO, fluorescence minus one. **(B)** Frequency and absolute count of OVA-specific B cells in IC- and A4RA-treated mice. **(C)** Representative contour plot of CD38 and GL7 expression in OVA-specific B cells, identifying three populations: memory/naïve (black), transitional (blue), and GC (orange) cells. **(D-F)** Summary plots of $CD38^+$ $GL7^-$ (D), $CD38^+$ $GL7^+$ (E), and $CD38^-$ $GL7^+$ (F) as frequency of OVA^+ B cells and of total cells at three days post-re-exposures. **(G)** Immunofluorescent images of GCs in inguinal lymph nodes harvested at three days post-re-exposures. Tissues were stained with CD3 (cyan), IgD (yellow), and GL7 (magenta). Scale bars, 100 μM. **(H)** Frequency of OVA^+ B cells

expressing $CD38^+$ $GL7^+$ (blue) and $CD38^-$ $GL7^+$ (orange) from three to 28 days post-re-exposures. Data are plotted as individual mice (B, D-F) or mean (H) ± SEM. ** and *** represent $p < 0.01$ and < 0.001, respectively.

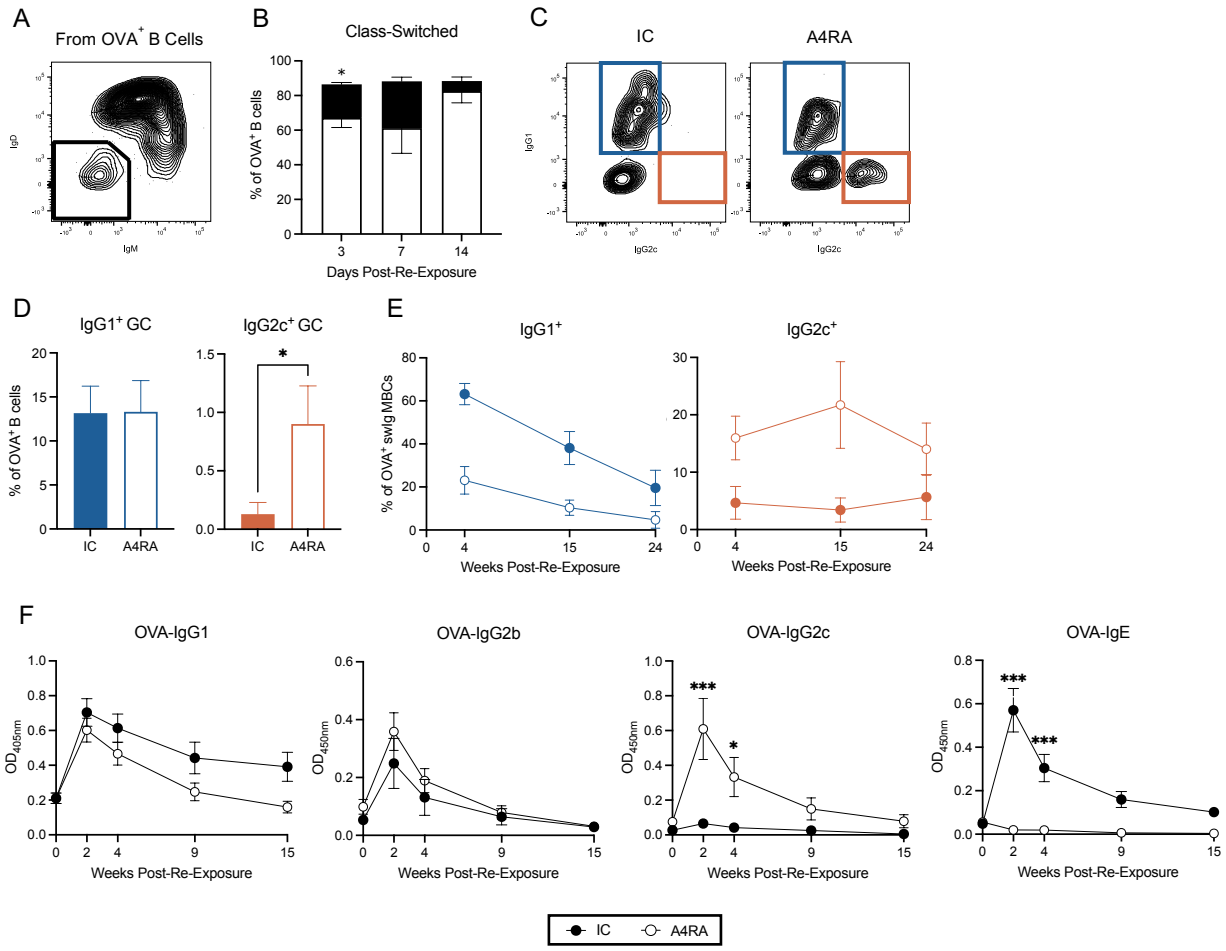


Figure 2. IL-4Rα blockade induces IgG2c responses in allergic mice.
(A) Representative contour plot of class-switched (IgD$^-$ IgM$^-$) OVA$^+$ B cells in pooled inguinal lymph nodes and spleens of epicutaneously-sensitized mice. Events were pre-gated as live, singlet, CD3$^-$ F4/80$^-$ B220$^+$ OVA$^+$. **(B)** Frequency of OVA$^+$ B cells that are IgD$^-$ IgM$^-$ from three to 14 days post-re-exposures. **(C)** Representative contour plots of IgG1 (blue) and IgG2c (orange) expression in class-switched OVA-specific B cells. **(D)** Summary bar graphs of IgG1 and IgG2c-expressing OVA$^+$ GC B cells at seven days post-re-exposure. **(E)** Frequency of OVA-specific MBCs expressing IgG1 or IgG2c up to 24 weeks post-re-exposures in pooled spleens, inguinal lymph nodes, and bone marrow. **(F)** ELISA quantification of serum OVA-specific immunoglobulins after tape-stripping (0) to 15 weeks post-re-exposures. Data are plotted as mean ± SEM. * and *** represent $p < 0.05$ and < 0.001, respectively.

