ADAPTIVE TYPE 2 IMMUNITY IN FOOD ALLERGY

PATHWAYS OF ADAPTIVE IMMUNE ACTIVATION DURING SENSITIZATION AND RECALL IN FOOD ALLERGY

By JOSHUA FRANK ERNEST KOENIG, BHSc.

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AUTHOR: Joshua Frank Ernest Koenig, BHSc. (McMaster University)

SUPERVISOR: Dr. Manel Jordana

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ABSTRACT

Approximately 10% of North Americans have at least one food allergy. Allergic reactions to foods are mediated by IgE antibodies which, upon allergen exposure, are crosslinked on the surface of mast cells and basophils, resulting in degranulation. The granule contents of mast cells and basophils are responsible for the diverse signs and symptoms of food allergy, including hives, itchiness, edema, vomiting, diarrhea, and a potentially lethal form of systemic shock called anaphylaxis. The clinical recommendation for food allergic Canadians is strict allergen avoidance, and emergency epinephrine use upon an accidental exposure. There are presently no disease modifying therapeutic options for allergic Canadians.

The work in this PhD thesis focuses on the pathways which result in the production and memory of allergen-specific IgE. Using murine models of food allergy and anaphylaxis, we have delineated pathways of early allergic sensitization which may occur prior to allergic patients presenting in the clinic during the first allergic reaction. We have demonstrated that $CD4^+$ T cell activation can occur in the absence of B cell activation, but can hold the memory of IgE responses persistently, potentially for a lifetime. Upon re-exposure to the allergen, $CD4^+$ T cells can activate naïve B cells, which pursue either direct or sequential isotype switching to IgE, both resulting in clinical reactivity against food allergens. Our findings will inform future diagnostic and prognostic tests in pre-allergic patients, and to design novel therapeutics with disease modifying capacity in humans.

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with, she is an endless source of laughter, of cute pictures of her children, and of stories. Tina probably has the best set of scientific hands that exist on the planet, especially for animal work. If she says "you *could* do it that way…", what you're doing is stupid and you should ask Tina how she would do it. Trust me. I have not listened to her in the past, and it goes poorly every time.

One of my major take-aways from my PhD is the impact that mentorship can have. Of course, I mean this from a strictly selfish perspective- the impact that each of my mentees has had on me. The first student I trained was Siyon Gadkar, who then became a graduate student in the lab. I had no idea what I was doing, but luckily that didn't phase Siyon. He made me realize the responsibility I had as a mentor and showed me that persistent people will prevail regardless of how bad their mentor is. Luckily, these days I just call Siyon my friend, and he helps me de-stress with video games. Adam Wade-Vallance, on the other hand, will always be my mentee, regardless of how successful he becomes at UCSF. I own you, Adam. I made you what you are today. Remember that. Jokes aside, Adam taught me how to help curiosity blossom. I am blessed that Adam is now a far superior scientist than I am, and I get the opportunity to pick his brain frequently about how the immune system works. Also, video games. Emily Grydziuszko is an absolute powerhouse and is truly my right hand. Emily's excitement for science is more infectious than the COVID pandemic she endured for most of her undergrad in the lab. I am not kidding when I say that Emily, as an undergraduate student, took the lead on most of my major sacrifices in the last 2 years of my degree. More importantly, Emily has the biggest heart of anyone I know. She can connect with anyone at a deep level. Emily provides me sage advice about life despite the fact that she's a good 6 years younger than me. Maggie Chopra improved my mood every single time I was in her presence. She turned the lab into our own little dance club and rewrote pop songs to be about science on the fly. Youssef El-Sayes taught me about precision, and how to support someone who sought to take a different path than I took. Jake Colautti has reinvigorated my love for science and showed me that we can do even the most complex experiments if we are determined. Malcolm Davidson is the coolest guy I know, he showed me all the best music, we coordinated our outfits, and threw frisbees together. The most solid guy to have in any room. Owen Baribeau reminded me how dangerous UV could be, I hope your eyes are okay. Thank you, all of the Jordana Lab members past and present.

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LIST OF ABBREVIATIONS AND SYMBOLS

AF	Alexa Fluor
AID	Activation Induced Cytidine Deaminase
Alum	Aluminum Hydroxide
BCR	B Cell Receptor
BLG	β-Lactoglobulin
BSA	Bovine Serum Albumin
CSR	Class Switch Recombination
CT	Cholera Toxin
CTV	CellTrace Violet
fDC	Follicular Dendritic Cell
FITC	Fluorescein Isothiocyanate
FRET	Fluorescence Resonance Energy Transfer
GC	Germinal Center
H+L	Heavy + Light Chain
HDM	House Dust Mite
Ig	Immunoglobulin
IgG1-def	IgG1-deficient
MHC	Major Histocompatibility Complex
NP	4-hydroxy-3-nitrophenyl
OAS	Original Antigenic Sin
OIT	Oral Immunotherapy
OVA	Ovalbumin
PC	Plasma Cell
PE	Phycoerythrin
PN	Peanut
RBL	Rat Basophil Leukemia
Tfh	T Follicular Helper
Tfr	T Follicular Regulatory
Th2	T Helper 2
QoL	Quality of Life
SA	Streptavidin

DECLARATION OF ACADEMIC ACHIEVEMENT

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I directly performed >90% of the work in Chapter 3. The tetramer enrichment platform was established by me and Allyssa Phelps, with support from Dr. Justin Taylor (Fred Hutchinson Cancer Research Center). The Ara h 1 tetramer data in Figure 8 was generated by Allyssa Phelps. The small intestine enrichment data generated in Figure 5A was generated by Emily Grydziuszko. Technical support was given by Tina Walker.

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CHAPTER 1

INTRODUCTION

Food allergies are IgE-mediated adverse reactions to specific foods which occur immediately following ingestion. Signs and symptoms of food allergic reactions vary. Some patients experience reactions localized to specific organs, like the gastrointestinal tract (cramps, diarrhea), the lung (wheezing, cough), or the skin (urticaria). An allergic reaction is typically categorized as anaphylaxis when two or more organ systems are involved, or if hypotension occurs[1,2]. Food-induced anaphylaxis is a hypotensive shock that requires emergency treatment as it can be lethal[3]. Presently, the clinical recommendation for food allergic patients is strict avoidance of the offending allergen and the use of an epinephrine auto-injector should a serious reaction occur. No curative therapies exist for food allergy. Oral immunotherapy (OIT), in which incrementally increasing quantities of allergen are ingested until unresponsiveness is achieved, is an experimental therapy, albeit FDA-approved in the US, that remains fraught with safety and efficacy concerns[4]. Patients on OIT are more likely to have anaphylactic reactions than those avoiding allergen, many cease therapy because of adverse events, and clinical reactivity returns if allergen consumption ceases[4,5]. Present research is focused on

understanding the basic biological mechanisms that underly food allergy to identify novel diagnostic, prognostic, and therapeutic targets.

1. Food Allergies

1.1. The Burden of Food Allergy

The prevalence of food allergy has steadily increased over the past 20 years. Accurate prevalence data are difficult to collect as studies frequently rely on self-reporting of diagnosis. A poor understanding of what constitutes a food allergy among the general public typically results in overestimation of the prevalence of the disease, as food intolerances and food preference are often misidentified as food allergy. The prevalence of self-reported food allergy among Canadians has increased from 7.1% to 9.3% between 2010 and 2017, a 32.4% increase[6]. In the United States, screened self-reports for probable food allergy found that 10.8% of individuals (>26 million) have at least 1 food allergy[7]. This study also found that 19% of people perceive they have a food allergy[7]. A screen of electronic medical records found that physician reported food allergy occurs in 2.5% of Canadians, much lower than self-reports[8]. Canada has designated the most common food allergens as "priority allergens," requiring them to be identified on food products. These allergens are peanut (PN) (1.2% of Canadians of all ages), tree nuts (1.4%), shellfish (1.1%), milk (1.1%), eggs (0.8%), fish (0.6%), wheat (0.4%), soy (0.3%), and sesame (0.2%)[6,9]. Among the priority allergens, PN is responsible for the most food-induced anaphylactic reactions and most food allergy-related deaths[3,10].

Food allergies significantly impact the quality of life (QoL) of allergic patients and their families. Twenty years of investigation have uniformly reported that food allergic patients report a QoL worse than many rheumatological diseases, as well as type 1 diabetes mellitus[11–14]. Those with multiple allergies or allergies against PN, soy, and fish tend to report worse QoL. QoL questionnaires have revealed that constant vigilance about avoiding allergens contributes to decreased health related QoL[13]. Caregivers and family members of children with food allergy report similarly impaired QoL[13]. One cause of decreased QoL is anxiety surrounding the possibility of having an anaphylactic reaction if allergen is accidentally ingested. Such anxiety is not unfounded, 14% of allergic patients are accidentally exposed yearly[15]. These exposures are particularly common on treat-centered holidays like Easter and Halloween[2]. There has been a 214% increase in emergency room visits for food-induced anaphylaxis, despite increased awareness about the disease[10]. In total, food allergies cost the United States medical system >$25 billion dollars annually[16].

1.2. Mechanism of Food Allergic Reactions

Allergic reactions to food are mediated by food-specific immunoglobulin (Ig) E antibodies. Allergen-specific IgE is elevated in allergic patients, while being absent or present in nominal amounts in non-allergic patients. Allergen-specific IgE has a very short half-life (2-3 days) in circulation[17]. Its half-life is dramatically extended (~100 days) when bound to its high-affinity receptor, FcεRI, on the surface of granulocytes like mast cells and basophils[18]. Allergen crosslinking of two

surface IgE molecules causes mast cells and basophils to degranulate with the ensuing rapid secretion of their granule contents[19]. These granules contain pre-formed vasoactive mediators which cause the signs and symptoms of an allergic reaction. A canonical example, histamine ligates histamine receptors on endothelial cells causing vasodilation and the decrease in blood pressure associated with severe anaphylactic responses[20]. Upon allergen-encounter, mast cells and basophils rapidly synthesize other allergic mediators, like platelet activating factor, which is the primary mediator of allergic signs and symptoms[21–23]. Other mediators include tryptases, chymases, heparin, prostaglandins, leukotrienes, among others, which together with histamine and platelet activating factor influence a wide range of cells[24]. These include airway smooth muscle, epithelial, and mucous secreting cells to induce airway constriction, cough, and increased mucous production; neurons in the skin to cause itching sensations; systemic endothelial cells to cause vasodilation and edema; and intestinal smooth muscle, epithelial cells, and goblet cells resulting in excess mucous production, cramping and diarrhea[24]. The rapid degranulation or synthesis of bioactive molecules explains how allergen ingestion causes immediate reactions in allergic patients.

Following the immediate reaction, up to 50% of allergic patients experience localized or systemic inflammation referred to as a late phase response[25–27]. This response is primarily mediated by allergen-specific T helper 2 (Th2) cells, which secrete various chemotactic factors to recruit immune cells or induce their local differentiation. Th2 cells secrete IL-4, IL-5, and IL-13, which influence the local

environment in various ways. Th2-derived IL-5 causes eosinophilic inflammation, especially at sites where allergen is encountered[26,28]. Th2-derived IL-13 causes goblet cell hyperplasia in the lungs and small intestine, resulting in increased mucous production[29]. The late-phase response is typically not life-threatening, but when it occurs chronically can have deleterious effects like tissue remodelling, as is the case in allergic asthma[30,31].

The morbidity and mortality associated with food allergies comes from the immediate phase reactions, those which are mediated by allergen-specific IgE. Allergic reactions are tameable using epinephrine auto-injectors (EpiPen) and additional care in hospital. It is clear that the potential to trigger allergic reactions persists as long as the capacity to produce IgE remains and, therefore, current research seeks to understand the origin, persistence, and memory of IgE responses with the objective to develop therapeutic strategies which eliminate allergen-specific IgE production.

1.3. Persistence of Food Allergy and Antigen-Specific IgE

Food allergies against some allergens, like milk and eggs, peak in prevalence in early life and about 50% of these allergies are outgrown by 5 years of age, increasing to 80% by age 16[32]. Titres of allergen-specific IgE decline in those who outgrow the allergy, often to undetectable levels. In comparison, allergies against PN, tree nut, and shellfish resolve in only ~20% of cases[32–34]. These lifelong allergies are characterized by the persistent capacity to produce

allergen-specific IgE. It remains unclear why some allergies are persistent and why others typically resolve.

Allergen-specific IgE antibodies are produced by allergen-specific IgE^+ plasma cells (PCs), which are terminally differentiated members of the B cell lineage. Persistent antibody titres are typically ascribed to long-lived PCs. In the context of vaccination, long-lived PCs home to bone marrow niches where they produce antibody and survive for lifespans estimated to be longer than 200 years in some contexts[35]. This paradigm has been incorrectly extrapolated to food allergy[36,37]. Multiple lines of evidence indicate that allergen-specific IgE titres most frequently do not persist. In patients with seasonal allergic rhinitis, levels of allergen-specific IgE decline rapidly outside of the allergy season, only to re-emerge during the next allergy season[38]. Patients sensitized to αGal from tick bites experience a decline of αGal-IgE when tick bites are avoided[39]. Patients allergic to the fish parasite Anisakis Spp. experience a decline in specific-IgE from >100 kU/L to <3 kU/L when fish is avoided for at least 10 months, but these levels re-emerge when fish is consumed anew[40].

In murine models of food allergy, we have demonstrated that IgE levels are transient following allergic sensitization, declining to undetectable levels by 6 months post-sensitization[18]. These levels were maintained while an active primary immune response was ongoing; germinal center (GC) activity lasted for ~60 days, during which time IgE^+ PCs were generated[18]. Allergen-specific IgE^+ PCs only persisted 1-2 months longer than the GC, indicating that these cells are transient.

Anaphylaxis upon allergen challenge subsided around 100 days following the loss of allergen-specific IgE^+ PCs, once surface IgE on mast cells and basophils was recycled[18]. Our findings are consistent with evidence that IgE^+ PCs are short lived[41]. These cells retain a plasmablast phenotype and localize in transient niches in secondary lymphoid organs[41]. They are generally not found in long-lived niches in the bone marrow. IgE-expressing cells are restricted by tonic signalling of their B cell receptor (BCR), which drives a short-lived phenotype and is reported to induce apoptosis[42,43]. In both humans and mice, significant evidence supports that the long-lived PCs paradigm of antibody production does not explain persistent IgE titres, and therefore an alternate mechanism must maintain the titre of allergen-specific IgE.

Long-term titres of allergen-specific IgE appear to be mediated by memory B cell (MBC) responses. Re-exposing desensitized mice with non-sensitizing intragastric administrations of allergen alone results in the rapid re-emergence of allergen-specific IgE and clinical reactivity upon allergen challenge[18]. This allergic memory appears to self-renew and is not easily shifted away from IgE generation. Food allergic patients undergoing OIT see a decline in allergen-specific IgE over the course of therapy, associated with an increase in the threshold of allergen tolerability. However, as alluded to earlier, upon cessation of therapy, allergen-specific IgE re-emerges and the threshold for allergen consumption lowers back to pre-immunotherapy levels, indicative of a memory IgE response[5]. A recent report in mice showed that chronic exposure to the aeroallergen house dust mite (HDM)

resulted in the accumulation of bone marrow long-lived IgE^+ PCs[44]. These PCs are likely derived from memory re-activation, which may play a role in diseases where allergen exposure is chronic. However, accidental exposures to food are so infrequent that this is unlikely to occur in food allergic patients. Understanding how pro-allergic memory is generated and re-activated is therefore critical to generating novel therapeutics to control the disease.

1.4. Allergic Sensitization: Food Allergy as a Phasic Disease

The mechanism of allergic sensitization in humans, understood as the process of generating allergen-specific IgE, remains poorly understood. Allergic sensitization occurs early in life, most often before the age of 7[45]. Allergic patients typically report to the clinic upon their first allergic reaction, which is frequently following the first known exposure to the offending food[46,47]. Indeed, 25% of food-induced anaphylactic reactions in Canadian children occurred in patients who were not diagnosed with food allergy[48]. The process of allergic sensitization is, therefore, clinically silent, which renders study of these events uniquely challenging. It is difficult to predict which patients will become allergic, therefore candidate recruitment to study early biological mechanisms is exceedingly difficult. Consequently, the immunological progression of food allergy is very poorly understood, especially the events that occur over time before the first clinic visit.

The development of the immune system is consistent among children, especially during their first 100 days of life[49]. This development includes rapid changes to the B and T cell repertoire, proposed to be driven by microbial

interactions. Recent work from the CHILD cohort study found that children at risk of asthma had transient gut microbial dysbiosis during the first 100 days of life[50]. From the same cohort, patients with food allergy had an altered microbiome composition compared to healthy controls[51]. These data indicate that early environmental factors may contribute to allergic sensitization, during a period of rapid immunological development. A landmark study identified that introduction of PN between ages 4 and 11 months dramatically reduced the prevalence of PN allergy at 5 years of age compared to those who delayed introduction (13.7% vs 1.9% prevalence[52], confirming that controlled, early introduction of food allergens is critical for healthy immune development towards food allergens.

The temporal gap between early feeding and later clinic visits during the patient's first allergic reaction suggests a prolonged disease progression that remains largely unknown. It has been reported that the generation of allergen-specific IgG predates the emergence of allergen-specific IgE in patients allergic to aeroallergens[53]. We have also demonstrated that food allergic sensitization through the skin in mice progresses through an IgG1-dominant, IgE-absent phase that transitions to IgE production upon subsequent subclinical allergen exposures[54]. Some infants and very young children present with a non-IgE mediated food allergy-like disease called Food Protein Induced Enterocolitis Syndrome wherein patients experience vomiting and lethargy following ingestion of a specific food[55]. These patients do not have food-specific IgE titres initially, but ~25% will transition to IgE-mediated food allergy[56,57]. Further, a recent report found that abdominal pain

following food ingestion is mediated by local IgE production in the intestines[58]. Taken together, these data indicate that food allergies exist on a spectrum of maturity, wherein the end-stage of the disease involves overt allergen-specific IgE production which causes clinical reactivity upon food allergen ingestion. How allergic sensitization is initiated and the trajectory of the disease towards IgE production and clinical reactivity remain unknown.

2. Initiation of Adaptive Immunity Against Food Allergens

2.1. Activation of Allergen-Specific T cells

Activation of adaptive immunity occurs in the lymph nodes which drain the tissue where allergen-exposure occurs. Tissue dendritic cells (DC) encounter allergen in the context of other immune adjuvants in the mucosa[59]. They internalize and digest allergens into peptides which are then displayed on their cell surface loaded into major histocompatibility complex (MHC)-II. In response to the tissue microenvironment, DCs also upregulate costimulatory molecules and program their cytokine repertoire[60]. In the context of type 2 immunity, DCs downregulate the production of IL-12, and upregulate the costimulatory molecules CD80/86, CD40L, ICOSL, and OX40L[60]. DCs migrate from the mucosa to the T cell zone of the draining lymph node, where they interact with cognate naïve $CD4^+$ T cells. These naïve $CD4^+$ T cells will differentiate either into either Th2 cells or T follicular helper cells (Tfh)[60].

Classical immunology dictates that Th2 cells are responsible for providing help to B cells and inducing IgE production. However, contemporary research has

uncovered that Tfh cells are, in fact, the critical cell type responsible for IgE switching in B cells[61,62], while Th2 cells have crucial roles in local cytokine production that mediates eosinophil recruitment, goblet cell hyperplasia, and airway hyperresponsiveness in allergic asthma[63]. The fate decision between Tfh and Th2 cells is thought to be instructed during the DC/T cell synapse. Th2 polarization occurs when naïve T cells are signalled through CD28:CD80/86, CD40L:CD40 and OX40:OX40L interactions with DCs. Most notably, the OX40:OX40L interaction causes naïve T cells to upregulate GATA3 and produce IL-4, which acts in an autocrine manner to complete Th2 differentiation[64–67]. Tfh differentiation also involves T cell receptor:MHC, CD80/86, and CD40L signalling but Tfh differentiation is preferentially induced in in the presence of IL-21, when IL-2Rα signalling is weak, and in the presence of ICOS:ICOSL[68–70]. Upon activation, Tfh cells upregulate the lineage-defining transcription factor Bcl6, and the effector cytokines IL-21, and IL-4[71,72]. Tfh cells then migrate to the border of the T cell zone and the B cell follicle, where they provide help to antigen-stimulated B cells[73]. Tfh maturation continues during the T/B cell synapse, and the cells migrate together into the B cell follicle and seed GCs[72]. A recent report elegantly demonstrated that the Tfh population undergoes antigen-dependent selection throughout the GC reaction which results in the survival of Tfhs with high affinity T cell receptors[74]. The Tfh population is likely a heterogenous population comprised of Tfh cells with varying polarizations. Type 1-polarized Tfh cells are reported in infection models[75].

Multiple publications have reported the existence of a type 2-biased, IL-13 producing population of Tfhs which is involved in IgE responses[76–78].

The memory T cell repertoire is comprised of T cell clones which remain following contraction of the primary immune response. A variety of signals influence the survival and retention of effector memory Th2 cells, including their affinity for antigen, responsiveness to IL-2, and the robustness of the primary clonal expansion upon activation[79–81]. Some Tfh cells are also selected to persist into a memory fate[72]. Their survival depends on continued Bcl6 expression and ICOS signalling[82]. It is not known what role memory Tfh cells have during memory type 2 responses.

2.2. Activation of allergen-specific B cells

Naïve allergen-specific B cells express both IgM and IgD BCRs and reside within the B cell follicles of secondary lymphoid organs, including draining lymph nodes. Allergens arriving to the lymph node through lymph drainage are captured by subcapsular macrophages, which encapsulate B cell follicles. These cells pass captured allergens to naïve B cells in the follicle, which bind to cognate allergens through their BCR[83–85]. Activated B cells internalize the allergen, present allergen-derived peptides on MHC-II, and migrate to the border of the B cell follicle and the T cell zone[86]. There, allergen-activated B cells form cognate synapses with activated Tfh cells, as described above. Tfh cells provide co-stimulation through ICOS, SLAM, CD40L and cytokine secretion[87]. During this interaction, Tfh cells

are the critical source of IL-4 which is required to change the isotype of their BCR from IgM to IgE[61,62].

2.3. Isotype Switching

The effector functions of antibodies are distributed among various isotypes which differ in the sequence of the constant region of their heavy chains, and in their secondary modifications of the constant region. These differences allow antibodies to bind to distinct Fc receptors with different effector functions. As an example, the constant region of the IgG1 heavy chain has three domains and is lowly glycosylated, whereas IgE has four domains and is highly glycosylated[88]. The glycosylations of the IgE heavy chain are required for binding to its high-affinity receptor, FcεRI[89,90]. As all naïve B cells express IgM/IgD, these cells must change the constant region of their heavy chain to express surface BCRs of other isotypes, and to secrete them as antibodies when they differentiate into PCs. This process is referred to as isotype switching, or class switch recombination (CSR).

An antibody isotype is expressed when it is located adjacent to the V(D)J locus, which defines antibody specificity[88]. In naïve cells, this is always the IgM constant region. CSR is initiated in response to cytokine signals provided by Tfh cells during B cell activation[73]. IgG1 and IgE CSR are induced in response to Tfh-derived IL-4 binding to B cell IL-4Rα[91,92]. IL-4-signalling causes transcription of a region upstream of the IgG1 or IgE heavy chain loci, called a switch region[93]. At the same time, activated B cells upregulate activation induced cytidine deaminase (AID),

which causes double-stranded breaks in the switch region of the heavy chain targeted for switching, and in the switch region adjacent to IgM[93]. These double stranded breaks recombine, looping out the intervening DNA and placing the target isotype immediately adjacent to the V(D)J locus allowing for transcription of the new antibody isotype[93].

CSR to IgE occurs either in a single switching event directly from IgM, or through sequential switching events through an intermediate isotype[94–99]. Early during a primary immune response, most IgE-expressing cells are derived from direct CSR[99]. As the immune response progresses, especially following allergen re-exposures, the frequency of sequentially switched cells increases[99]. These data indicate that the sequential switching events are not immediate, but rather that cells first undergo CSR to an intermediate isotype, exist as a B cell of that isotype for some time, prior to switching again to IgE. In humans, variable region sequencing of peripheral blood and gastrointestinal B cells found IgE-expressing cells as clonal relatives to all upstream isotypes[100,101]. These data suggest that all isotypes can be intermediates in sequential switching in humans, but it remains unclear whether these cells are simply related clones or are direct descendants. In mice, the only reported intermediate isotype for sequential switching to IgE is IgG1[99].

2.4. Fate Decisions of Allergen-Specific B cells

Following the B/T cell synapse, the allergen activated B cell enters a transitional, multipotent activation stage where cell fate decisions are made[102]. Firstly, contrary to classical belief, recent evidence has shown that CSR occurs at

the T/B border following Tfh help rather than within the GC[73]. Tfh-derived IL-4 induces IgE and IgG1 CSR in allergen-specific B cells[61,62]. Secondly, these cells will pursue one of two fates: they will either migrate with the Tfh deep into the B cell follicle to establish or join a GC, where they will undergo additional maturation steps, or they will immediately differentiate into effector cells[103]. The latter pathway is referred to as the extrafollicular pathway. Allergen-activated B cells which differentiate extrafollicularly will either upregulate Blimp-1 and differentiate into antibody-secreting PCs or retain a B cell phenotype and return to a quiescent state as an MBC[104]. PCs generated via the extrafollicular pathway tend to reside in circulation or in the secondary lymphoid organs as short lived plasmablasts[105]. As mentioned previously, IgE^+ PCs tend to adopt a short-lived phenotype, and therefore it is postulated that the majority of IgE^+ PCs are generated via the extrafollicular pathway[41,106]. It is known that the extrafollicular pathway generates functional MBCs, as GC-deficient mice have similar memory responses to WT mice, but their exact role in the layers of immune memory remains poorly understood[107]. A recent report in a model of influenza infection demonstrated that MBCs seed the lung following mucosal infection, which they posit are early egressing cells from the lymph node, likely from the extrafollicular pathway, that encounter their cognate antigen locally in the lung and are retained there[108]. Extrafollicularly derived effector cells are generally lower affinity compared to those which are derived from the GC because GC B cells engage in an iterative process of mutation and selection which enhances the affinity of their BCR.

2.5. Affinity Maturation and Germinal Center Dynamics

GC B cells are a distinct B cell phenotype characterized by the expression of Bcl6[109]. GC B cells follow follicular DC (fDC)-derived CXCL13 gradients via CXCR5, guiding these cells towards the GC[110]. The GC is a collection of activated B cells and other accessory cells within the lymphoid follicle. Early histology experiments observed that the GC was divided into two zones, a dense clustering of B cells referred to as the dark zone and a more dispersed clustering of B cells and non-B cells referred to as the light zone[111]. Incoming GC B cells upregulate CXCR4 to enter the dark zone where they divide numerous times in quick succession[112]. These cells upregulate AID, which causes point mutations in the antibody specificity-defining variable region of their BCR[103,113]. These point mutations may alter the amino acid sequence of the variable region, influencing the affinity of the BCR for its cognate antigen. These mutations could either confer greater affinity for the antigen, have no effect on affinity, or impair affinity. Cells which mutate an amino acid encoding codon into a stop codon die by apoptosis, which represents the majority of cells which die within the dark zone of the GC[114,115]. After a pre-encoded number of divisions, dark zone GC B cells lose CXCR4 expression and migrate to the light zone, where BCR affinity is tested and selected.

fDC and Tfh cells reside within the light zone of the GC and are the machinery for antigen-driven selection. B cells incoming from the dark zone bind antigen complexes located on the surface of fDCs. The affinity of the GC B cell's BCR is

tested through mechanical force; the cell will pull on its BCR to remove the antigen from the surface of the fDC[103,116]. Most antigen-BCR interactions are broken in this process, only high affinity interactions succeed in capturing antigen[117]. The GC B cell then internalizes and presents antigen-derived peptides on MHC-II and engages in competition for help from Tfh cells. GC B cells which have the highest affinity are able to capture the most antigen, present the highest density of protein:MHC, and therefore are the most competitive for Tfh help[118]. Tfh cells provide CD40L stimulation, which enhances GC B cell survival[119]. Without CD40L stimulation, unfit cells die by neglect. Those which are selected will either re-enter the dark zone or exit the GC as an MBC or a PC[118]. Cells which re-enter the dark zone are stimulated to divide a defined number of times based on the strength of T cell help, once again undergoing AID-mediated somatic hypermutation to increase antibody affinity[120]. This iterative cycle happens over many generations of the same clone, and at each cycle effector cells are output from the GC, resulting in a series of clones with increasing affinity which can be assembled into clonal lineage trees.

The clonodynamics of the GC is an area of active investigation which seeks to explain the process of exporting effector cells from the GC. In the early stages of a GC, there is a wide diversity of clones competing for survival signals[121]. This permissive system allows for an early stage of clonal selection prior to affinity maturation. Activated cells which are not competitive for survival signals are exported as early MBCs[122]. Stochastically, about 10% of GCs will purify to a single successful clone, which typically occurs as a result of a clonal burst facilitated by a

single mutation which provides a substantial improvement in affinity and allows for dominance in the competitive environment[121]. As these cells improve their affinity, a temporal switch occurs where the late GC exports primarily PCs, with a smaller percentage of cells exported as MBCs[122]. PCs that are exported from the GC tend to be long-lived, residing in niches in the bone marrow[123]. Intriguingly, despite the majority of MBCs being exported early in the GC, upon antigen re-exposure it is the late-exported MBCs which are activated during the secondary response[124]. Other factors contribute to the output of cells from the GC. A recent report demonstrated that a subset of regulatory Tfh cells (Tfr) express the neuropeptide neuritin, which acts to suppress PC differentiation in GC B cells[125]. Furthermore, neuritin negatively regulates IgE^+ cells, though it is unclear whether this occurs in the GC or prior to GC B cell entry[125]. Another report identified IL-13-expressing Tfh cells which enhance the survival of IgE-expressing cells within the GC and potentiate affinity maturation in IgE^+ GC B cells[76].

IgE-expressing cells survive poorly within the GC[126]. These cells peak early during the GC response, and are absent from the late GC[41,126]. They tend to reside within the dark zone where they are more likely to die by apoptosis[126]. Though IgE-expressing cells accumulate a similar number of affinity-enhancing mutations, they do not appear to contribute significantly to the secreted IgE repertoire. These cells do not emerge as winner clones within a GC (personal communication G. Victora), solidifying the argument that they are not competitive participants of the GC reaction. Instead, it has been proposed that high affinity IgE^+ PCs are derived from

$IgG1^+$ GC B cells, which undergo sequential switching to IgE[127]. As previously mentioned, more recent reports indicate that CSR does not occur within the GC, therefore it is not clear whether nor how high affinity IgE^+ PCs are a product of the GC. We have demonstrated that persistent IgE-titres persist as long as GC activity continues, which suggests that IgE^+ PCs are indeed a product of the GC[18]. None-the-less, high affinity MBCs are a product of the GC, which may switch to IgE during a memory response.

3. Unique Memory of IgE Responses

The memory of an antibody response is typically held within the MBC repertoire of the same isotype. For example, if an $IgG1^+$ MBC is stimulated by its cognate antigen, it will proliferate, some of its progeny will become $IgG1^+$ PCs and others will be reseeded as $IgG1^+$ MBCs. However, we and others have demonstrated that IgE^+ MBCs are extremely rare or non-existent in both mice and humans[41,128]. As stated earlier, tonic signalling of the IgE BCR causes the upregulation of Blimp-1 in IgE-expressing cells, forcing their differentiation into PCs and away from the memory program[42,43]. Despite the absence of IgE MBCs, allergen re-exposures result in the rapid emergence of secondary IgE, indicating that an alternative pathway of memory exists[18].

Analysis of IgE-expressing cells which emerge during a memory response reveal that an increasing proportion display evidence of sequential CSR from IgG1 with additional re-exposures[99]. Adoptive transfer of $IgG1^+$ MBCs yields a robust secondary IgE response upon antigen re-exposure, demonstrating their capacity for

sequential switching upon recall[127]. We have demonstrated that IgE titres wane in a model of PN sensitization, and that IgE-expressing cells are no longer detectable 6 months post-sensitization. However, until the latest timepoint studied (15 months post-sensitization) antigen-specific $IgG1^+$ MBCs remain[18]. Upon allergen re-exposure, IgE titres rapidly re-emerge confirming IgG1 as a prominent reservoir of secondary IgE. Whether other isotypes of MBC hold the memory of IgE responses is not known. Among $IgG1^+$ MBCs, highly mature $CD80^+$, $CD73^+$, $PDL2^+$ cells rapidly generate secondary IgE responses, while other less mature populations ($CD80^+$, $CD73^-$ or $CD80^-$ $CD73^-$) can also contribute to the secondary IgE pool[127]. However, the highly mature MBC phenotype is thought to originate from the mature GC, and this population of cells is reported to be the primary memory reservoir selected during all recall responses[102,124]. It remains unclear whether other subsets of MBCs preferentially hold IgE memory.

Memory IgE responses critically require T cell help. This is in contrast to classical memory experiments, in which adoptive transfer of MBCs into RAG KO mice (which lack T and B cells) had intact memory humoral responses upon antigen challenge[129]. However, memory IgE responses require additional CSR, which is only known to occur in the context of T cell help. Curiously, *in vitro* culture of allergic splenocytes with allergen in the presence of a CD40L-blocking antibody did not impact the emergence of secondary IgE, an interaction thought to be required for CSR[18]. Adoptive transfer of allergic MBCs with T cells allowed for a memory IgE response upon challenge, but only if those T cells expressed IL-4[130].

Consistently, we have shown that secondary IgE responses in a human *in vitro* culture system and in mouse models are completely inhibited in the presence of an IL-4Rα blocking antibody[131]. These data indicate that IL-4, presumably from T cells, is required for memory IgE responses. Interestingly, adoptive transfer of allergic B cells with naive T cells was also sufficient for a secondary IgE response, perhaps indicating that MBCs do not require help from type 2 polarized memory T cells for a secondary IgE response[132].

4. Present Therapeutic Strategies

In Canada, there is no approved therapeutic strategy for food allergy. The clinical recommendation is strict allergen avoidance, and the administration of rescue epinephrine in the case of an accidental exposure to the allergen. In principle, strict allergen avoidance could avoid all possible morbidity and mortality from food allergy. In practice, allergen avoidance is complicated by contamination, and some allergens are present in a broad variety of foods. Indeed, as mentioned previously, 14% of patients are accidentally exposed each year[15]. If strict allergen avoidance is observed, a rapid decline in allergen-specific IgE would likely occur, as evidenced in Anisakis *spp.* infection and αGal allergy[39,40]. However, some allergies (eg. shellfish, peanut) are lifelong in most individuals, suggesting that avoidance alone is not sufficient to manage the disease. Rescue epinephrine counteracts the signs and symptoms of anaphylaxis but does not address the underlying pathology of food allergy.

As mentioned previously, OIT is approved in the US. Daily ingestion of allergen is found to increase the threshold of allergen which can be tolerated in a single sitting. This is accompanied by a decrease in allergen-specific IgE production and an increase of allergen-specific IgG4, the latter is thought to be protective against allergic reactions in food allergic individuals[133]. OIT is a viable option for those who can tolerate the therapy and who can adhere to daily ingestion indefinitely. A recent systematic review found that patients undergoing OIT were at a higher risk of anaphylaxis and other adverse outcomes and were more likely to drop out of therapy due to adverse reactions than those on placebo[4]. The increased tolerance threshold resulting from OIT is transient, the majority of individuals who cease OIT will return to clinical reactivity 1 year following therapy[133,134]. There is not yet a complete mechanistic explanation of how OIT improves allergen tolerance. It is not clear whether the increase in IgG4 nor the reported induction of T regulatory (Treg) cells is directly protective against allergic reactions[133]. It is, however, logical that cessation of therapy results in the re-emergence of reactivity, as memory B cells are stimulated with antigen, and can therefore regenerate allergen-specific IgE levels. In summary, there is a large population who cannot tolerate OIT, and the benefits of the therapy appear to only manage the disease but are not curative.

Numerous biologics have been tested in food allergy, but as of yet none have induced a clinically meaningful level of desensitization. The rationale behind omalizumab, a monoclonal antibody against the domain of IgE with binds to FcεRI,

is that it will bind serum free-IgE to limit its ability to sensitize granulocytes[135]. However, its uses in food allergy have been limited by safety concerns and a lack of efficacy[135]. The perpetual generation of allergen specific IgE by IgE^+ PCs would necessitate that this therapy be continued indefinitely. In order to be curative, omalizumab would have to reprogram allergic memory, which has not yet been demonstrated. We have recently demonstrated that dupilumab, an IL-4Rα antibody, has the potential to inhibit the *de novo* regeneration of allergen-specific IgE from memory, and its effects may last beyond the course of treatment[131]. Dupilumab is presently in clinical trials for food allergy as a monotherapy, and in combination with OIT.