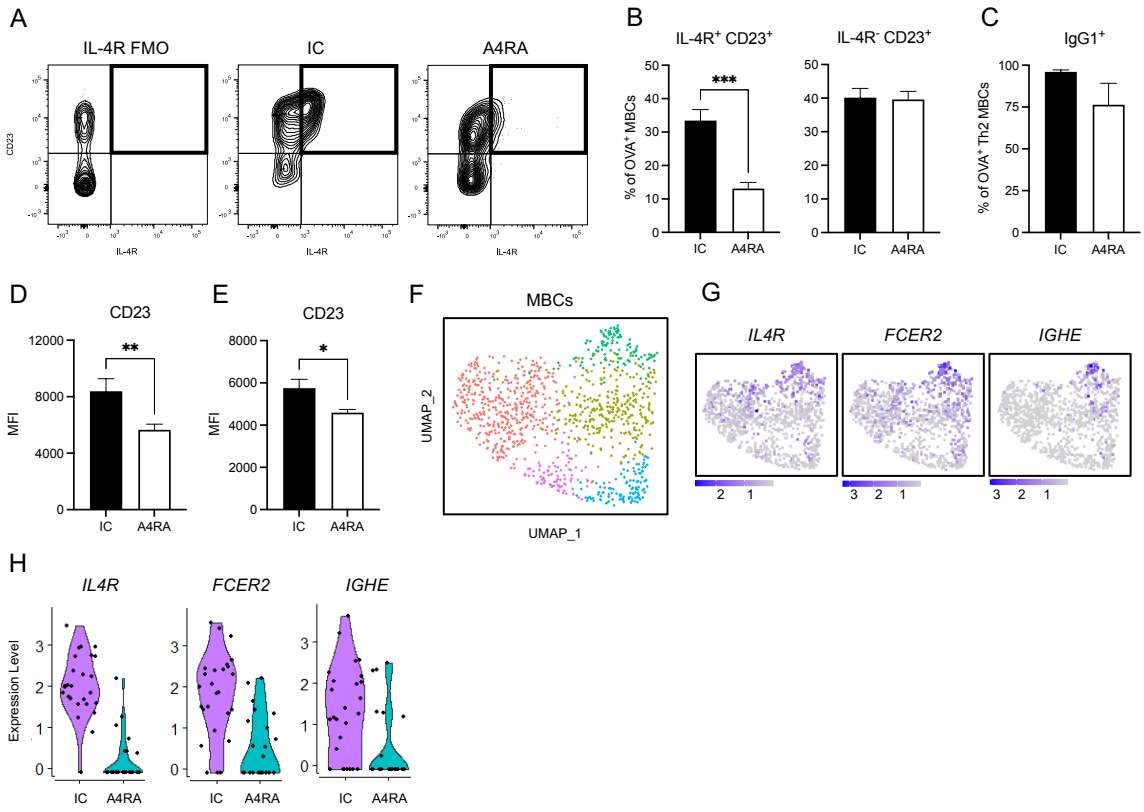


Figure 3. IL-4 signaling is required to maintain Th2-skewing in allergen-specific MBCs.

(A) Representative contour plots of IL-4R and CD23 expression in OVA$^+$ MBCs in pooled inguinal lymph nodes and spleens of epicutaneously-sensitized mice. Events were pre-gated as live, singlet, CD3$^-$ F4/80$^-$ B220$^+$ OVA$^+$ CD38$^+$ GL7$^-$ IgD$^-$ IgM$^-$. FMO, fluorescence minus one. **(B)** Summary plots of IL-4R$^+$ CD23$^+$ and IL-4R$^-$ CD23$^+$ populations from OVA$^+$ MBCs. **(C)** Percent of Th2-polarized MBCs expressing IgG1. **(D and E)** Mean fluorescence intensity (MFI) of surface CD23 expression on OVA-specific (D) and non-specific (E) MBCs. **(F)** Uniform manifold approximation and projection (UMAP) or subclustered MBCs from peanut-allergic human PBMCs. **(G)** Feature plots of *IL4R, FCER2,* and *IGHE* expression within MBC subclusters. **(H)** Violin plots depicting level of transcript expression in subcluster *. Data are plotted as mean ± SEM (B-C). *, **, and *** represent $p < 0.05$, 0.01, and < 0.001, respectively.