5. The Present Work

The research in the present PhD thesis sought to understand the origins and trajectory of allergen-specific IgE, and the reactivation of allergic memory. To enable our investigations of allergen-specific MBCs, we extensively optimized an allergen-tetramer enrichment platform which allowed us to reliably detect cells as rare as allergen-specific naïve B cells in unimmunized mice (Chapter 3). We applied this technology in the two other projects presented here. In Chapter 4, we sought to understand the importance of sequential CSR in generating anaphylactic IgE in models of food allergy. We investigated the importance of sequential switching during both the primary and secondary IgE response, ultimately determining that the IgM-expressing cells are redundantly capable of all

mechanisms typically ascribed to IgG1-expressing cells. In Chapter 5, we used mouse models to overcome limitations to the investigation of the early events in human allergic sensitization to foods. We sought to understand the trajectory of B cell activation during the earliest incipient stages of allergic sensitization, and therefore we established a model of the clinically silent stage of allergic sensitization in humans. This stage is characterized by the absence of B cell activation where the memory of IgE responses appears to be held for a lifetime within memory T cells.

6. Hypothesis

The work in this thesis was driven by the overarching hypothesis that food allergy displays a phasic disease trajectory characterized by a clinically silent pre-sensitization phase which matures to a clinically reactive phase. We hypothesize that multiple redundant pathways can initiate IgE production and keep the memory of IgE responses.

Chapter 4 was driven by the central hypothesis that direct CSR and sequential CSR are redundant mechanisms which both lead to anaphylactic IgE production.

Chapter 5 was driven by the hypothesis that a clinically silent sensitization phase precedes IgE production following adverse exposures to allergen. Our initial hypothesis was that MBCs are initiated early in during the process of sensitization, in the absence of IgE-producing PCs.

CHAPTER 2

MATERIALS AND METHODS

1. Ethics Statement

All animal procedures were approved by McMaster University's Animal Research Ethics Board.

2. Mice

Age, sex, and vendor matched controls were used for all experiments. Where possible, littermate controls were prioritized. IgG1 KO mice (B6.B129P2(Cg)-Ighg1^{tm3Cgn}) were provided by Dr. Amy Kenter (University of Illinois). Verigem mice (B6.129P2(Cg)-Igh-7$^{tm1.2Cdca}$) were provided by Dr. Christopher D.C. Allen (University of California San Francisco). C57Bl/6 mice were purchased from Charles Rivers. μMT (B6.129S2-Ighm^{tm1Cgn}/J) and CD45.1 (B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ) were purchased from the Jackson Laboratory. In general, female mice at 6-8 weeks of age were used for experiments.

3. Genotyping

Ear notches were boiled at 90°C for 10 minutes in digestion buffer (10 mM Tris-HCL pH 8.0, 50 mM KCL, 0.5% NP40, 0.5% Tween20), prior to adding 20 μg Proteinase K (MilliporeSigma, Cat: 3115836001), incubating at 54°C and vortexing

every 30 minutes until the ear notch was dissolved. Proteinase K was inactivated in a second 10-minute boiling step at 90°C. Non-genomic debris was pelleted by centrifugation at max speed for 30 seconds, prior to storing the DNA at -20°C.

PCR amplification was performed using ExTaq (Takara Bio Inc., Cat: R001A) following supplier recommendations. Optimal annealing temperatures were determined by gradient PCR. In general, the DNA was amplified using an Eppendorf Mastercycler PRO S (Eppendorf AG, Cat: 6325) using the following program:

94°C – 3 minutes
94°C – 10 seconds ┐
58°C – 20 seconds ├ 30 cycles
72°C – 5 minutes ┘
72°C – 5 minutes
4°C – Indefinite

IgG1-deficient mice were genotyped using a common primer (CCTACCCTAAGACATTCCCTAGAAGT), a primer for the WT allele (TCCAAGTCCTGAGGGCCAAG) and a primer for the IgG1-deficient allele (TGTGCTCGAAGGCGATTAAG)

Amplicons were visualized by electrophoresis on a 1.5% agarose (ThermoFisher Scientific, Cat 16500100) gel in TAE buffer (BioShop Canada Inc., Cat: TAE222) with 1:10000 GelRed Nucleic Acid Stain (Biotium Inc., Cat: 41003). Samples were loaded in 1x Gel Loading Dye Purple (New England BioLabs Ltd., Cat: B7024S) and compared against the GeneRuler 100 bp DNA Ladder (ThermoFisher

Scientific, Cat: SM0241). Gels were visualized using an AlphaImager (Alpha Innotech) using a 595 nm filter and images were analyzed using ImageJ[136].

4. Antibody Treatments

To block IgG-mediated anaphylaxis, 500 µg anti-CD16/32 (Clone 2.4G2; Bio X Cell, Cat: BE0307) in 500 µl PBS was administered by intraperitoneal injection 24 hours prior to challenge. To deplete T cells, three intraperitoneal injections of 200 µg anti-CD4 (GK1.5; Bio X Cell, Cat: BE0003-1) in 500 µl PBS were administered 2 days apart. T cell depletion was maintained by weekly intraperitoneal injection of 100 µg anti-CD4 (GK1.5; Bio X Cell, Cat: BE0003-1) in 500 µl PBS until mice were euthanized. Maintenance doses are required when re-exposing mice to antigen as residual T cells rapidly proliferate in response (data not shown). Anti-CD40L (MR-1; Bio X Cell, Cat: BE0017-1) was administered as previously described[122]; three 350 µg intraperitoneal injections in 500 µl PBS, 2 days apart followed by weekly intraperitoneal maintenance injections of 125 µg in 500 µl PBS. Polyclonal Armenian hamster IgG (Bio X Cell, Cat: BE0091) was used as an isotype control for anti-CD40L. To block IL-4 signalling, 1 mg of anti-IL-4Rα (M1, generated in house) was injected intraperitoneally 24 hours before sensitization and/or each subsequent re-exposure. Rat IgG2a anti-trinitrophenol (2A3; Bio X Cell, Cat BE0089) was used as an isotype control for anti-IL-4Rα. Anti-OX40L (RM134L, Bio X Cell, Cat BE0033) and anti-ICOSL (HK5.3, Bio x Cell, Cat BE0028) were both administered intraperitoneally at 200 µg every other day, either 3 times prior to sensitization or starting 5 days prior to re-exposure until sacrifice. Appropriate

isotype controls (ICOSL: rat IgG2a, 2A3, Bio x Cell, Cat BE0089; OX40L: rat IgG2b, LTF-2, Bio X Cell, Cat BE0090).

5. Production of anti-IL-4Rα

The rat IgG2a anti-mouse IL-4Rα (M1) antibody producing hybridoma was a gift from Dr. Fred Finkelman (University of Cincinnati, Ohio, USA). The hybridoma was grown in serum-supplemented media and then transferred into serum free media and maintained in a spinner flask for up to 10 days. Culture supernatants were collected, and antibody was isolated using a Protein G-Sepharose column (GE Healthcare, Cat: 17061801).

6. Conventional Model of Food Allergic Sensitization and Challenge

Mice were sensitized using either 3.75 mg All-Natural Smooth PN Butter (Kraft Heinz, Northfield, USA), 1 mg ovalbumin (OVA) (MilliporeSigma, Cat: A5378), or 1 mg 4-hydroxy-3-nitrophenyl (NP)-OVA (LGC Biosearch Technologies, Cat: N-5051-100) intragastrically in the presence of 5 µg cholera toxin (List Biological Labs, Cat: 100B) suspended in 0.5 ml PBS. Oral gavages were performed using a 60 mm long, 1.22 mm diameter straight metal gavage needle (Delvo SA, Bien, Switzerland) once per week for 4 weeks. At various time points, generally 2 weeks but up to 12 months in some experiments, mice were bled retro-orbitally into gel-separated serum collection tubes (BD, Cat: 365967) for serum Ig analyses. One day later, mice were challenged intraperitoneally with 0.1-5 mg of either OVA, crude PN extract (Stallergenes Greer USA, Cat: XPF171D3A25), NP-OVA, or NP-

bovine serum albumin (BSA) (LGC Biosearch Technologies, Cat: N-5050H-100). Core temperature was measured at 10-minute intervals over 40 minutes using a rectal probe (VWR International, Cat: 23226-656) and hematocrit assessed using the HemataSTAT II centrifugation device (VWR International, Cat: 14221-620). Clinical signs were scored according to Figure 12F. The ratio of NP-conjugated reagents depended on availability from the supplier, in general NP-OVA was conjugated with 16-22 NP molecules, and NP-BSA with 27-30.

7. Adapted Early Events Model of Allergic Sensitization

Mice were primed with a single intragastric administration of either 3.75 mg All-Natural Smooth PN Butter (Kraft Heinz, Northfield, USA), 1, 10 or 100 mg OVA (MilliporeSigma, Cat: A5378), or 1 mg NP-OVA (LGC Biosearch Technologies, Cat: N-5051-100) in the presence of 5 µg cholera toxin (List Biological Labs, Cat: 100B) suspended in 0.5 ml PBS by gavage (as described above). Mice were then rested for 1 - 9 months prior to intragastric re-exposure with 1 mg of either 3.75 mg All-Natural Smooth PN Butter (Kraft Heinz, Northfield, USA), 1 mg OVA (MilliporeSigma, Cat: A5378), 1 mg NP-OVA (LGC Biosearch Technologies, Cat: N-5051-100), or NP-BSA (LGC Biosearch Technologies, Cat: N-5050H-100) without cholera toxin. In flow cytometry experiments, mice were sacrificed 6 days following the final gavage unless otherwise specified. Challenge experiments were performed as described above.

8. Detection of Antigen-Specific Immunoglobulin

All ELISA protocols used 96-well flat bottom absorptive plates (VWR Cat: 439454). ELISAs were blocked using 5% skim milk in PBS, and unless otherwise specified, reagents were prepared in 1% skim milk in PBS. Washes were done using PBS with 0.05% TWEEN 20 (Sigma, Cat: P-1379) using a Tecan Hydroflex (Tecan Trading AG). All ELISAs were read using a Thermo Scientific Multiskan FC plate reader and analyzed in Excel. No-sample controls were included in all assays, the average optical density of which was subtracted from all samples prior to plotting.

Allergen-specific IgG1 and total Ig: Plates were coated with either 4 µg/ml of OVA (Sigma, Cat: A5378), NP-BSA (LGC Biosearch Technologies, Cat: N-5050H-100), or crude PN extract (Greer, XPF171D3A25) in 100 µl of carbonate bicarbonate buffer (Sigma, Cat: C-3041), sealed with an adhesive cover (ThermoFisher, Cat: 3501) and incubated overnight in the fridge. The next day, the plates were blotted entirely and blocked for 2 hours at room temperature. Plates were washed 3 times and incubated with samples at indicated dilutions overnight in the fridge. The next day, plates were washed 3 times, and incubated with either 0.25 µg/ml biotinylated anti-mouse IgG1 (Southern Biotech, Cat: 1070-08), or biotinylated anti-mouse IgG (H+L) (Poly4053, Biolegend, Cat: 405301) for 2 hours at room temperature. Plates were washed 3 times and incubated with 1:1000 streptavidin (SA) alkaline phosphatase diluted in 0.3% BSA (Sigma, Cat: A4503) for 1 hour at room temperature and covered from light. After 3 washes, plates were developed using 4-nitrophenyl phosphate (Sigma, Cat: N-9389) dissolved in 1x diethanolamine

substrate buffer (ThermoFisher, Cat: 34064) in dH_2O until either signal was detected in the no-sample control wells or in the highest sample dilution. Plates were stopped with 25 µl 1N NaOH Samples were read at 405 nm.

Allergen-specific IgE: Plates were coated with 50 µl of 2 µg/ml anti-mouse IgE (R35-72, BD, Cat 553413) in PBS overnight in the fridge. The next day, plates were washed 3 times and blocked for 1 hour at 37°C. Plates were washed 3 times and samples were added at the indicated dilutions and incubated overnight in the fridge. The next day, plates were washed 5 times, incubated with 50 µl of 300 ng/ml OVA, 150 ng/ml CPE, or 300 ng/ml NP_{27}BSA conjugated to digoxigenin following supplier recommendations (ANP Technologies, Cat: 90-1023-1KT) for 90 minutes at room temperature. Plates were washed 5 times and incubated with 1:5000 anti-digoxigenin POD fragments (Roche, Cat: 11 633 716 001) in 0.3% BSA-PBS for 1 hour at room temperature, covered from light. Plates were washed 5 times, then detected using 50 µl TMB (Sigma, Cat: T0440) and stopped using 25µl 2N H_2SO_4. Samples were read at 450 nm.

Thiocyanate Elution: An additional thiocyanate elution step was added after sample incubation. After washing, wells were incubated for 15 minutes with the indicated concentrations of thiocyanate at room temperature. Plates were washed immediately, and the ELISA continued as written above.

9. Tissue Collection and Processing

Mesenteric lymph nodes and spleens were collected into HyClone Hank's Balanced Salt Solution (Hanks; GE Healthcare, Cat: SH30015.03) and kept on ice until processing. Mesenteric lymph nodes were crushed between frosted slides into a single cell suspension in Hanks and passed through a 40 µm cell strainer (Corning Inc., Cat: 352340). Spleens were crushed through a 40 µm cell strainer using the stopper of a 3 ml syringe (BD, Cat: 309657) into Hanks. In some experiments, red blood cells (RBC) were lysed from splenocyte preps by resuspending in 1 ml ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM NA_2EDTA) and agitating for 1.5 minutes. Lysis was stopped by diluting with at least 10 volumes of Hanks. Small intestines were flushed with approximately 10 ml PBS using a 60 mm long, 1.22 mm diameter straight metal gavage needle (Delvo SA, Bien, Switzerland), and then were stored in PBS on ice until processing. Small intestines were opened longitudinally and then cut into 2-3 mm wide pieces, transferred into 1mM DL-dithiothreitol (MilliporeSigma, Cat: 43816), 1-mM HEPES (MilliporeSigma, Cat: H4034)-PBS and placed in a 37°C shaker at 200 rpm for 10 minutes. Tissues were vortexed, passed through a metal sink strainer, collected into 5 mM EDTA (ThermoFisher Scientific, Cat: E468-500), 10 mM HEPES, 10% heat inactivated fetal bovine serum (FBS; ThermoFisher Scientific, Cat: 10437028)-PBS, and incubated in a 37°C shaker for 10 minutes. The tissues were washed and shook in EDTA-HEPES-FBS-PBS a total of 3 times. The tissues were then transferred into 0.5 mg/ml Collagenase A (F. Hoffmann-La Roche Ltd., Cat: 10103586001) and

100 µg/ml DNAse I (F. Hoffmann-La Roche Ltd., Cat: 10104159001) and shook for 60 minutes at 37°C. Digested tissues were crushed through a 70 µm filter (Corning Inc., Cat: 352350) using the plunger of a 3 ml syringe. Immune cells were enriched by resuspending cells in 40% Percoll (GE Healthcare, Cat: 17-0891-01) in RPMI (ThermoFisher Scientific, Cat: 31800089), underlaying 70% Percoll in RPMI, and spinning at 1000g for 30 minutes. Cells at the interface were collected and used for downstream analysis. To collect bone marrow, one or two hind legs per mouse were collected intact in PBS and kept on ice until processing. The femur and tibia were separated, the ends of the bone were cut, and the bone marrow was flushed with PBS using a 25G 5/8 in. needle (BD, Cat: 305122) attached to a 10 ml syringe (BD, Cat: 309604). The bone marrow was pipetted up and down to break up bone marrow chunks and passed through a 40 µm cell strainer.

10. Late Phase Inflammation and Cytokine Secretion

Late phase eosinophilic inflammation in the peritoneal cavity was assessed 72 hours post-challenge. Mice were euthanized and their peritoneum lavaged with 3 ml 10 mM EDTA (ThermoFisher Scientific, Cat: E468-500) in PBS. Lavages were passed through a 40 µm filter (Corning Inc., Cat: 352340) and eosinophils were quantified via flow cytometry.

Spleens were collected at 72 hours post-challenge for cytokine secretion in culture. $8x10^5$ RBC-depleted splenocytes per well were cultured in a 96-well plate in 200 µl of RPMI 1640 (ThermoFisher Scientific, Cat: 31800089) supplemented with 10% FBS (ThermoFisher Scientific, Cat: 10437028), 1% penicillin and

streptomycin (ThermoFisher, Cat: 15140148), 1% L-Glutamine (BioShop Canada Inc., Cat: GLU102.1), and 1x β-mercaptoethanol (ThermoFisher Scientific, Cat: 21985023). Splenocytes were either stimulated with 25 µg OVA (MilliporeSigma, Cat: A5378) or crude PN extract (Stallergenes Greer USA, Cat: XPF171D3A25) or media alone. Samples were incubated for 5 days at 37°C, 5% CO_2 prior to collection and storage of supernatants at -20°C. Cytokines were detected using a Luminex MagPix kit including IL-4, IL-5, IL-10, IL-13, IFN-γ (MilliporeSigma, Cat: MCYTOMAG-70K-05).

11. Degranulation Assay

Serum samples from allergic mice were diluted to 100 ng/ml based on total IgE. For heat inactivated samples, serum was heated to 56°C for 1 hour. Rat Basophil Leukemia cells (RBL-2H3, ATCC, Cat: CRL-2256) were sensitized with diluted serum samples. Sensitized RBL-cells were plated in a 96 well flat bottom and equilibrated at 37°C for 10 minutes. Cells were stimulated with the indicated concentrations of allergen and incubated at 37°C without CO_2 for 30 minutes. Cells were pelleted and 50 µl of supernatants was incubated with p-nitrophenyl N-acetyl-β-D-glucosamide in citrate buffer to measure secreted β-Hexosaminidase. Cells were lysed with 0.1% Triton X-100 to measure total β-Hexosaminidase. Plates were read using a Thermo Scientific Multiskan FC plate reader at 405 nm. Percent degranulation was calculated as 100x(supernatant content)/(supernatant+lystate content)[137].