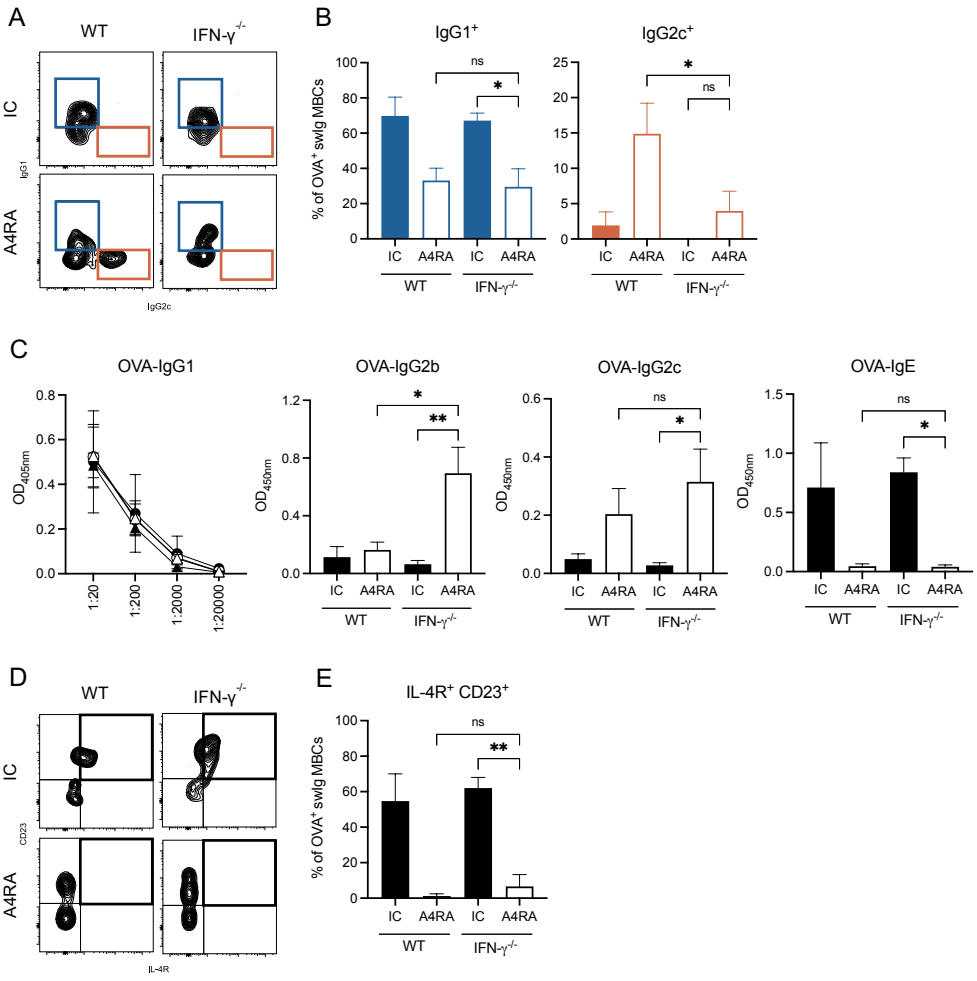


Figure 4. Generation of IgG2c$^+$ MBCs is IFN-γ-dependent.
(A) Representative contour plots of IgG1/IgG2c expression in OVA$^+$ MBCs from pooled inguinal lymph nodes and spleens of epicutaneously-sensitized mice. Events were pre-gated as live, singlet, CD3$^-$ F4/80$^-$ B220$^+$ OVA$^+$ IgD$^-$ IgM$^-$. **(B)** Summary bar graphs of IgG1 and IgG2c-expressing OVA$^+$ MBCs at 14 days post-re-exposure. **(C)** ELISA quantification of serum OVA-specific immunoglobulins after 14 days post-re-exposures. **(D)** Representative contour plots of IL-4R/CD23 expression in OVA$^+$ MBCs from pooled inguinal lymph nodes and spleens of epicutaneously-sensitized mice. Events were pre-gated as live, singlet, CD3$^-$ F4/80$^-$ B220$^+$ OVA$^+$ IgD$^-$ IgM$^-$. **(E)** Frequency of IL-4R$^+$ CD23$^+$ cells from class-switched OVA-specific MBCs in pooled spleens and inguinal lymph nodes. Data are plotted as mean ± SEM. *, **, and *** represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

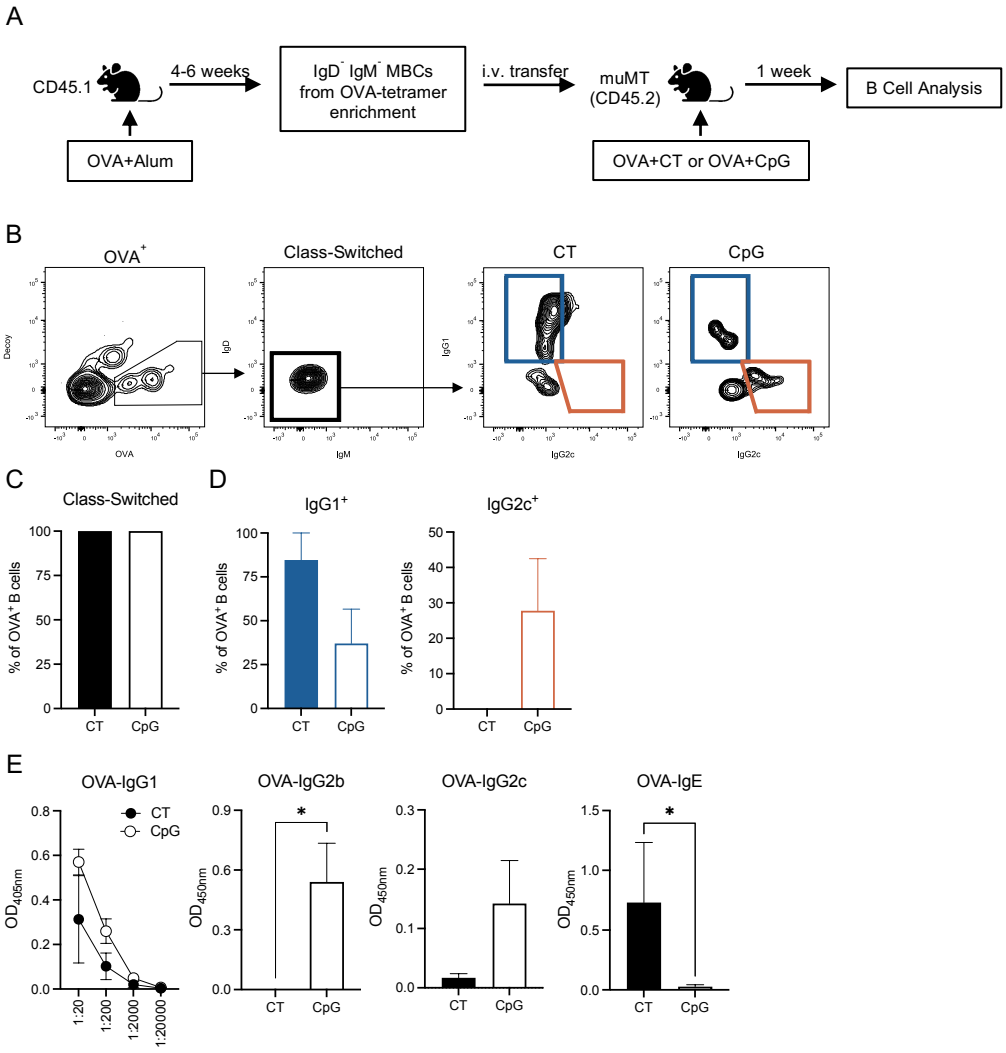
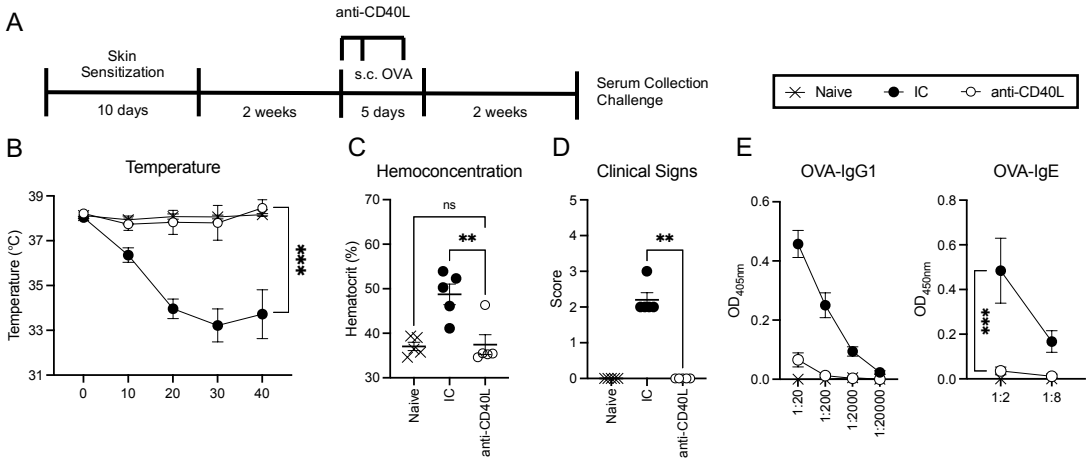