12. Flow Cytometry

Cells were transferred into 2% FBS (ThermoFisher Scientific, Cat: 10437028), 2 mM EDTA (ThermoFisher Scientific, Cat: E468-500) PBS (FACS Buffer). All steps were performed using this media as a base unless otherwise specified. ≤ 3 x 10^6 cells were plated into a 96-well u-bottom plate (Corning Inc., Cat: 353077) and spun for 5 minutes at 200g at 4°C. Cells were resuspended in 25 µl FACS buffer with anti-CD16/32 (Clone 93, Biolegend, Cat 101302) to block antibodies binding to Fc receptors, and incubated on ice for 15 minutes, covered from light. 25 µl of extracellular antibody cocktail was added on top of the Fc Block, and the cells were incubated for 30 minutes on ice, covered from light. Staining was stopped with the addition of 200 µl FACS buffer prior to centrifugation. Cells were washed with an additional 200 µl of FACS, and then resuspended in a final volume of 200 µl of FACS for flow cytometry. In panels including both anti-IgM and GL-7, anti-IgM was included in the extracellular cocktail and GL-7 was included in a second 30-minute staining step to avoid free anti-IgM binding to the rat IgM GL-7 antibody. If intracellular staining was required, after the second wash step the cells were resuspended in 100 µl BD Cytofix/Cytoperm (BD, Cat: 554714) for exactly 20 minutes, stopped with 150 µl BD Perm/Wash (BD, Cat: 554714), washed once with 200 µl BD Perm/Wash, and resuspended in 50 µl intracellular staining cocktail in BD Perm/Wash. Cells were stained for 30 minutes on ice, covered from light. Staining was stopped with 200 µl BD Perm/Wash, washed once with 200 µl BD Perm Wash, once with 200 µl FACS, and then resuspended in 200 µl FACS for

analysis. IgE was detected intracellularly to visualize bona-fide IgE-producing cells; unlabeled anti-IgE (RME-1) was included in the Fc Blocking stage to block signal for FcεRI/II bound IgE[41]. A list of antibodies used for flow cytometry is included in Appendix 1. Data was collected on a BD LSRFortessa (BD, Franklin Lakes, USA) and analyzed using FlowJo v10.7 (FlowJo LLC, Ashland, USA).

13. Tetramer Construction and Antigen Enrichment

OVA (MilliporeSigma, Cat: A5503), β-Lactoglobulin (MilliporeSigma, Cat: L3908), NP-OVA (Biosearch LTD, N-5051) was incubated at a 1.5:1 ratio with Sulfo-NHS-LC-Biotin (ThermoFisher, Cat: A39257) for 2 hours on ice. Protein was purified using a 10 kDa size-exclusion filter (MilliporeSigma, Cat: UFC901008). The concentration of biotinylated OVA was assessed by mixing it with decreasing concentrations of SA-Phycoerythrin (PE, Agilent Technologies, Cat: PJRS25), separating the mixture by electrophoresis on a 4-15% gradient Tris/glycine gel (Bio-Rad Laboratories, Cat: 456-1084) and blotting with SA-Alexa Fluor 680 (SA-AF680; ThermoFisher Scientific, Cat: S21378). Blots were read using a LI-COR Odyssey CLx (LI-COR Biosciences, Lincoln, USA) at 700 nm. The concentration at which SA-PE no longer saturated the biotinylated OVA, and therefore had a visible band when detected with SA-AF680, reported the concentration of biotinylated OVA. This concentration was used to incubate biotinylated OVA at a 4:1 ratio with SA-PE, generating an OVA -PE tetramer. To exclude B cells which bound SA or PE, a decoy molecule was generated following the same protocol but loading the tetramer with an irrelevant protein (β-lactoglobulin, MilliporeSigma,

Cat: L3908). SA-PE was labeled with Dylight594 (ThermoFisher Scientific, Cat: 46413) following supplier recommendations, which allowed the decoy to be detected using the PE-CF594 filter, while the tetramer was detected using the PE filter. Appendix 2 lists tetramer reagents used in this thesis.

To enrich on OVA -specific cells, processed tissues were first incubated with 1 µl of 1 µM PE-Dylight594 decoy for 5 minutes at room temperature prior to adding 1 µl of 1 µM OVA -PE and staining on ice for 30 minutes, covered from light. Staining was stopped by adding 15 ml FACS buffer. Optionally, $1/40^{th}$ of the sample was removed to analyze an unenriched fraction. Cells were pelleted and resuspended in 25 µl anti-PE microbeads (Miltenyi Biotec, Cat: 130-105-639) and incubated on ice for 15 minutes, covered from light. The samples were topped up to 5 ml with FACS buffer and passed through a magnet-mounted LS column (Miltenyi Biotec, Cat 130-042-401). The columns were washed with 5 ml FACS, which was collected with the initial 5 ml and kept to analyze the flow through fraction. LS columns were removed from the magnet and enriched cells were eluted by flushing twice with 5 ml FACS buffer. The anti-PE microbeads bind PE with a mouse IgG1 antibody, and as a result all bead labeled cells will appear $IgG1^+$. When detecting $IgG1^+$ B cells by flow cytometry, we blocked surface IgG1 using an unlabeled anti-IgG1 (RMG1-1) and stained intracellularly for IgG1.

14. Adoptive Transfer

OVA-enriched cells from a single donor were resuspended in PBS and adoptively transferred into μMT recipients via tail vein injection. In some experiments, cells were stained with Cell Trace Violet (ThermoFisher, Cat: C34557) following manufacturer recommendations prior to transfer.

CHAPTER 3

TETRAMER-BASED STRATEGY FOR THE HIGH-RESOLUTION DETECTION OF ALLERGEN-SPECIFIC B CELLS.

Introduction

In food allergic individuals, allergen-specific B cells and antibody secreting cells have many isotypes and phenotypes. These include the IgE-secreting PCs which produce circulating allergen-specific IgE, and MBCs of varying isotypes and levels of maturity which maintain IgE memory. B-lineage cells make up ~50% of lymph node and spleen cells but, among those, the antigen specific repertoire is only a very small proportion. As a result, investigation of the bulk B cell repertoire has not provided a deep understanding of the mechanisms of B cell activation and differentiation.

A richer understanding of B cell dynamics has come from studying antigen-specific B cells. A number of approaches have been used to study these cells. One approach involves genetically modifying mice to dramatically increase the number of cells which are antigen specific. The first BCR transgenic strains were generated by injecting two full-length plasmids encoding recombined V(D)J-IgM, and VJ-light chains which integrated at a different locus than the endogenous V(D)J site[138]. As a result, these mice can not undergo CSR and are, therefore, not suited for the

study of IgE. Second generation BCR transgenics inserted a recombined V(D)J proximal to the endogenous IgH locus, defining the antibody specificity of the B cells[139]. These models can undergo CSR and somatic hypermutation and remain commonplace in studying B cell biology. However, these models do not have a perfectly monoclonal repertoire, primarily because the transgenic heavy chain can recombine with both kappa and lambda light chains, resulting in multiple specificities. Importantly, using BCR-transgenic mice to study allergic models requires a transgenic BCR specific for the model allergen studied. Further, several reports have indicated that the frequency of naïve B cells specific for an antigen biases B cell differentiation, and therefore may not be representative of physiological B cell responses[140,141]. Lastly, B cell responses in allergic individuals start from a polyclonal pool of B cells with varying V(D)J recombinations of varying affinities for allergen. It has been shown that polyclonal responses against complex antigens are more permissive than those against restrictive repertoires or against antigens with only one antibody-binding epitope[142]. As a result, strategies to study the antigen-driven response in the endogenous polyclonal BCR repertoire of wildtype (WT) mice are required.

Another approach to studying antigen-specific B cells utilizes their capacity to bind their cognate antigen in its native conformation through their BCR. These approaches generally involve conjugating the antigen of interest to a fluorescent probe, then incubating the reagent with a single-cell suspension from a given organ[143,144]. Cells which have bound to the antigen-fluorochrome conjugate are

detectable by flow cytometry and considered antigen-specific. This technique works particularly well with haptens, as they can be conjugated to high ratios on large fluorescent carriers like PE or allophycocyanin (>100 haptens:carrier). The high valency of these molecules allows low affinity binders to capture enough fluorescent antigen for detection. However, in this approach, complex multi-epitope antigens are typically conjugated as monomers to small fluorescent molecules. As a result, low affinity cells or those which downregulate their surface BCR-expression, like $IgG1^+$ PCs, may not capture enough fluorescent monomer to be detected[41]. This approach is limited in contexts where antigen-specific cells are very rare. B cells with BCRs specific for the fluorescent conjugate can be equal in number to those which are specific for the model antigen[102]. This issue is amplified when the fluorochrome is a large, immunogenic molecule such as is the case with PE and allophycocyanin. Furthermore, the rarity of antigen-specific cells means that many cells must be analyzed to detect a reasonable population. Often, a minimum of 1 million events are collected at the flow cytometer, and in the case of very rare populations like antigen-specific IgE^+ cells, some groups routinely collect 3-10 million events[41]. As a result, the approach is costly, results in long flow cytometry sessions especially when multiple mice or conditions are being compared. Further, the approach excludes most antigen specific cells, which are found in the remainder of cells which are not stained nor run on the cytometer.

In this chapter, I describe the in-depth validation of a modified tetramer-based approach to fluorescent antigen staining for use in the study of IgE-expressing cells

and in food allergy models. This approach involves constructing allergen-tetramers around a fluorescent molecule to generate a higher-valency reagent to detect antigen specific cells. A large pool of cells from candidate tissues are enriched for antigen-specificity using anti-fluorophore magnetic particles and a magnetized column. This approach has been used previously to study an unprecedented number of antigen-specific cells in mice that have been immunized with a model antigen and in some models of infection[102,144–148]. Here, I evaluated the capacity of antigen-tetramer enrichment to detect all populations of interest when studying allergic disease. Further, I describe the generation of tetramers of multiple novel specificities. This chapter introduces and validates a set of technologies that have driven the discovery in Chapters 4 and 5 of this thesis.

Results

Comparison of monomeric and tetrameric antigen staining to detect antigen-specific B cells.

Our group has previously detected allergen-specific B cells in a model of intragastric sensitization using monomeric OVA conjugated to fluorescein isothiocyanate (FITC) or Alexa Fluor 647 (AF647). Extracellular staining with fluorescent OVA yielded a detectable population of OVA-specific $CD19^+$ B cells which comprised up ~0.5% of alive singlet cells in mice intragastrically sensitized with OVA and cholera toxin (CT; Figure 1A). In naïve mice, a small population of OVA^+ cells was detected, though at a proportion approximately 10-fold greater than

a previous enumeration of 4000 OVA^+ naïve B cells per mouse (~0.004% of cells in a sample, Figure 1A)[145]. Indeed, a large proportion of OVA^+ $CD19^+$ cells lacked expression of IgM and IgD, which indicates that they were not naïve B cells and instead had been activated and undergone CSR (Figure 1B). Monomeric OVA staining is therefore not a reliable tool to study rare or naïve B cells.

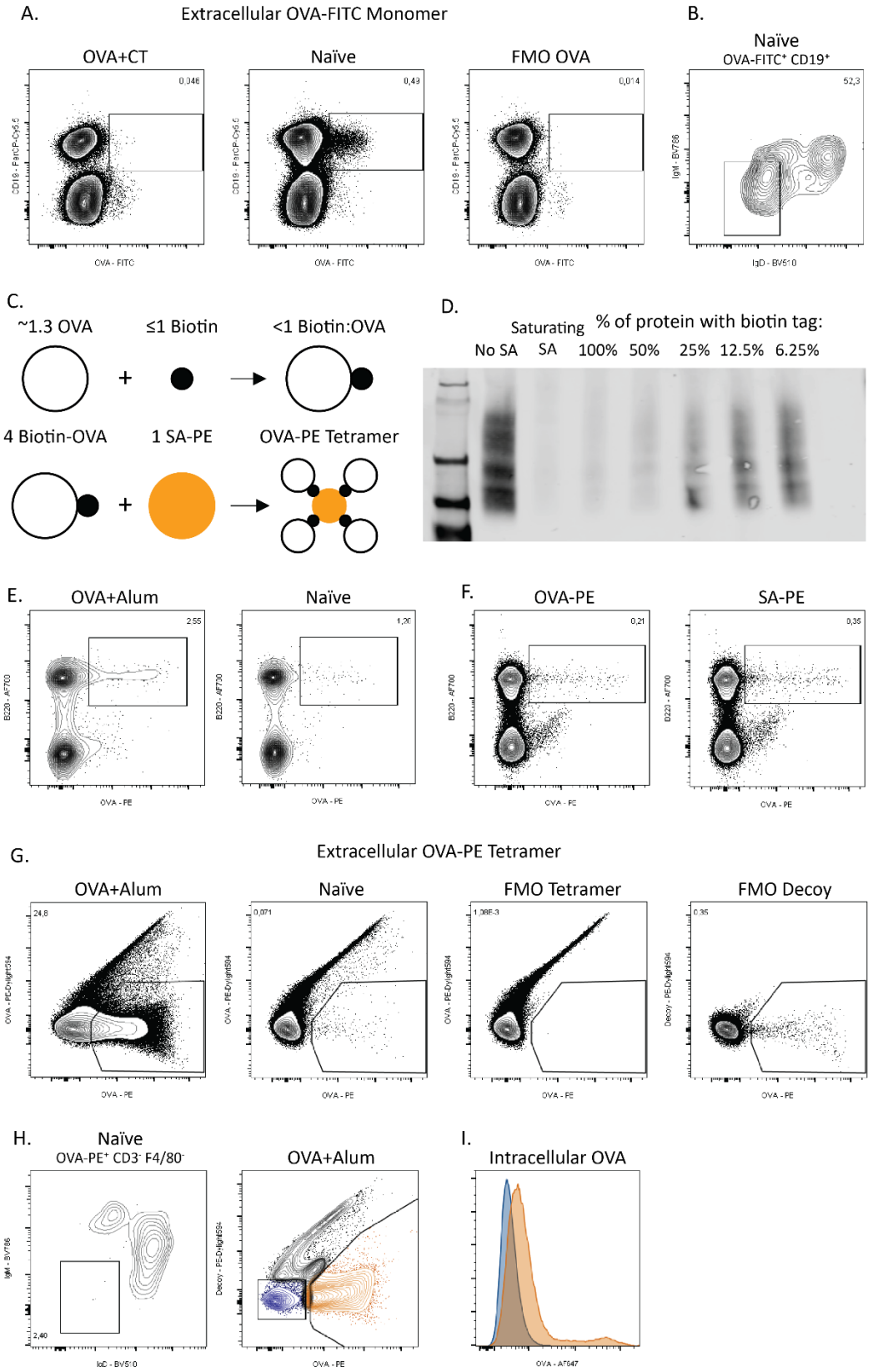
Staining B cells with monomeric antigens is reported to underestimate the B cell repertoire because monomers are poorly captured by low-affinity B cells[149]. To improve upon our detection of antigen-specific B cells, we constructed fluorescent OVA tetramers by biotinylating monomeric OVA at a <1biotin:1protein ratio and polymerizing it around PE-conjugated SA[145]. SA binds four biotin molecules, resulting in an OVA-tetramer (Figure 1C). To determine that OVA was conjugated at a <1:1 ratio, we incubated biotin-OVA with decreasing amounts of SA-PE and then separated the mixture using native gel electrophoresis. We transferred the separated protein onto nitrocellulose and blotted using SA-Alexa Fluor 680 (AF680) to detect free biotinylated-OVA. Numerous bands were detected when biotin-OVA, without SA-PE incubation, was probed with SA-AF680 (Figure 1D). No band was detected in saturating conditions (0.5 SA (2 binding sites) per protein), indicating that all available biotin-OVA was bound and therefore could not be detected with SA-AF680. Further, the absence of detectable biotin-OVA anywhere in the lane indicated that polymerized biotin-OVA did not have multiple biotin binding sites and therefore concluded that there was, on average, fewer than one biotin per OVA molecule (Figure 1D). The bands re-emerged when incubated at a

0.0625 SA (0.25 binding sites) per protein, revealing that 25% of the OVA had a biotin tag. This ratio was used to determine the molar concentration of biotin-OVA within the total amount of OVA, and this concentration was used to incubate biotin-OVA at a 4:1 ratio with SA-PE to generate a tetramer.

Consistent with previous reports, OVA-PE tetramers revealed a robust population of OVA^+ cells in mice immunized intraperitoneally with OVA and the robust Th2-adjuvant aluminum hydroxide (Alum), and a smaller but detectable population in naïve mice (Figure 1E). However, a population of similar size bound to the SA-PE backbone of the tetramer, revealing that the majority of putative OVA^+ naïve B cells instead were specific for non-OVA components of the OVA-PE tetramer (Figure 1F). To exclude the non-OVA-specific cells, we generated a decoy reagent which included all components of the OVA-PE tetramer except OVA^{145}. We conjugated SA-PE to Dylight594, which through fluorescence resonance energy transfer (FRET), can be detected in the same channel as PE-CF594 or PE-Dazzle594 and therefore can be visualized separately from OVA-specific cells. We loaded the SA-PE-Dylight594 backbone with an irrelevant protein biotinylated using the same kit as the OVA-PE tetramer, ensuring that B cells which bind to the linker and biotin molecules added during biotinylation would also be excluded. Samples were pre-stained with decoy-PE-Dylight594 before adding OVA-PE. When decoy-binding cells were excluded, a much smaller population of OVA^+ cells consistent with previous reports was detected (Figure 1G). Importantly, nearly all OVA^+ cells were *bona fide* naïve B cells based on

expression of IgM and IgD (Figure 1H). As expected, a much larger population of OVA^+ cells was detected upon OVA-Alum immunization. OVA-PE-binding cells also bound monomeric OVA-AF647 when stained intracellularly, while those which were not bound by the OVA-PE tetramer did not stain intracellularly for OVA-AF647 monomer (Figure 1I). Together, these data demonstrate that decoy-PE-Dylight594 exclusion and OVA-tetramer staining reliably detects antigen-specific B cells with sufficient specificity to investigate rare MBCs and naïve B cells. However, meaningful investigation of these populations remains limited by the low number of antigen-specific cells that are collected in million or even multi-million cell samplings at the cytometer.