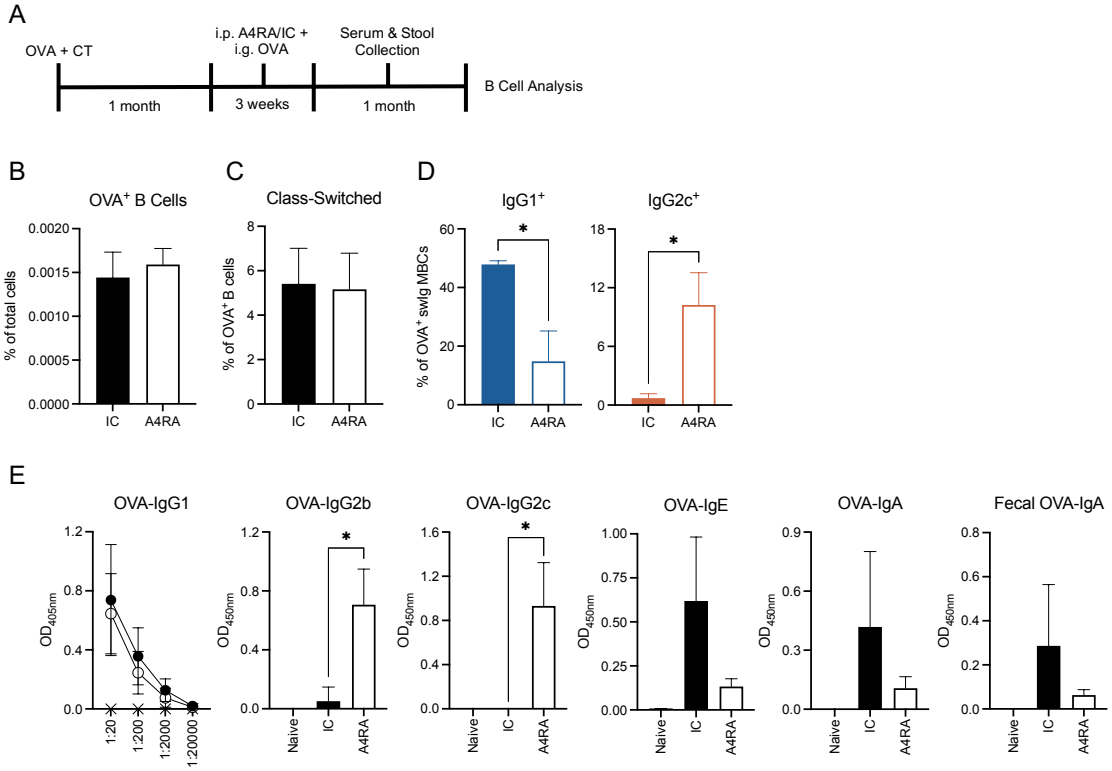
Figure 5. IL-4 signaling is required to maintain Th2-skewing in allergen-specific MBCs.

(A) Schematic depicting adoptive transfer experiments to evaluate the plasticity of class-switched OVA$^+$ MBCs. **(B)** Gating strategy to identify donor IgG1$^+$/IgG2c$^+$ OVA-specific B cells in recipient mice. Events were pre-gated as live, singlet, CD3$^-$ F480$^-$ CD45.1$^+$ B220$^+$. **(C and D)** Summary plots of IgD$^-$ IgM$^-$ cell frequency from OVA$^+$ B cells (C) and IgG1$^+$/IgG2c$^+$ cells from class-switched OVA$^+$ B cells (D). **(E)** ELISA quantification of serum OVA-specific immunoglobulins in recipient mice one week post-immunization. Data are plotted as mean ± SEM. * represents $p < 0.05$.



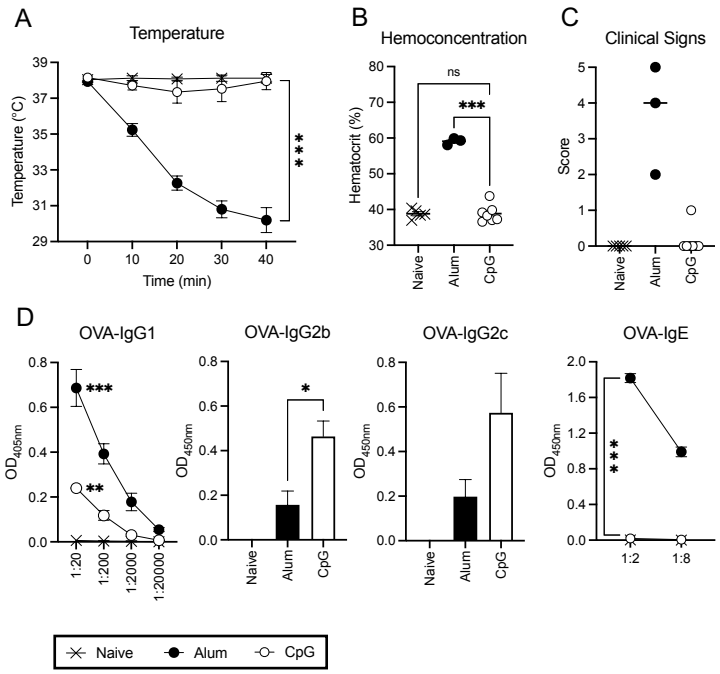
Supplementary Figure 1. Secondary IgE responses in epicutaneously-sensitized mice require CD40-CD40L interactions.

(A) Schematic of anti-CD40L treatment prior to and during subcutaneous (s.c.) allergen re-exposures in epicutaneously-sensitized mice. **(B)** Core body temperature measured in ten minute intervals following systemic challenge with 5 mg OVA. **(C)** Quantification of hemoconcentration at 40-minutes post-challenge. **(D)** Highest clinical sign recorded throughout 40 minute allergen challenge. Scale as follows: 0 = no signs; 1 = in-ear scratching; 2 = reduced activity; 3 = motionless; 4 = no whisker response; 5 = seizure/moribund/death. **(E)** ELISA quantification of serum OVA-specific IgG1 and IgE at two weeks post-re-exposures. Data are plotted as mean (B and E) or individual mice (C and D) ± SEM. ** and *** represent $p < 0.01$ and < 0.001, respectively.



Supplementary Figure 2. IgG2 responses are induced following IL-4Rα blockade in orally sensitized mice.

(A) Schematic of oral sensitization with OVA and cholera toxin (CT), followed by A4RA/IC treatment and intragastric (i.g.) re-exposures to OVA. Sera and stool were collected two weeks after the final re-exposure. Spleens and mesenteric lymph nodes were collected one month following i.g. OVA. **(B)** Percent of OVA^+ B cells from total pooled spleens and mesenteric lymph nodes. **(C)** Frequency of IgD^- IgM^- cells from OVA^+ B cells. **(D)** Percent of IgD^- IgM^- OVA^+ MBCs expressing IgG1 and IgG2c. **(E)** ELISA quantification of OVA-specific immunoglobulins. Data are plotted as mean ± SEM. * represents $p < 0.05$.



Supplementary Figure 3. Anti-IL-4Rα-skewed response mirrors primary adaptive immune response with Th1 adjuvant.
(A) Core body temperature measured in ten-minute intervals following systemic challenge with 5 mg OVA. **(B)** Quantification of hemoconcentration at 40-minutes post-challenge. **(C)** Highest clinical sign recorded throughout 40-minute allergen challenge. Scale as follows: 0 = no signs; 1 = in-ear scratching; 2 = reduced activity; 3 = motionless; 4 = no whisker response; 5 = seizure/moribund/death. **(D)** ELISA quantification of serum OVA-specific immunoglobulins two weeks after sensitization. Data are plotted as mean (A and D) or individual mice (B and C) ± SEM. *, **, and *** represent $p < 0.05$, 0.01, and < 0.001, respectively.

Chapter 5: Discussion

5.1 The Past: A plateau in therapeutic advancement

Historically and to this day, the standard of care for food allergy is strict allergen avoidance and rescue epinephrine in acute anaphylaxis, with allergen immunotherapy as the most extensively trialed treatment. The core principles of allergen immunotherapy were established a century ago, long preceding our modern perspective on the underlying immunological principles.[100] The evolution of allergen immunotherapy or other therapeutics, however, has been greatly outmatched by the growing prevalence of food allergies – first in Western countries, and now globally.

In the early 1990s, the first observation of IgE sequential class switch through IgG1 was made, though it would be over 20 years later before allergen-specific IgG1$^+$ B cells gained recognition for their contribution to allergic pathology.[69,70,101] This was followed by two seminal studies in 2017 demonstrating a critical role of IgG1$^+$ MBCs in the maintenance of lifelong IgE responses in mice.[57,72] The work presented in this dissertation builds upon this knowledge to further our understanding of how lifelong IgE-mediated allergies are maintained and how they may be reprogrammed.

5.2 The Present: Chapter summaries

5.2.1 Identifying the target

In Chapter 2, we developed a novel two-step procedure for the detection of *bona fide* human IgE^+ MBCs involving a sequential flow cytometry gating strategy followed by single-cell genetic validation.[102] Briefly, this involved a standard gating strategy to identify class-switched MBCs ($CD19^+$ $CD38^{lo}$ $CD27^+$ IgM^- IgD^-), followed by an improved sequential exclusion of each BCR isotype (pan-IgG, IgA, and IgE). Importantly, our enhanced staining approach substantially reduced the number of false positive events in the IgE gate, which likely were low BCR-bearing IgG^+ MBCs harboring CD23-bound IgE.[66] To confirm the genetic identity, putative IgE-expressing MBCs were single-cell sorted, immunoglobulin heavy chains were PCR amplified, and PCR products were sanger sequenced.

Employing this two-step method on PBMCs from cohorts of peanut-allergic and non-allergic individuals, we found that circulating IgE^+ MBCs were undetectable irrespective of allergic status. With the hypothesis that IgE^+ MBCs may preferentially localize to inductive mucosal tissue sites, we also sampled tonsillar mononuclear cells from healthy individuals and, even here, found an absence of IgE^+ MBCs. Since antibody titers may positively correlate with circulating antigen-specific MBC frequency, we expanded our analysis to include PBMCs from patients with atopic dermatitis – a disease characterized, in part, by abnormally high serum IgE levels.[36,103] In this cohort where total serum IgE was 22-times higher than our peanut-allergic cohort (4477 vs. 197 kIU/L), *bona fide* IgE^+ MBCs were present at a frequency of 0.0015% of total B cells.

As a whole, our study clarifies pre-existing discrepancies regarding the existence/rarity of human IgE^+ MBCs. While perhaps below our level of detection in peanut-allergic patients, the frequency was at least < 0.0015% from total B cells, which is orders of magnitude less than previous reports.[66,67,104] Moreover, the complete absence of IgE^+ MBCs in our sampling of over one billion mononuclear cells discourages the proposition of using IgE^+ MBCs as a biomarker of food allergy and/or therapeutic efficacy.[66,104]

Since the release of our publication, two insightful papers that expanded on this work were made available. Firstly, Hoh *et al.* obtained esophageal, gastric, and duodenal biopsies from peanut-allergic patients to investigate IgE clonality by high-throughput DNA sequencing.[68] Of the various mucosal sites evaluated, the stomach and duodenum were identified as primary reservoirs of IgE^+ B lineage cells. Through morphological and immunofluorescent analyses, however, all appeared to be IgE-secreting cells, as indicated by the co-expression of CD138. Thus, IgE^+ MBCs appear to be absent even in mucosal tissues of allergic patients, though the strength of this conclusion remains limited due a small sampling size. Moreover, the authors identified clonal overlap between B cell isotypes within the same tissues, suggestive that allergen-specific MBCs can reside in mucosal tissue and undergo local class switch recombination to IgE. Secondly, Zghaebi *et al.* utilized allergen tetramers to detect Bet v 1-specific IgE^+ MBCs in PBMCs of birch-allergic patients by flow cytometry.[105] The authors did not use a sequential gating strategy to exclude contamination from cytotropic IgE-expressing IgG^+/IgA^+ B cells, nor did they perform genetic validation, however, they did use an anti-IgE clone which they report binds only to membrane IgE and

not to receptor-bound IgE. In this study, the average frequency of putative Bet v 1-specific IgE^+ MBCs was reported to be 2.14% of circulating class-switched MBCs.