Figure 1: Comparison of monomer and tetramer staining for ovalbumin-specific cells.
A) Representative plot of OVA-monomer binding cells from the mesenteric lymph nodes of mice 6 days post-sensitization with 4 gavages of OVA and CT. B) Isotype switched cells among OVA-monomer binding B cells from naïve mice. C) Schematic of OVA-PE tetramer construction. D) Biotin-OVA was separated by gel electrophoresis and blotted using SA-AF680. In lanes 3-8, Biotin-OVA was pre-incubated with decreasing concentrations of SA-PE prior to electrophoresis to determine biotinylation ratio. E) Representative plot of OVA-PE tetramer binding cells from pooled spleen and mesenteric lymph nodes of mice 7-9 days post- i.p. immunization with OVA-Alum. F) OVA-PE tetramer- and SA-PE backbone- binding cells from pooled spleen and mesenteric lymph node cells from naïve mice. G) Representative plot of decoy tetramer and OVA-PE tetramer binding cells from OVA-Alum immunized mice. Pre-gated on $CD3^-$ $F4/80^-$. H) Isotype switched cells among OVA-tetramer binding B cells from naïve mice. I) Intracellular OVA-binding of extracellular $OVA\text{-}PE^+$ or $OVA\text{-}PE^-$ cells from OVA-Alum immunized mice. *(Figure on next page)*


A.
Extracellular OVA-FITC Monomer
OVA+CT
Naïve
FMO OVA
0,046
0,49
0,014
CD19 - FarCP-Cy5.5
OVA - FITC
B.
Naïve
OVA-FITC+ CD19+
52,3
IgM - BV786
IgD - BV510
C.
~1.3 OVA
≤1 Biotin
<1 Biotin:OVA
4 Biotin-OVA
1 SA-PE
OVA-PE Tetramer
D.
Saturating
% of protein with biotin tag:
No SA
SA
100%
50%
25%
12.5%
6.25%
E.
OVA+Alum
Naïve
2,55
1,20
B220 - AF700
OVA - PE
F.
OVA-PE
SA-PE
0,21
0,35
G.
Extracellular OVA-PE Tetramer
OVA+Alum
Naïve
FMO Tetramer
FMO Decoy
24,8
0,071
1,08E-3
0,35
OVA - PE-Dyl ight594
Decoy - PE-Dyl ight594
H.
Naïve
OVA-PE+ CD3- F4/80-
OVA+Alum
2,40
IgM - BV786
IgD - BV510
Decoy - PE-Dyl ight594
I.
Intracellular OVA
OVA - AF647

Using magnetic enrichment to increase the number of detectable antigen-specific cells in flow cytometry.

Investigation of rare B cell populations typically requires collection of many millions of events at the flow cytometer. These samplings generally represent <10% of the cells that could be potentially analyzed from larger organs like the spleen. In these contexts, the majority of antigen-specific B cells remain unanalyzed. To improve the resolution of antigen-specific B cells, we employed a previously reported strategy of incubating decoy and OVA-PE tetramer-stained cells with anti-PE magnetic beads and enriching bound cells using a magnetic column (Figure 2A)[144]. Antigen enrichment increased the proportion of antigen-specific cells and decoy$^+$ cells in both naïve and immunized mice, though a significant number of cells which bound neither the decoy nor OVA tetramer remained (Figure 2B-D). Importantly, on average 20-times the number of antigen-specific cells were collected in a single sampling, allowing for greater resolution of the antigen specific repertoire and the potential to sub-phenotype these cells based on their protein expression (Figure 2B). In naïve mice, the number of antigen-specific B cells closely approximated previous reports of their frequency (~8000 vs ~4000)[145], likely differing due to the method of cell enumeration. Previous reports have used counting particles at the flow cytometer, while we calculated the number of cells based on their frequency and the total cell count of the enriched fraction. In immunized mice, OVA-PE binding cells remained in the flow through fraction,

which indicates that during robust immune responses the flow through fraction may need to be analyzed in parallel depending on the research question (Figure 2B-D).

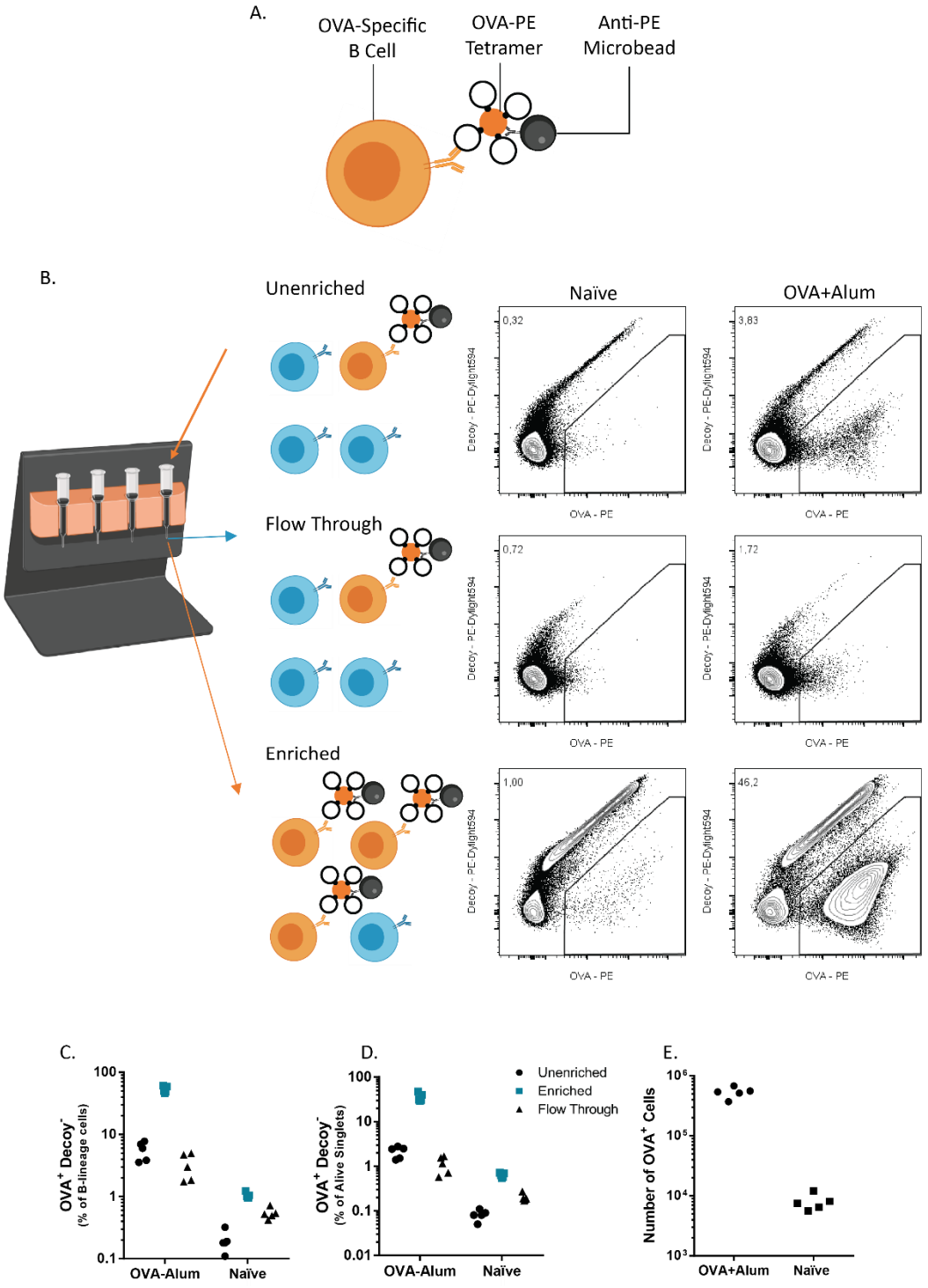


Figure 2: Enrichment of OVA-PE binding cells using anti-PE magnetic microbeads. A) Schematic of OVA-PE tetramer and anti-PE microbead binding. B) Schematic of antigen enrichment strategy and representative plots from the spleen and mesenteric lymph nodes of mice 7-9 days post-OVA-Alum immunization or naïve mice. Pre-gated on $CD3^-$ $F4/80^-$ cells. C-D) Proportion of OVA-specific cells in the unenriched, enriched, and flow through fractions of OVA-Alum immunized and naïve mice from C) B-lineage cells ($CD3^-$ $F4/80^-$ and expressing one or both of CD138 or B220) or D) alive singlet cells. E) Quantification of the number of OVA-specific cells in the enriched fraction of OVA-Alum immunized or naïve mice. *(Figure on previous page)*

Validation of the Phenotype and Isotype of Antigen-Enriched B-lineage Cells.

Antigen-enrichment to study the antigen-specific B cell repertoire has been previously reported but has not been thoroughly validated[145–148,150]. First, we asked whether the tetramer staining and enrichment technology excluded antigen-specific B cells. We enriched OVA-specific cells from the spleen and mesenteric lymph nodes of OVA-Alum immunized mice, then stained intracellularly with monomeric OVA-AF647. B cells which bound the OVA-PE tetramer in the enriched fraction also bound intracellular OVA-AF647 again affirming that these cells are *bona fide* OVA-specific B cells (Figure 3A). As expected, OVA-specific $CD138^+$ PCs stained brightly for antigen intracellularly due to their high antibody production, while OVA-specific B cells stained dimly due to low quantities of membrane-bound antibody being present inside the cell (Figure 3A). OVA-PE^- cells which were retained in the enriched fraction did not bind intracellular OVA-AF647, indicating that these are contaminants and not antigen-specific B cells and therefore should not be included in future analyses (Figure 3A). Importantly, the frequency of OVA-AF647 binding cells which stained lowly for the OVA-PE tetramer was similar

before enrichment and in the flow through fraction (Figure 3B). This indicates that cells which likely bound a low number of magnetic beads are pulled down during enrichment and are not systematically excluded. We next asked whether this strategy could pull down various B-lineage cells. This is relevant as, for example, PCs are reported to downregulate surface BCR expression and therefore may not be enriched using this technology[151]. The decoy$^-$ OVA$^+$ population included B220$^+$ B cells with intermediate total Ig staining, detected using an anti-mouse heavy and light chain antibody (H+L, Figure 3C). We also detected H+L^{bright} cells which differentially expressed B220. These cells co-expressed CD138 and Blimp-1 and were therefore identified as antibody secreting cells (plasmablasts/PCs, Figure 3D, E). Within the B cell population, we detected GL-7$^+$ CD38$^-$ cells which co-expressed Bcl-6 and were therefore identified as germinal center (GC) B cells (Figure 3F). GL7$^-$ CD38$^+$ cells and GL-7$^+$ CD38$^+$ Bcl6$^-$ cells were also detected and identified as memory B/naïve B cells and transitional B cells, respectively (Figure 3G). Finally, a population of B220$^-$ H+L^{int} non-B lineage cells were also detected. These are likely cells which express Fc receptors loaded with antigen-specific antibodies (Figure 3C, orange arrow), as they do not express canonical B-lineage markers like Bcl6 or CD138 (data not shown). In summary, antigen-enrichment allows for the high-resolution detection of the major B-lineage cell types.

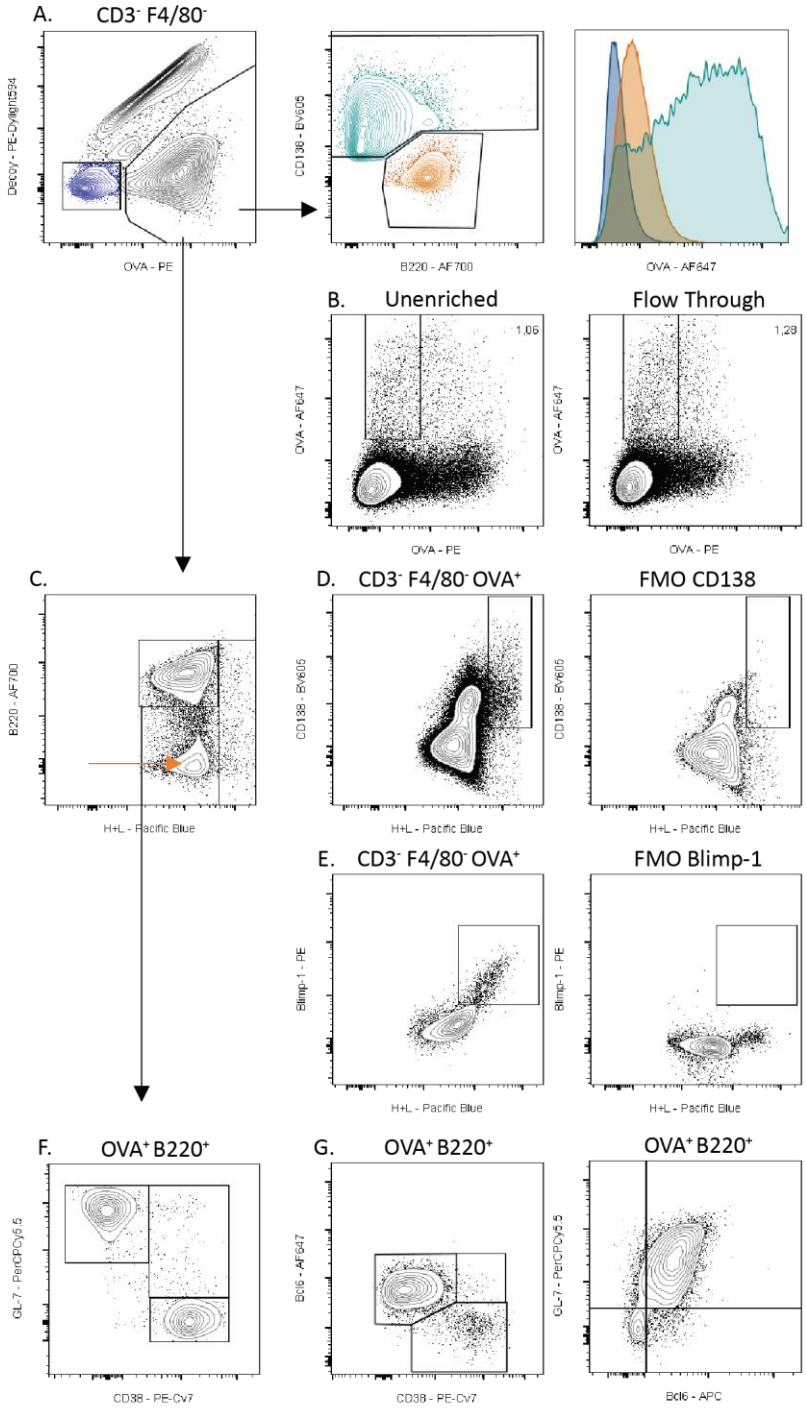


Figure 3: OVA-PE enrichment includes major B lineage phenotypes. A) Intracellular OVA-AF647 monomer binding of extracellular OVA-PE-binding B cells and plasma cells from the enriched fraction of OVA-Alum immunized mice. B) Proportion of OVA-PE^{low}, OVA-AF647-binding cells in the unenriched and flow through fractions of OVA-Alum

immunized mice. C) B220 and anti-mouse antibody heavy and light chain (H+L) staining of OVA-specific cells from OVA-alum immunized mice. D) CD138 and E) Blimp-1 expression of $H+L^{high}$ OVA-specific cells. F) GL-7 and CD38 expression of OVA-specific B cells. G) Bcl6 expression of OVA-specific B cells.

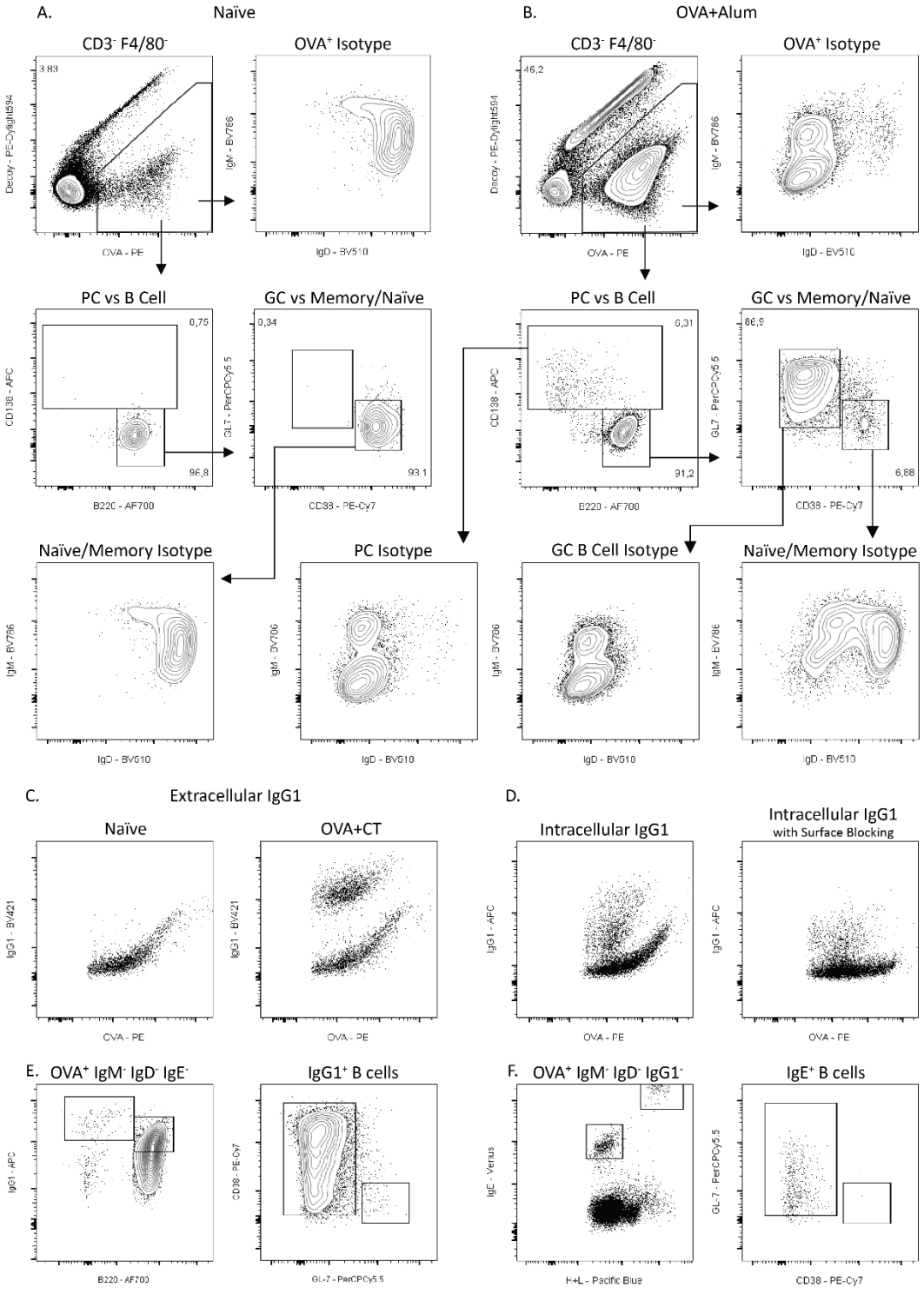
We next sought to determine whether B cells of relevant isotypes to the study of food allergy are represented in the antigen-enriched fraction. To our knowledge, antigen-enrichment has not yet been used for in-depth analyses of B cell subsets of specific isotypes. This is important in the study of food allergy, as the disease is mediated by antigen specific IgE. Antibody isotype differentially regulates B cell dynamics. As an example, tonic signalling of the IgE BCR promotes PC differentiation, which is thought to limit the existence of IgE^+ MBCs[41–43]. Indeed, IgE^+ MBCs are nearly undetectable in humans and mice. IgE^+ GC B cells downregulate membrane-Ig expression, which could impact the ability for antigen-tetramers to capture these cells during enrichment[126]. We first sought to determine the isotype of OVA-specific B-lineage cells enriched from naïve and OVA-Alum immunized mice. As demonstrated above, OVA-enrichment from naïve mice yielded near-exclusively IgM^+ and IgM^+ IgD^+ cells. These cells were $B220^+$ $CD138^-$ $CD38^+$ $GL7^-$, a phenotype consistent with naïve B cells (Figure 4A). In immunized mice, a robust population of switched IgM^-IgD^- cells were detected along with a smaller population of IgM^+ IgD^+ B cells which may be remaining naïve B cells or potentially a population of IgM^+ IgD^+ MBCs (Figure 4B). Further, immunized mice had a population of antibody secreting cells ($CD138^+$ $B220^{+/-}$) and GC B cells ($GL7^+$ $CD38^-$) which were absent in naïve animals, the majority of which were

isotype switched (Figure 4B). Lastly, switched MBCs ($GL7^-$ $CD38^+$ IgM^- IgD^-) were detectable only in immunized mice (Figure 4B).

Next, we sought to determine if B-lineage cells of varying isotypes were present among these switched cells. The presence of $IgG1^+$ and IgE^+ cells was of principal importance for the work presented in this thesis and, therefore, we started with these isotypes. The magnetic beads used for enrichment use a mouse IgG1 anti-PE antibody and, as a result, all tetramer- and decoy-binding cells stained positive for IgG1, even in unimmunized mice. Cells which captured a large density of OVA-PE bound many magnetic beads, indicated by their colinear IgG1 staining (Figure 4C). IgG1-expressing cells could be properly visualized by gating IgG1 against OVA, though this strategy worked poorly for IgG1-ASCs which downregulated surface BCR (Figure 4C, data not shown). This limitation was overcome by blocking surface IgG1 and staining for intracellular IgG1 (Figure 4D). Using this strategy, OVA-specific $IgG1^{hi}$ $B220^-$ ASCs and $IgG1^{int}$ $B220^+$ B cells were detected in the enriched fraction. Among the $IgG1^+$ B cells, both GC B cells and a small population of $IgG1^+$ MBCs were detected (Figure 4E). To detect IgE-expressing cells, we utilized mice which have a Venus reporter inserted at the IgE constant region locus[41]. $Venus^{high}$ HL^{high} ASCs and $Venus^{int}$ HL^{int} B cells were detected at a resolution far higher than previous reports (Figure 4E). Consistent with these reports, all IgE^+ B cells had a GC phenotype, and IgE MBCs were absent from the enriched fraction (Figure 4 D-E). Given the sensitivity of this technique, these data provide firm support that IgE-expressing MBCs do not exist. All considered, the

OVA-enrichment platform allows for the sensitive detection of OVA-specific cells of all isotypes and B-lineage phenotypes.

Figure 4: Isotype distribution of various OVA-specific B-lineage populations following OVA-PE enrichment. A-B) Isotype of OVA-PE binding cells, OVA^+ PCs, OVA^+ GC B cells, and OVA^+ memory/naïve B cells from the spleen and mesenteric lymph nodes of OVA-Alum immunized and naïve mice. C) Extracellular IgG1 staining of OVA-PE specific cells from 4x OVA-CT immunized mice. Anti-PE microbeads use a mouse IgG1 antibody which contaminates real IgG1 staining. D) Intracellular IgG1 staining of OVA^+ cells from OVA-Alum immunized mice with or without blocking surface IgG1 using an unlabeled antibody of the same clone (RMG1-1). E) Left: Intracellular IgG1 staining of OVA-specific cells from OVA-Alum immunized mice. Pre-gated on IgM^- IgD^- IgE^-. Right: GC vs MBC phenotype of $IgG1^+$ cells. F) Left: IgE expression of IgM- IgD- IgG1- OVA-specific B cells from OVA-Alum immunized Verigem mice, which have a Venus reporter inserted downstream of Cε. Right: GC vs MBC phenotype of IgE^+ cells.*(Figure on next page)*


A.
Naïve
B.
OVA+Alum
CD3⁻ F4/80⁻
OVA⁺ Isotype
PC vs B Cell
GC vs Memory/Naïve
Naïve/Memory Isotype
PC Isotype
GC B Cell Isotype
C.
Extracellular IgG1
Naïve
OVA+CT
D.
Intracellular IgG1
Intracellular IgG1 with Surface Blocking
E.
OVA⁺ IgM⁻ IgD⁻ IgE⁻
IgG1⁺ B cells
F.
OVA⁺ IgM⁻ IgD⁻ IgG1⁻
IgE⁺ B cells

Antigen-Enrichment from the Small Intestine and Bone Marrow.

Antigen-enrichment has been used here and by others in secondary lymphoid organs. The enrichments shown in Figures 2-4 were performed on a pool of the spleen and mesenteric lymph nodes, known inductive sites for intragastric and intraperitoneal immunization. This nascent technology has not yet been used across multiple tissues to study B cells resident to these sites. We tested the use of antigen enrichment in the lamina propria of the small intestine and in the bone marrow. B cell immunity in the lamina propria has been extensively studied against commensals[152], but the involvement of local immunity during intragastric allergic sensitization has not been investigated thoroughly. We separately pooled the lamina propria of 10 naïve mice and 5 mice intragastrically sensitized with 4 administrations of OVA and CT prior to OVA-enrichment. In naïve mice, OVA-enrichment revealed a very small population of local naïve B cells (Figure 5A, top row). In allergic mice, much larger populations of antigen-specific B cells and PCs were detected (Figure 5A, bottom row). By contrast to experiments in the secondary lymphoid organs, the majority of OVA-specific cells were ASCs, rather than B cells and ~98% of the OVA-specific cells localized to the small intestine were class switched.

The bone marrow houses long-lived PCs which secrete antibodies into circulation for decades in the context of viral infection and immunization[153]. While IgE-expressing cells generally are short lived, a recent report has renewed interest in the bone marrow as a site of long-lived IgE^+ PCs which emerge in response to

chronic aeroallergen stimulation[44]. To test the feasibility of using antigen-enrichment to study these cells, we OVA-enriched cells from the bone marrow from two hind legs of naïve mice and those sensitized with 4 administrations of OVA+CT. As expected, enrichment from naïve mice revealed a population of unswitched B cells which may represent developing B cells in the bone marrow (Figure 5B, top row). In allergic mice, a larger population of PCs and a robust population of switched cells emerged (Figure 5B, bottom row). This technique improves resolution of the switched MBC repertoire in the bone marrow of immunized mice and is feasible to study long lived PCs and developing B cells.

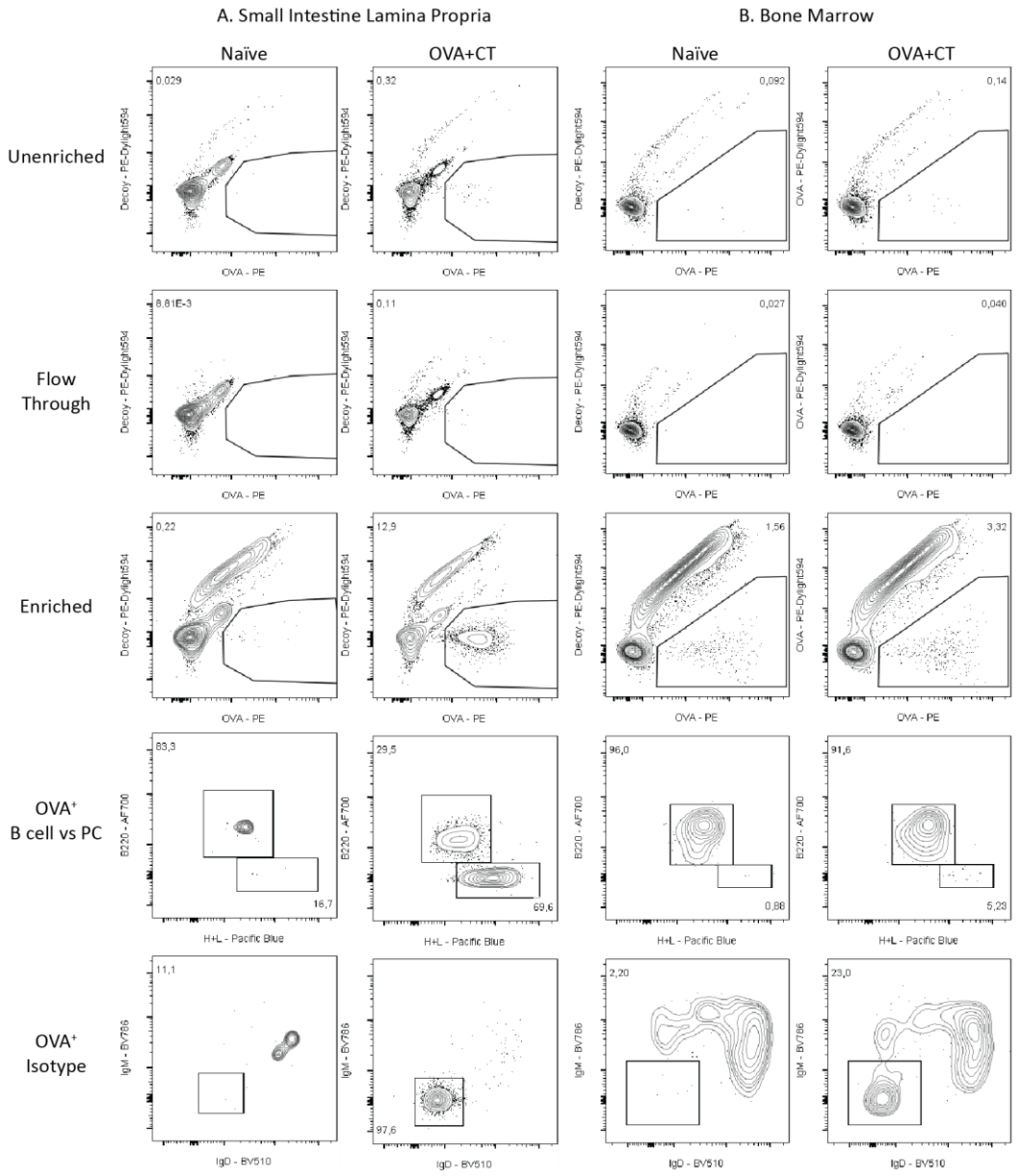


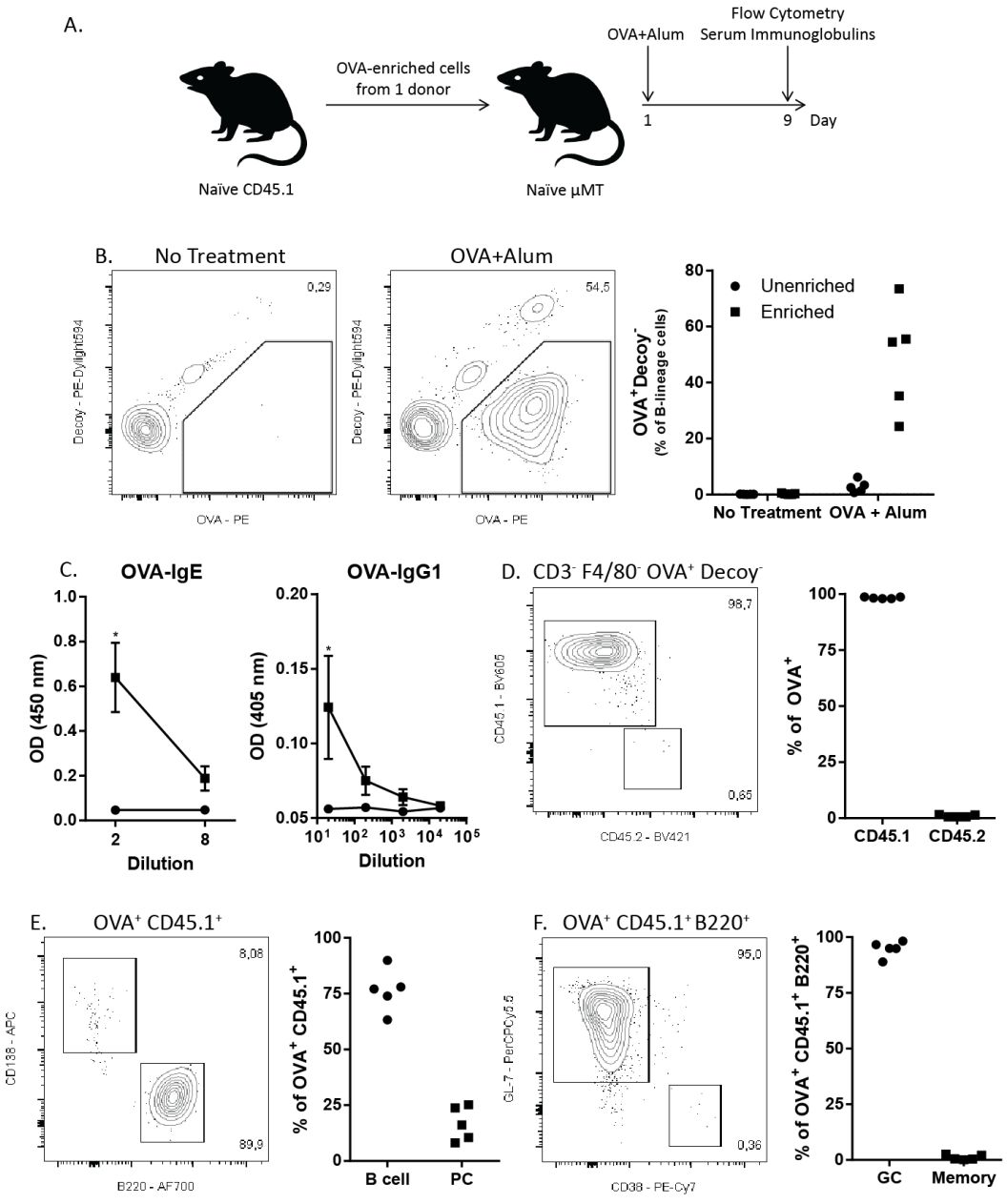
Figure 5: Antigen-enrichment from the small intestine and the bone marrow. A) OVA-PE enrichment from 10 pooled small intestinal lamina propria samples from naïve mice (top) or 5 pooled small intestinal lamina propria samples from mice receiving 4 gavages of OVA and CT (bottom). B) Representative plots of OVA-PE enrichment from the bone marrow from two hind legs of naïve or OVA and CT gavaged mice. Within OVA-specific cells B cells ($B220^+$) and plasma cells ($H+L^{hi}$) and isotype (IgM vs IgD) are shown. Pre-gated on $CD3^-$ $F4/80^-$.

Antigen-Enriched Cells in Adoptive Transfer

Adoptive transfer is a useful tool for tracing B cells during an immune response. We sought to determine if cells bound through their BCR and enriched through a magnetic column could be adoptively transferred and used to study type 2 immunity and IgE responses. We enriched OVA-specific cells from naïve CD45.1 donors and adoptively transferred cells into naïve CD45.2 B cell deficient μMT recipients at a one donor to one recipient ratio. The exact number of antigen-specific cells transferred was not assessed but based on our yield OVA-specific cells from naïve mice we likely transferred 5000 – 10 000 OVA-specific cells (Figure 2E). $Decoy^+$ and $Decoy^-$ OVA^- cells were also transferred. Recipients were either immunized with OVA-Alum or left untreated (Figure 6A). While OVA-specific cells were nearly undetectable in unimmunized recipients, immunized recipients had robust proliferation of OVA-specific cells (Figure 6B). Immunized mice produced serum OVA-specific IgE and small but detectable quantities of OVA-specific IgG1 (Figure 6C). Greater than 98% of the OVA-specific cells in immunized mice were donor derived (Figure 6D), demonstrating that the limited B cell repertoire in μMT mice did not contribute to the OVA-specific response. Among donor-derived OVA-specific cells, populations of both $CD138^+$ PCs and $B220^+$ B cells were detected (Figure 6E), and among B cells a large population of GC B cells and a smaller but detectable population of MBCs were detected (Figure 6F). The paucity of MBCs in this model was likely due to the early time point of evaluation (9 days post-immunization), where GC-derived MBC production is underway, but these cells

have not had sufficient time to accumulate[122]. Together, these data demonstrate that OVA-enriched cells from naïve mice are multipotent, seeding populations of all major B cell lineages and capable of isotype switching to IgE and IgG1. Adoptive transfer of OVA-enriched cells is a feasible tool to trace OVA-specific B cell responses in allergic models.