These studies highlight two important considerations for future directions. Firstly, evidence of IgE class switching occurring locally emphasizes the need to investigate recall responses within tissues. While PBMCs are the common choice due to ease of access, heterogeneity between circulating and tissue-resident MBCs is apparent and thus, the cellular interactions which facilitate a recall response may differ.[106–108] Next, antigen tetramerization protocols have become readily available and easily implemented, enabling the study of antigen-specific B cells rather than bulk populations.[109] As allergen-specific B cells are rare (< 0.03% of circulating B cells), important changes may go undetected with conventional techniques.[77] Indeed, our own research has evolved to prioritize the use of fluorochrome-conjugated allergen tetramers (when appropriate) to evaluate the status of allergen-specific MBCs, as reflected in Chapter 4. We surmise that the use of allergen tetramers may enable a more precise estimate of IgE^+ MBC frequency in humans.

5.2.2 Disabling the target

With it made apparent that IgE^+ MBCs are likely too rare to independently facilitate efficient recall responses, we next sought to establish a means by which to interrupt the recall response facilitated by non-IgE MBCs. In Chapter 3, we developed a novel human PBMC culture platform to concurrently investigate allergen-induced B and T cell memory responses.[110] Here, we performed scRNA-seq to gain insight as to the identity of B and T cells involved in the recall response and, in particular, to identify candidate biological

processes/molecules involved in MBC reactivation. This analysis revealed a cluster of MBCs that expanded upon peanut stimulation with a prominent IL-4 responsive gene signature.

The role of IL-4 as it pertains to primary Th2 responses is well described in various mouse models (*e.g.* allergic asthma, helminth, etc.), though its requirement was not well elucidated in the context of recall responses to food allergens.[24,28,111,112] Using a mouse model that enables temporal control over the IgE response, we found that antibody-mediated blockade of IL-4Rα inhibited IgE responses from allergen-specific $IgG1^+$ MBCs and provided complete protection from IgE-mediated anaphylaxis. The same protection from IgE responses was observed when treating human PBMCs with anti-IL-4Rα prior to peanut stimulation. Strikingly, in both the mouse and human systems we found that IL-4Rα blockade was able to overcome the Th2 biased T cell response, promoting Th1 and regulatory responses (*e.g.* IL-10, IFN-γ, etc.). The IFN-γ response and absence of IgE was even sustained following anti-IL-4Rα clearance and tertiary allergen re-exposures. Collectively, the work conducted in Chapter 3 illustrates a critical requirement of IL-4 signaling to regenerate IgE responses. Clinically, this provides strong evidence in support of anti-IL-4/IL-4Rα use in the next generation food allergy therapeutics, with the potential to – at least transiently – disable allergen-specific MBCs.[113]

Dupilumab, the human anti-IL-4Rα monoclonal antibody, has already produced great success in the treatment of atopic diseases, including moderate-to-severe asthma, atopic dermatitis, and chronic rhinosinusitis with nasal polyposis.[114–116] In all diseases, the

majority of patients exhibit improved clinical score. Interestingly, Bangert *et al.* recently identified that atopic dermatitis patients who achieve clinical remission with dupilumab retain a population of skin-resident Th2A cells which they hypothesize may mediate disease relapse.[74] It should be noted, however, atopic dermatitis pathology is mediated by a diversity of Th subsets (*e.g.* Th17), not only Th2.[117] The observation of persisting Th2A cells aligns with our scRNA-seq data which identified a cluster of Th2A cells which was maintained with anti-IL-4Rα.[110] Likewise, OIT diminishes, but does not eliminate, Th2A cells, which may explain the transient nature of sustained unresponsiveness.[73,75] In contrast with our work, however, the atopic dermatitis study did not report significant skewing away from the pathogenic Th2 response.[74] For example, transcriptomic and proteomic analysis revealed a sustained upregulation of IL-13 after one year of dupilumab treatment, whereas our data demonstrate a loss of IL-13 upregulation and a concomitant increase in type 1 and regulatory cytokines. This apparent potential for T cell reprogramming, which was sustained beyond anti-IL-4Rα clearance, raises the question as to whether this can also influence MBC responses, particularly in a way that negates their propensity for IL-4 signaling and an IgE fate.

5.2.3 Reprogramming the target

Chapter 4 presents our current investigation of allergen-specific MBC plasticity in the context of IL-4Rα blockade. Specifically, this work sought to address whether inhibition of IL-4 signaling simply prevented IgE class switch recombination from MBCs while maintaining a pathogenic trajectory, or if MBCs were removed from this trajectory as

represented by a change in phenotype and isotype. We found that B cell activation in a recall response proceeded in the absence of IL-4 signaling and, at variance with previous work, secondary GCs were formed at a similar magnitude.[118,119] While a small subset of allergen-specific IgG1$^+$ MBCs were retained, anti-IL-4Rα gave rise to long-lived IgG2c$^+$ MBCs and a transient serum OVA-IgG2c and OVA-IgG2b response. The profile of serum antibodies closely matched that of mice immunized with the Th1 adjuvant, CpG ODN, suggesting that a single administration of anti-IL-4Rα was adequate to deviate the response towards Th1. Moreover, IL-4 signaling was necessary to maintain the IL-4-responsive phenotype of allergen-specific MBCs described in Chapter 2 (co-expression of CD23 and IL-4Rα). Through adoptive transfer, we established that class-switched MBCs from allergic mice could facilitate IgG2b/c responses, suggesting that the Th1 B cell skewing does not necessarily arise from a *de novo* response. Taken together, these results identify plasticity within the MBC compartment which could be harnessed by IL-4Rα blockade to deviate away from an IgE fate.