Figure 6: Adoptive transfer of OVA-enriched cells. A) Schematic of transfer experimentation. Spleen and mesenteric lymph nodes from naïve CD45.1 mice were pooled prior to OVA-PE tetramer staining and enrichment. Cells were pooled and adoptively transferred into the same number of recipients, resulting in each recipient getting a number of cells equivalent to one donor. Mice were immunized with OVA-Alum and analyzed at day 9. B) Representative plot of OVA-binding cells in the enriched fraction at day 9 and quantification of OVA-specific cells as a percentage of B-lineage cells ($CD3^-$, $F4/80^-$ and positive for one or both of CD138 and B220). C) Serum OVA-IgE and IgG1 at day 9, measured by ELISA. D-F) Representative plot and quantification of the phenotype of OVA-specific cells in OVA-Alum immunized mice. D) Donor vs recipient-derived cells as a percentage of OVA^+ cells. E) B cells ($B220^+$) vs plasma cells ($CD138^+$) as a percentage of OVA^+ $CD45.1^+$ cells. F) GC B cells ($GL7^+$) vs MBCs ($CD38^+$) as a percentage of OVA^+ $CD45.1^+$ B cells. * $p<0.05$ compared to no treatment group using repeated measures t test with Bonferroni correction. *(Figure on next page)*



Construction of Novel B Cell Tetramer Specificities

We have significantly expanded upon previous validations of B cell tetramer enrichments to study antigen-specific B cells. We therefore sought to generate novel tetramer specificities for a variety of applications. Below, we report the

construction of four new tetramer specificities and demonstrate their use in a simple antigen and alum immunization system. We constructed a tetramer of β-lactoglobulin (BLG), a major allergen in cow's milk, which is intended for use in models of milk allergy and in the PBMCs of patients allergic to milk (Figure 7). We also commonly use BLG for our decoy specificity. We constructed a tetramer of the major PN allergen Ara h 1 to study PN specific immunity in animal models and allergic patients (Figure 8). Construction of tetramers of other major PN allergens, like Ara h 2, 3 and 6 is ongoing in our laboratory. We constructed a tetramer of using NP-conjugated OVA (NP-OVA), which will be used to study B cell responses against NP, a common hapten model antigen (Figure 9). OVA-PE-Dylight594 was used as the decoy in these experiments to exclude OVA-specific cells in the analysis. Lastly, we report upon the construction of a tetramer of recombinantly-expressed receptor binding domain of the SARS-COV-2 spike protein for use in pre-clinical vaccination studies and to study B cell response to the virus (Figure 10).

Each antigen was biotinylated at a ratio that resulted in <1 biotin per protein. None of the proteins demonstrated evidence of multiple biotins per protein, as evidenced by the disappearance of detectable antigen in the presence of sufficient SA to block all biotin sites (Figure 7-10A). Each antigen had a biotin tag on ~12.5% to 25% of molecules, which was used to built tetramers at an appropriate 4:1 ratio of biotin-protein:SA-PE. Antigen-enrichment greatly improved the frequency of antigen-specific cells for analysis (Figure 7-10B). Each antigen had unique binding

characteristics. BLG and RBD had intermediate populations of cells between the decoy-specific population and antigen-specific population that was present at approximately equal proportions in naïve and immunized mice. All of these cells expressed IgM and IgD in both naïve and immunized mice, suggesting that they were not antigen specific (Figure 7,10B, orange arrow). However, for all tetramers the antigen-specific B cells in the enriched fraction from naïve mice were naïve B cells (IgM^+ IgD^+), while isotype switched cells were present only in immunized mice (Figure 7-10B). As an additional example of activation, GC B cells were only present in immunized mice for all tetramers (Figure 7-10B). The Ara h 1 tetramer was constructed using an APC backbone, rather than PE, as has been done previously (Figure 8)[144]. Both the Ara h 1- and NP- tetramers bound a higher frequency of naïve B cells than may be expected, future research should determine whether all of these IgM^+ IgD^+ B cells are antigen specific (Figure 8-9). Altogether, these tetramers are reliable tools for the detection of antigen-specific B cells in both naïve and immunized mice and are feasible for use in the various contexts described above.

Figure 7: Construction of β-lactoglobulin tetramer. A) ratio of biotinylated protein to total protein determined by gel electrophoresis as described in Figure 1D. B) BLG-PE enrichment from naïve mice (left) and mice immunized with BLG-Alum (right). BLG-PE-binding cells in unenriched, flow through and enriched fractions. (Bottom) Example phenotype and isotype of BLG-specific B cells.

Figure 8: Construction of Ara h 1 tetramer A) ratio of biotinylated protein to total protein determined by gel electrophoresis as described in Figure 1D. B) Ara h 1-APC enrichment from naïve mice (left) and mice immunized with crude peanut extract+Alum (right). Ara h 1-binding cells in unenriched, flow through and enriched fractions. (Bottom) Example phenotype and isotype of Ara h 1-specific B cells.

Figure 9: Construction of NP-tetramer A) ratio of biotinylated protein to total protein determined by gel electrophoresis as described in Figure 1D. B) NP-OVA-PE enrichment from naïve mice (left) and mice immunized with NP-OVA-Alum (right). NP-PE-binding cells in unenriched, flow through and enriched fractions. (Bottom) Example phenotype and isotype of NP-specific B cells.

Figure 10: Construction of receptor binding domain of SARS-COV-2 spike protein tetramer. A) ratio of biotinylated protein to total protein determined by gel electrophoresis as described in Figure 1D. B) RBD-PE enrichment from naïve mice (left) and mice immunized with RBD+Alum (right) RBD-PE-binding cells in unenriched, flow through and enriched fractions. (Bottom) Example phenotype and isotype of RBD-specific B cells.

Figure 7

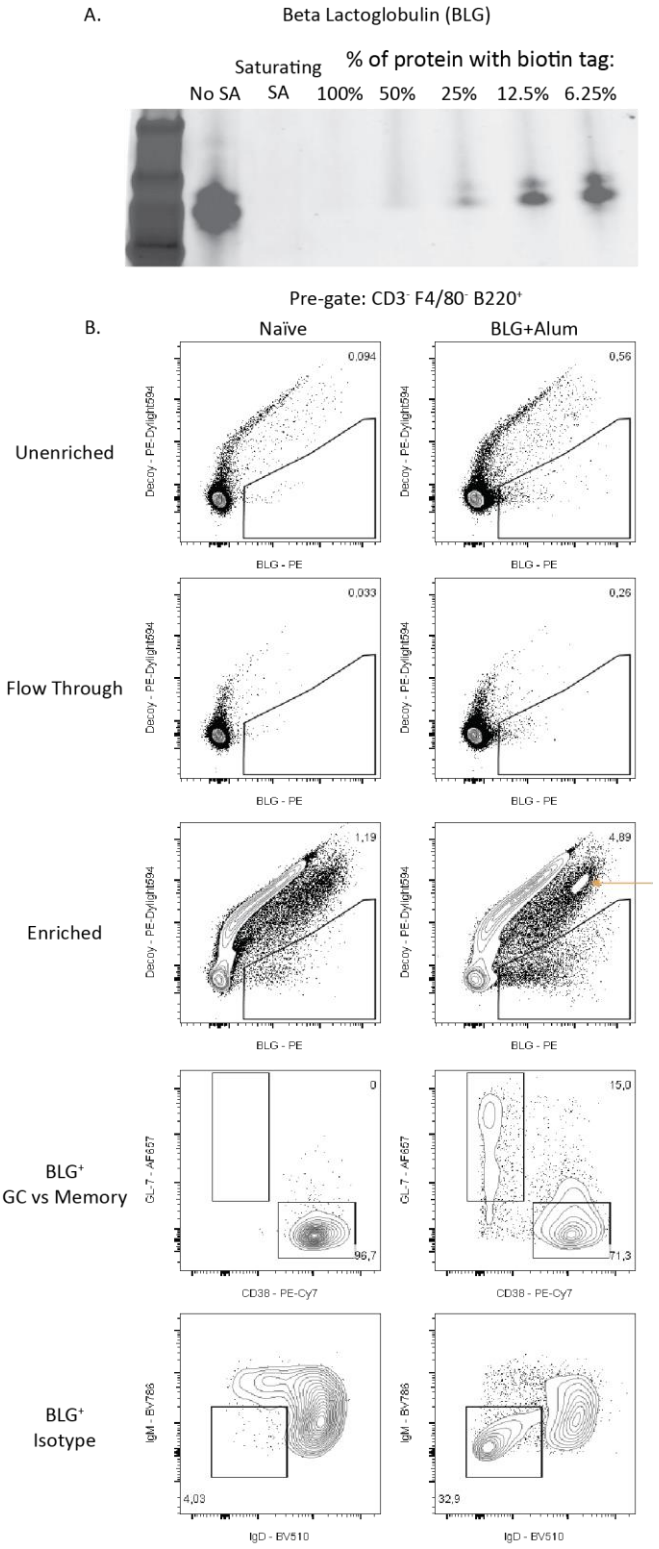

A.
Beta Lactoglobulin (BLG)
Saturating
% of protein with biotin tag:
No SA
SA
100%
50%
25%
12.5%
6.25%
B.
Pre-gate: CD3⁻ F4/80⁻ B220⁺
Naïve
BLG+Alum
Unenriched
Flow Through
Enriched
BLG⁺ GC vs Memory
BLG⁺ Isotype
Decoy - PE-Dylight594
BLG - PE
GL-7 - AF647
CD38 - PE-Cy7
IgM - Bv786
IgD - BV510
0,094
0,56
0,033
0,26
1,19
4,89
0
96,7
15,0
71,3
4,03
32,9

Figure 8

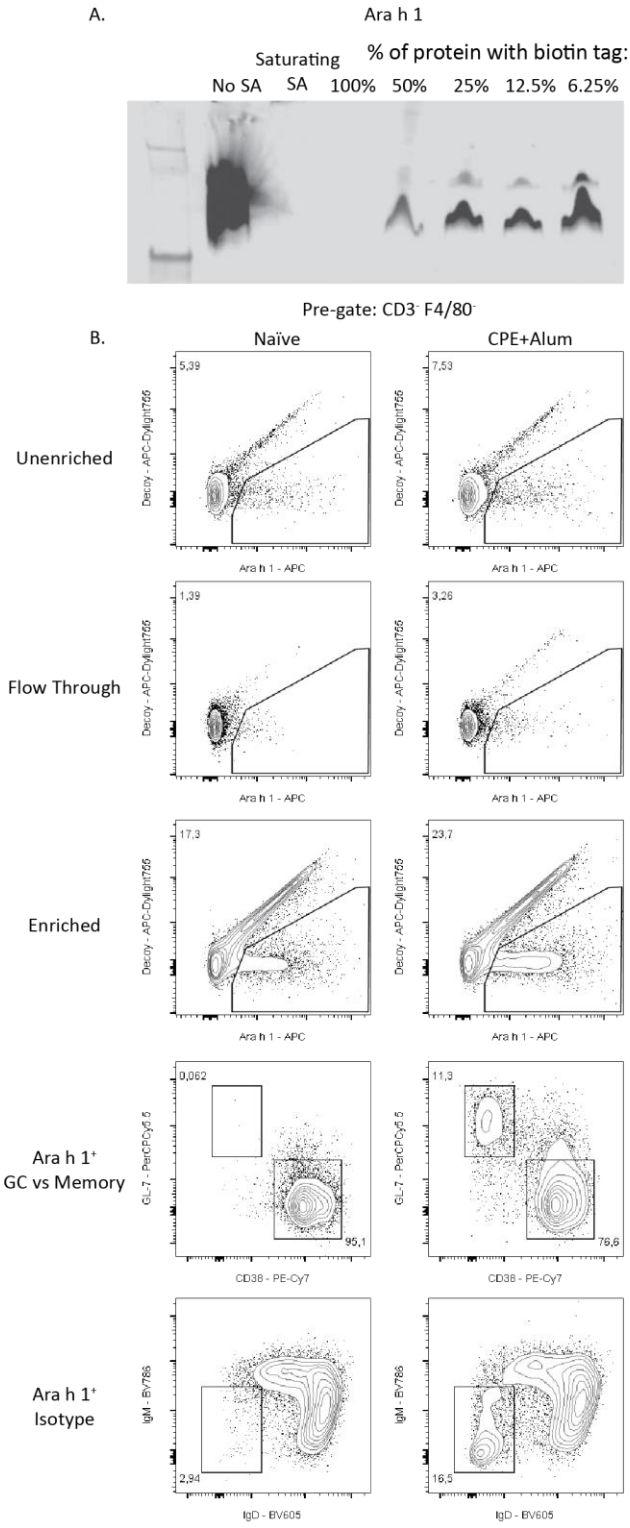

A.
Ara h 1
% of protein with biotin tag:
Saturating
No SA
SA
100%
50%
25%
12.5%
6.25%
Pre-gate: CD3- F4/80-
B.
Naïve
CPE+Alum
Unenriched
Flow Through
Enriched
Ara h 1+ GC vs Memory
Ara h 1+ Isotype
5,39
7,53
1,39
3,26
17,3
23,7
0,062
95,1
11,3
76,6
2,94
16,5
Decoy - APC-Dylight755
Ara h 1 - APC
GL-7 - PerCPCy5.5
CD38 - PE-Cy7
IgM - BV786
IgD - BV605

Figure 9

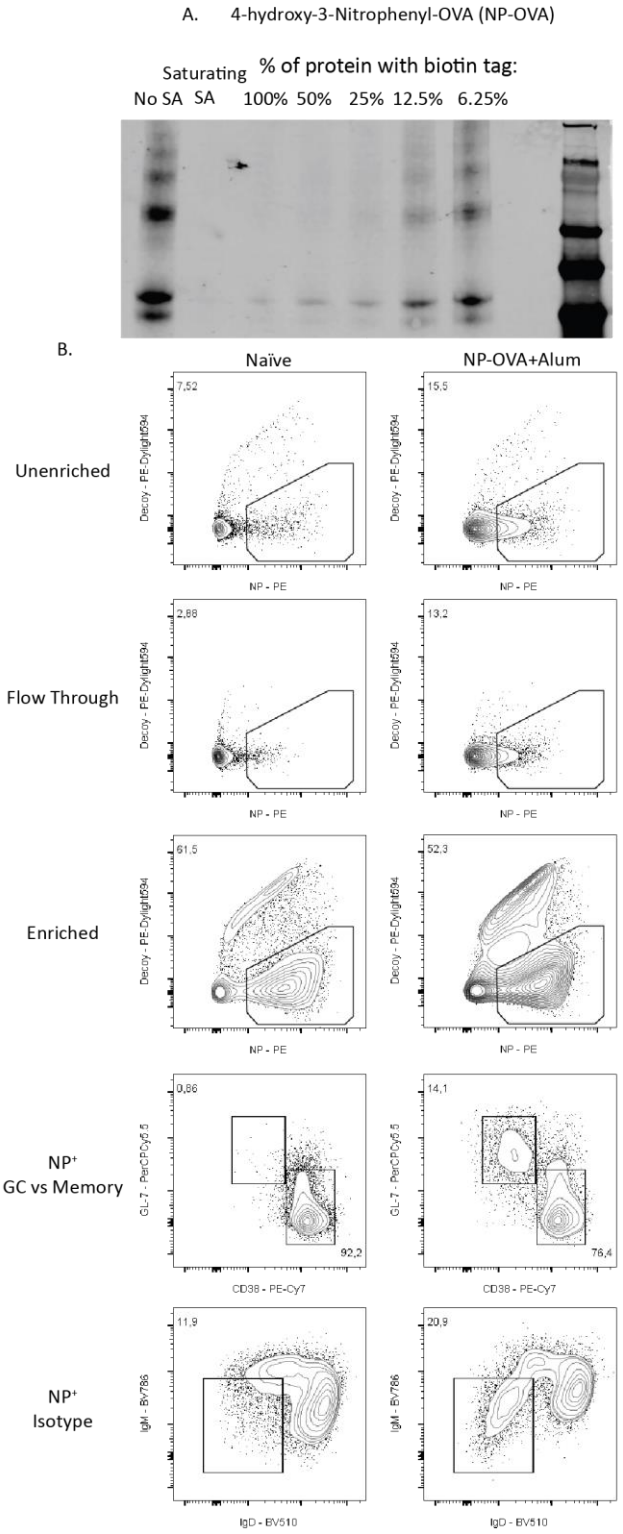
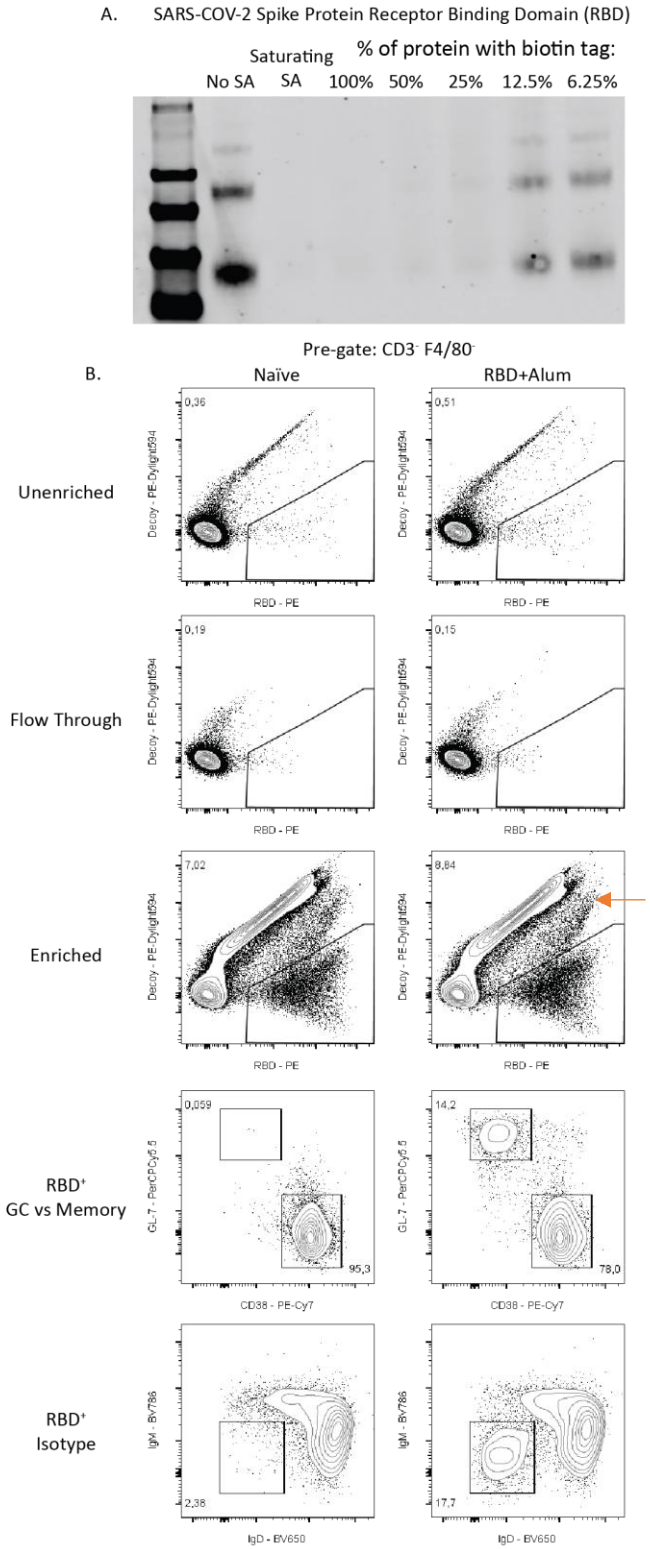

A. 4-hydroxy-3-Nitrophenyl-OVA (NP-OVA)
Saturating
No SA SA
% of protein with biotin tag:
100% 50% 25% 12.5% 6.25%
B.
Naïve
NP-OVA+Alum
Unenriched
Flow Through
Enriched
NP+ GC vs Memory
NP+ Isotype
7,52
15,5
2,88
13,2
61,5
52,3
0,86
14,1
92,2
76,4
11,9
20,9
Decoy - PE-Dylight594
NP - PE
GL-7 - PerCPCy5.5
CD38 - PE-Cy7
IgM - BV786
IgD - BV510

Figure 10


A.
SARS-COV-2 Spike Protein Receptor Binding Domain (RBD)
Saturating
% of protein with biotin tag:
No SA
SA
100%
50%
25%
12.5%
6.25%
B.
Pre-gate: CD3- F4/80-
Naïve
RBD+Alum
Unenriched
Flow Through
Enriched
RBD+
GC vs Memory
RBD+
Isotype
Decoy - PE-Dylight594
RBD - PE
GL-7 - PerCPCy5.5
CD38 - PE-Cy7
IgM - BV786
IgD - BV650
0,36
0,51
0,19
0,15
7,02
8,84
0,059
95,3
14,2
78,0
2,38
17,7

Discussion

Understanding B cell biology requires tools to study rare antigen-specific B cells with sufficient resolution to detect even rarer sub-phenotypes. This need is amplified when studying IgE responses in food allergy due to the rare and transient nature of IgE-expressing cells. To this end, we have thoroughly validated a methodology to analyze the allergen-specific repertoire with a resolution that is unparalleled in allergy research. We have validated that the methodology enriches all populations of interest to food allergy, including IgE-expressing GC B cells and PCs. Indeed, previous experiments have run up to 10 million events per sample, and not achieved the number of allergen-specific IgE^+ cells in the 1 million cell samplings that we have demonstrated in the enriched fractions above (Figure 4F).