Important questions, beyond the scope of this manuscript, remain unanswered:

1) As the activities of MBCs are directly influenced by cognate interactions with CD4 T cells, to what extent are allergen-specific T cells reprogrammed in this context? While a limited number of studies have investigated Th2A cells in atopic diseases, the presence of a terminally differentiated phenotype is apparent.[73,74,110,120] The consequences of being unable to reprogram their phenotype, however, are uncertain considering that the actions of TFh cells, not effector Th2 cells, are what largely influence IgE B cell responses.[27,28]

Whether the persistence of the Th2A population will perpetually maintain Th2-skewed TFh cells (TFh2 and TFh13) is not known.

Relatedly, 2) to what extent must pathogenic lymphocytes (*e.g.* Th2A cells and Th2-polarized MBCs) be eliminated or reprogrammed to achieve sustained allergen tolerance? In Chapter 3 and 4 we identified residual populations of Th2A cells and allergen-specific Th2-polarized MBCs, respectively. While we and others have speculated that these residual pathogenic cells may rationalize instances of disease relapse, this has not been explicitly shown.[74,75,110] Indeed, it would seem unnecessary to wholly eliminate pathogenic Th2 cells, as homeostasis is typically a balance various subsets, and pathology arises when said balance is lost. Thus, it is more probable that a certain threshold of tolerogenic/non-Th2 cells must be met to adequately suppress or outcompete pathogenic cells.

Lastly, 3) Is protection beyond induction of IgG2b/c exerted by anti-IL-4Rα? OIT trials have demonstrated that desensitization to allergens involves, not only IgG2 and IgG4, but also suppressive functions of Tregs and Bregs. In food allergy, conventional FoxP3$^+$ CD25$^+$ Tregs acquire a Th2-skewed phenotype, including the Th2-associated transcription factors GATA-3 and IRF-4, and production of IL-4 and IL-13.[88] In mice, selective deletion of *Il4* and *Il13* in FoxP3$^+$ cells restores the inhibitory functions of Tregs.[88] Both naturally- and therapeutically-acquired tolerance to cow's milk is associated with an upregulation of Breg-related genes in allergen-specific B cells.[121] Aside from universal expression of IL-10, Bregs are phenotypically and functionally heterogenous, as is their origin.[122,123] A role of

IL-4 – should one exist – in mediating Breg development and/or function remains to be investigated.

5.3 The Future: How do we create disease-transforming therapeutics?

5.3.1 Conceptualizing an ideal food allergy therapeutic

Collectively, the work presented in this dissertation in concert with existing literature, identifies the pathway through which allergen-specific MBCs replenish the short-lived IgE^+ PC pool as the critical therapeutic target. Here, we identify IL-4/IL-13 signaling as a critically required process to facilitate such process, though continued efforts are necessary to identify other targets with disease-transformative potential.

We consider a "disease-transforming" therapy as one which can permanently restore allergen tolerance.[124] In this instance, "tolerance" epitomizes any non-pathogenic response to allergens, whether this be an active regulatory response as seen in healthy individuals, an anergic response, or any other non-Th2 response that suppresses pathogenicity. In theory, this could be achieved by elimination or exhaustion of allergen-specific B and T cells (Fig. 2A), neutralization/blockade of secreted or membrane-bound molecules which are critical for the recall response (Fig. 2B and C), or by actively providing/removing signals which counteract/maintain Th2 polarization such that the underlying immunological response is reprogrammed (Fig. 2D).[113]

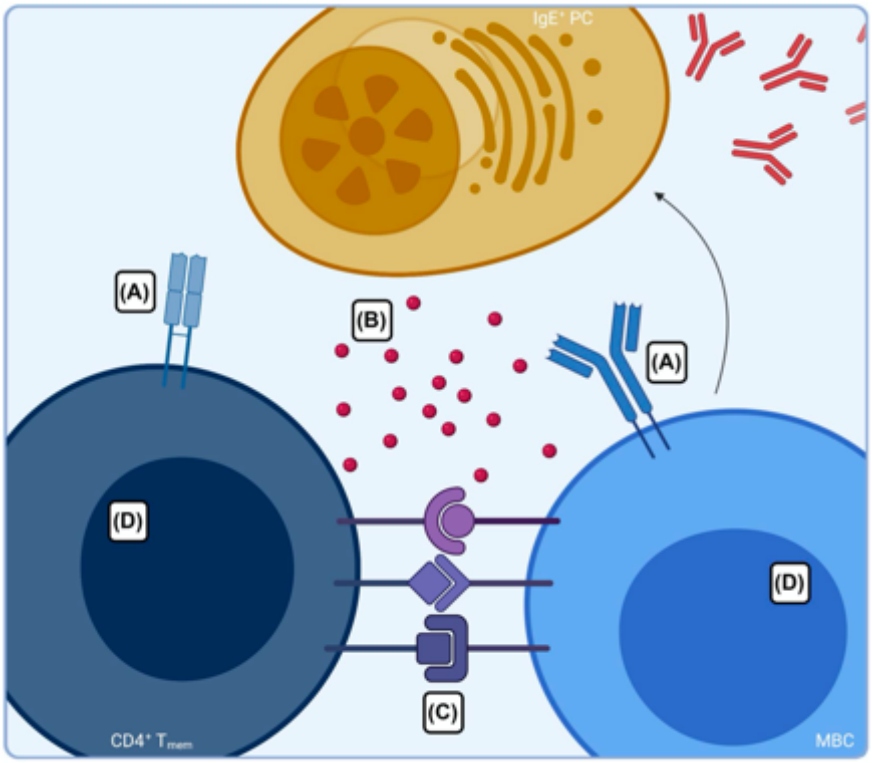


Figure 2. Potential biological targets to disarm allergic recall responses.
(A) Elimination and/or inhibition of allergen-specific lymphocytes utilizing therapeutics that engage with allergen-specific T cell or B cell receptors. (B) Inhibition of allergic recall responses with therapeutics targeting key cytokines/chemokines and/or their cell-surface receptors. (C) Inhibition of allergic recall responses through blockade of co- stimulatory interactions. (D) Harnessing plasticity of allergen-specific cells to reprogramming the pathogenic lymphocytes.

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5.3.2 Steps to get there

1) Rationally define targets of interest. Recall responses to antigen require a complex interplay between various cell types. While flow cytometry permits a high throughput screen of various targets at the single-cell level, it requires that cells are removed from their native tissue structure, therefore sacrificing insight gleaned from spatial organization. Simply defining the space in which allergen-specific lymphocytes reside and the proximal cells is likely to aid in further uncovering the processes that facilitate a recall response. Immense progress has been made in the histology field, where detection of six fluorescent channels was once the limit, and now technologies exist that permit over 65 markers – exceedingly more than that obtained by flow cytometry.[125,126] Moreover, in our era of single-cell transcriptomics, a number of algorithms have been developed which infer cell-cell communication networks from single-cell datasets by evaluating expression of known receptor-ligand pairs.[127] Whether taking a spatial approach or through computational inference of transcriptomic data, these methodologies are likely to reveal candidate cellular phenotypes and molecules involved in the recall response and/or maintenance of the pathogenic Th2 phenotype.

2) Test the targets in preclinical models. The human PBMC culture developed in Chapter 3, we believe, could be used to effectively screen the efficacy of various biologics. Though, as commented on in Section 5.2.1, use of PBMCs may come with inherent limitations in its applicability, as a recall response is not elicited in the circulation. A prospective alternative may be the use of human tissue. For example, human tonsil organoids accurately

recapitulated adaptive immune responses to influenza, including affinity maturation, antibody secretion, and even classical GC structure.[128] Any such tool is necessary to augment the transition of therapeutics from mice to men.

3) Establish biomarkers to aid in evaluating treatment success. OIT trials have made it evident that the response to therapy is heterogenous. This will likely be no different with next generation treatments. In the case of some OIT studies, allergen-specific IgE levels and basophil reactivity at baseline are inversely correlated with success in establishing desensitization.[96,129,130] Given, however, that those deemed "treatment failure" exhibited the same trend for decreased IgE and increased IgG4, just to a lesser extent, it is plausible that a longer duration of treatment would have facilitated successful desensitization. Thus, identification of quantitative measurements, such as these or others (*e.g.* ratio of allergen-specific Th2 polarized MBCs to non-Th2 MBCs), may help to monitor progress and differentiate between those who may require lifelong treatment and others who may be taken off.

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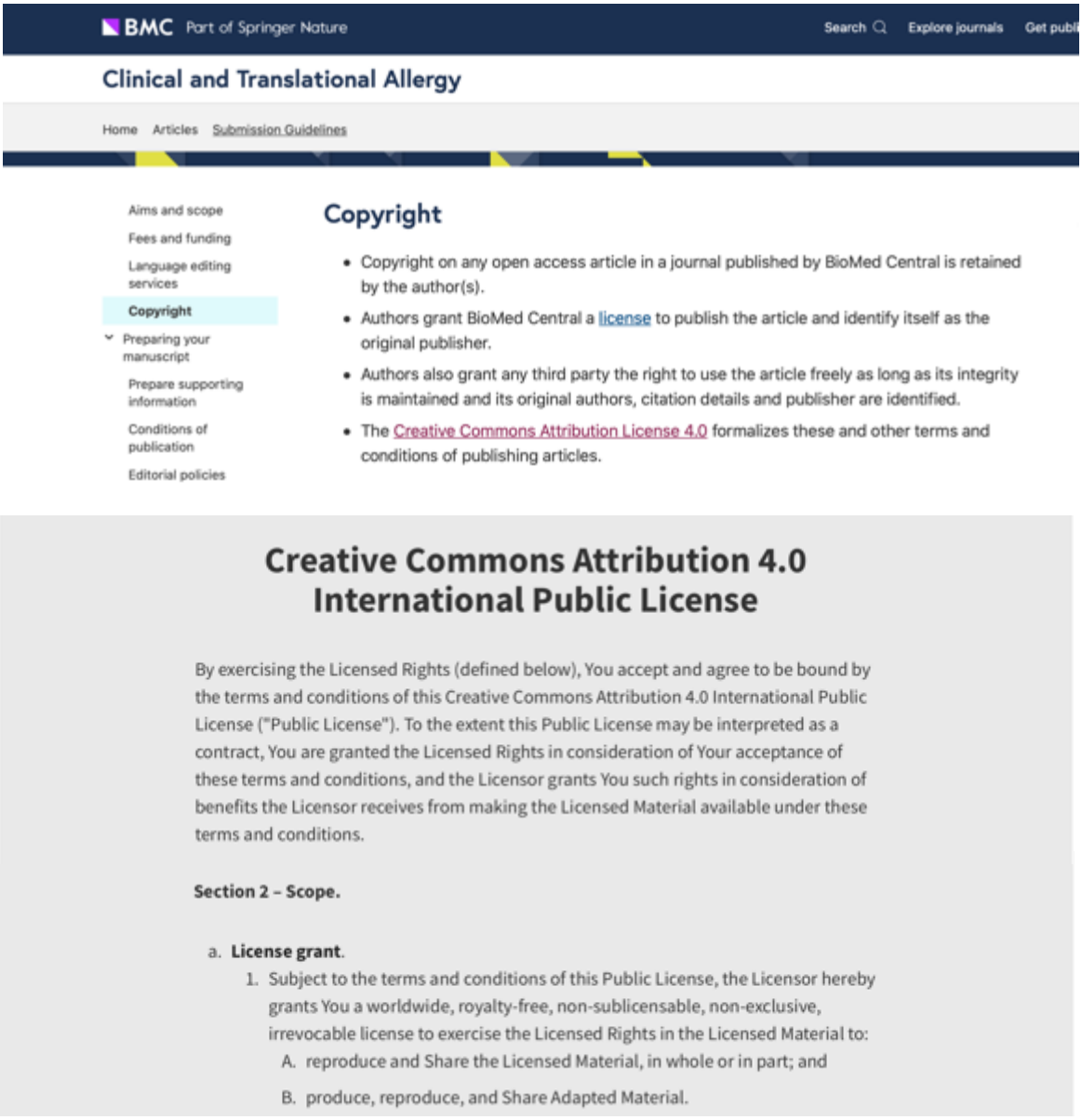

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