Limitations in detecting IgE B cells resulted in the incorrect conclusion that IgE-expressing cells are not present within GCs[154]. Sensitive methods to study these cells, such as the Verigem IgE reporter animals used here, have since proven the existence of IgE^+ GC B cells,[41,126] which are visualized at high resolution using the presented antigen-enrichment platform. Since then, our group and others have determined that IgE-expressing MBCs do not exist. The present work provides the strongest evidence yet of this notion, as even when enriching allergen specific B cells from >200 million spleen and lymph node cells, IgE^+ $CD38^+$ cells were undetectable (Figure 4F).

We demonstrated that OVA-tetramer enrichments were superior to staining with OVA-monomers when studying rare populations, as we have done

previously[18]. Specifically, our monomer staining approach yielded many antigen-specific cells from naïve mice that were class switched, indicating that they were not, in fact, naïve B cells (Figure 1B). Most studies which use monomer staining do not include a decoy molecule to exclude B cells which bind to the fluorescent molecule or linkers attached to the antigen during conjugation. These epitopes are likely the target of the contaminant switched B cells that are detected, as exclusion of these populations even prior to enrichment eliminated switched cells from naïve mice (Figure 1H).

In the later chapters of this thesis, we have used both monomer and tetramer staining to study antigen specific B cell responses. The validation in this chapter allowed us to study antigen specific B cells from the small intestine to study tissue local immunity, and in the bone marrow to study resident long-lived PC populations (Figure 5). Further, the observations that adoptively transferred cells from OVA-enrichment yielded IgE following adoptive transfer has unveiled avenues to study a polyclonal B cell repertoire in transfer experiments rather than using monoclonal cells from BCR transgenic animals (Figure 6). The antigen enrichment platform validated here should be used to study allergen-specific B cells from allergic patients, to study the localization and mechanisms of B cell activation in models of food allergy, and the kinetics and mechanisms of memory IgE responses.

Acknowledgement:

I directly performed >90% of the work in this chapter. The tetramer enrichment platform was established by me and Allyssa Phelps, with support from Dr. Justin Taylor (Fred Hutchinson Cancer Research Center). The Ara h 1 tetramer data in Figure 8 was generated by Allyssa Phelps. The small intestine enrichment data generated in Figure 5A was generated by Emily Grydziuszko.

CHAPTER 4

THE COMPETITIVE ADVANTAGE OF IgG1$^+$ CELLS EXPLAINS THE NON-CRITICAL ROLE OF SEQUENTIAL SWITCHING IN IgE RESPONSES AGAINST FOOD ALLERGENS.

Introduction

In order to produce secreted IgE, antigen-activated B cells must switch the constant region of their BCR isotype from IgM to IgE. Instructions to undergo isotype switching are given by Tfh and are thought to occur at the T/B border in lymph nodes prior to B cells differentiating extrafollicularly or entering a GC[61,62,125]. Tfh cells provide co-stimulation to antigen-activated B cells through CD40L, which causes the NFκB-dependent upregulation of AID that is required for isotype switching[155,156]. Tfh-derived cytokines drive B cells to transcribe the switch region that precedes the target isotype[93]. Switch region transcription improves chromatin accessibility for AID, which induces double stranded breaks in both the IgM switch region and the target switch region. The double stranded breaks in the IgM switch region recombine with those in the switch region of the target subclass, excising the intervening isotype constant regions as circular DNA. Tfh-derived IL-4 causes the transcription of both IgG1 and IgE switch regions, thereby inducing both IgG1 and IgE isotype switching[96,157,158].

Isotype switching to IgE occurs either directly from IgM to IgE, or sequentially through and intermediate isotype[94–99]. In mice, the only reported intermediate is IgG1, which is consistent with their shared induction by IL-4[99]. In humans, multiple isotypes are clonally related to IgE, but it remains unclear whether these relationships represent direct intermediates of IgE isotype switching. Among circulating IgE^+ cells, the most common clonal relatives are $IgG1^+$ cells[100]. In the intestines, IgA1 is the most common relationship[101]. Notably, IgA1 in humans is upstream of IgE and therefore can be an intermediate. In mice, IgA is downstream of IgE, and therefore cannot contribute to sequential switching.

It is possible that sequential switching of IgE is an event that only occurs at a molecular level and has minimal impact on the cell biology of IgE responses. In favour of this hypothesis, one report demonstrated that IgG1 and IgE switch regions can recombine prior to recombination with IgM and isotype switching[159]. However, three observations suggested that sequential switching involves a first isotype switch to IgG, a cellular lifespan as an $IgG1^+$ cell, and then a second isotype switch to IgE. First, the proportion of sequentially switched IgE^+ cells increased over the course of several immunizations of antigen and alum[99]. Second, IgE-expressing cells were found to be clonal descendants of $IgG1^+$ B cells which mutated within the GC[99,126]. Lastly, adoptively transferred $IgG1^+$ MBCs yielded IgE^+ PCs during a recall response[127]. These data suggest that there is a biological relevance for sequential switching in IgE responses.

Using a model of repeated hapten immunization using Alum as an adjuvant, Xiong *et al.* (2012) demonstrated that sequential switching was required to produce high affinity anti-hapten IgE antibodies[99]. These data suggest that antigen-activated B cells isotype switch to IgG1, enter the GC to affinity mature, and switch to IgE following affinity maturation. This model is consistent with the observation that IgE^+ GC B cells represent only a small fraction of GC B cells, and rapidly decline in number over the course of an immune response[41]. IgE^+ GC B cells are mostly localized within the dark zone of the GC, rather than undergoing positive selection in the light zone, and are prone to apoptosis[126]. Further, antigen-independent signalling of the IgE-BCR pushes IgE^+ cells towards PC differentiation, limiting their persistence as GC B cells[106]. The importance of sequential switching during primary IgE responses against complex antigens, like food allergens, has not yet been assessed.

Sequential switching of $IgG1^+$ MBCs to IgE^+ PCs is thought to be the primary pathway of IgE memory. A number of reports in both mice and humans have demonstrated that IgE^+ MBCs do not exist or are so rare that they are likely not relevant contributors to secondary IgE responses[41,160]. This has led to the conclusion that MBCs of another isotype hold the memory of IgE responses. Secondary IgE is found to overlap significantly with the IgG repertoire in both humans and mice, strongly suggesting that $IgG1^+$ MBCs are the reservoir of IgE memory[161]. However, there has not yet been a direct evaluation of whether $IgG1^+$ MBCs are critically

required to hold IgE memory or whether direct switching of IgM memory also contributes to secondary IgE.

In this chapter, I assessed the role of direct and sequential isotype switching to IgE in the context of allergic sensitization and memory to food allergens. In contrast to previous reports, sequential switching was not required for the clinical manifestations of food allergy. IgE derived from sequential switching had high polyclonal affinity for allergen and induced systemic anaphylaxis upon challenge. Direct switching maintained the memory of IgE responses for greater than 1 year, identifying IgM^+ MBCs as an additional layer of IgE memory. We observed that $IgG1^+$ cells were dominant among allergen-specific cells, explaining why sequential switching is the primary pathway of IgE generation, despite its lack of critical importance.

Results

To address whether sequential switching was required for food allergic sensitization, we acquired IgG1-deficient (IgG1-def) mice which have been previously used to study sequential switching[99]. These mice have a replacement of the IL-4-responsive promoter upstream of the IgG1 switch region with a human metallothionein II promoter[162]. The resultant mouse is incapable of IgG1 isotype switching at a molecular level, and therefore do not have detectable serum IgG1, $IgG1^+$ B cells nor $IgG1^+$ PCs. These mice are reported to have intact IgE production

which is derived exclusively from direct switching[99]. IgG1-def mice are therefore a functional knockout of sequential switching.

Direct switching generates anaphylactic IgE against food allergens.

We sensitized IgG1-def mice and WT C57Bl/6 controls using a well-characterized model of intragastric sensitization to foods. Mice were administered four intragastric exposures of allergen (OVA or PN) and the type 2 adjuvant CT. In WT mice, this administration protocol generates allergen-specific IgG1 and IgE[18]. Upon intraperitoneal challenge with allergen, sensitized mice experience a largely IgE-mediated systemic shock as measured by drop in core body temperature over 40 minutes and an increase in blood concentration indicative of vascular leakage. Furthermore, these mice exhibit clinical signs of anaphylaxis which range from scratching, to reduced activity, to seizure and death (Figure 12F).

IgG1-def mice had an expected lack of serum allergen-specific IgG1 following sensitization with either OVA or PN (Figure 11A, Figure 12A), and generated fewer allergen-specific Igs of any isotype (Figure 11B). However, the absence of sequential switching did not impact the production of allergen-specific IgE (Figure 11C, 12B), consistent with previous reports that direct switching is sufficient to maintain IgE levels[99]. Upon allergen challenge, IgG1-def and WT mice experienced a similar drop in core body temperature, increase in hematocrit, and severity of observed signs of anaphylaxis (Figure 11D-F, Figure 12C-E), demonstrating that direct switching is sufficient for the clinical manifestations of food allergy.

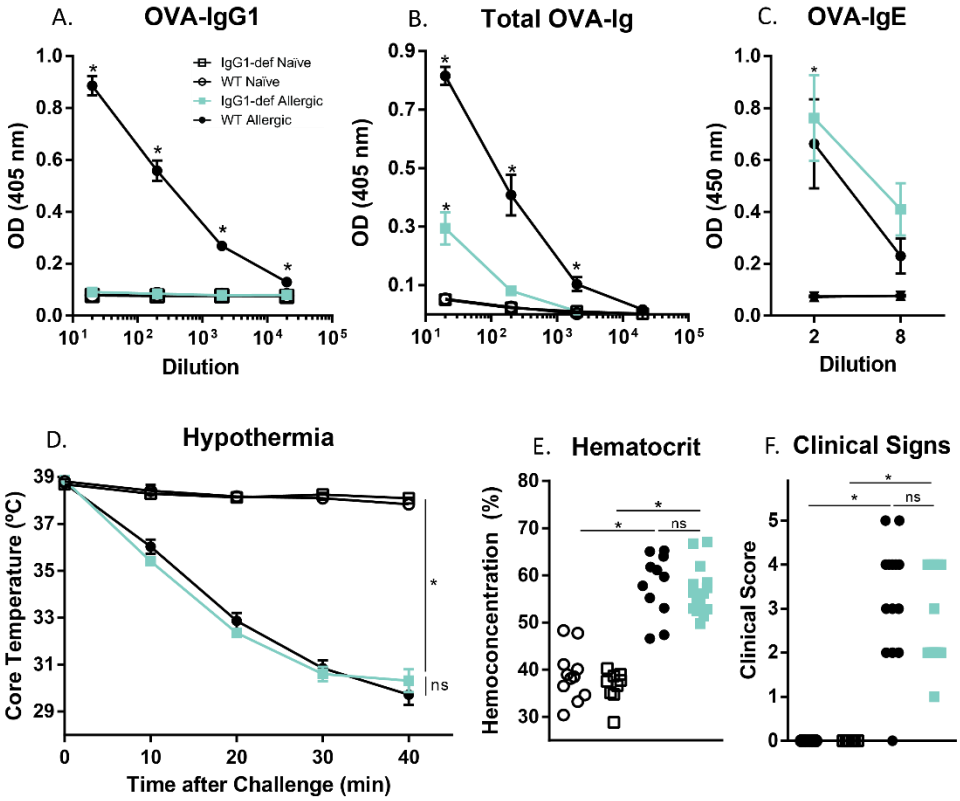
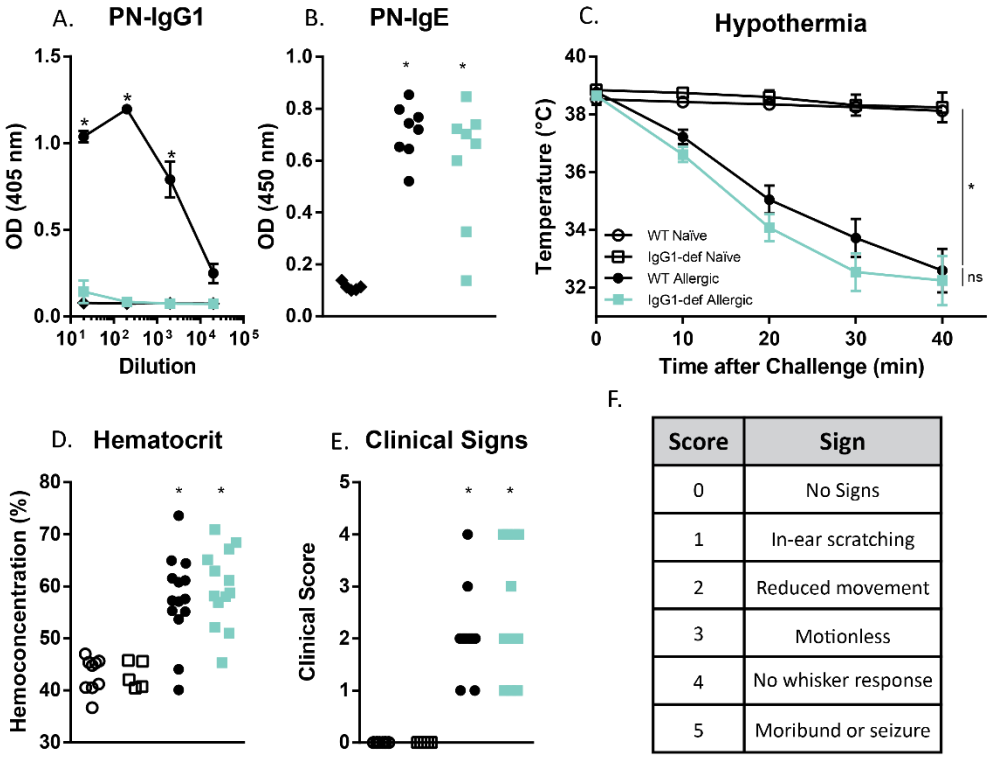


Figure 11: Direct switching is sufficient for the clinical manifestations of food allergy. IgG1-def and WT mice were sensitized with 4 intragastric exposures of OVA+CT and intraperitoneally challenged 2 weeks later with OVA. A-C) Evaluation of OVA-specific immunoglobulins by ELISA. A) IgG1, B) Total immunogloblin, C) IgE. D-F) Clinical assessment of anaphylaxis. D) Rectal measurement of core body temperature at 10-minute intervals over 40 minutes following challenge. E) Blood concentration at 40 minutes. F) Clinical signs of anaphylaxis measured over 40 minutes. N=12-15 per group. * $p<0.05$ measured by one- or two-way ANOVA. Unless specified, indicated statistical significance is compared to naïve.



Score	Sign
0	No Signs
1	In-ear scratching
2	Reduced movement
3	Motionless
4	No whisker response
5	Moribund or seizure

Figure 12: Direct switching is sufficient for the clinical manifestations of food allergy. IgG1-def and WT mice were sensitized with 4 intragastric exposures of PN+CT and intraperitoneally challenged 2 weeks later with PN. A-B) Evaluation of PN-specific immunoglobulins by ELISA. A) IgG1 B) IgE. C-F) Clinical assessment of anaphylaxis. C) Rectal measurement of core body temperature at 10-minute intervals over 40 minutes following challenge. D) Blood concentration at 40 minutes. E) Clinical signs of anaphylaxis measured over 40 minutes. F) Sign associated with each clinical score. N=12-15 per group. * $p<0.05$ measured by one- or two-way ANOVA. Unless specified, indicated statistical significance is compared to naïve.

Anaphylaxis in mice can be mediated either by cross-linking IgE bound through FcεRI to the surface of mast cells and basophils, a pathway known as the "classical pathway" or by IgG-allergen immune complexes which ligate FcγRII/III on the surface of macrophages, known as the "alternative pathway"[163]. We evaluated whether the anaphylaxis experienced by IgG1-def mice was mediated by IgE by blocking alternative anaphylaxis using an anti-FcγRII/III antibody. FcγRII/III-

blockade administered 1 day prior to challenge caused a ~45% decrease in temperature in WT mice at 40 minutes (Figure 13A), consistent with our previous report that the alternative pathway partially contributes to anaphylaxis in intragastrically sensitized mice[164]. IgG1-def mice were not significantly protected from anaphylaxis when treated with anti-FcγRII/III (Figure 13B-D). Taken together, these data demonstrate that direct switching generates anaphylaxis inducing IgE antibodies against food allergens.

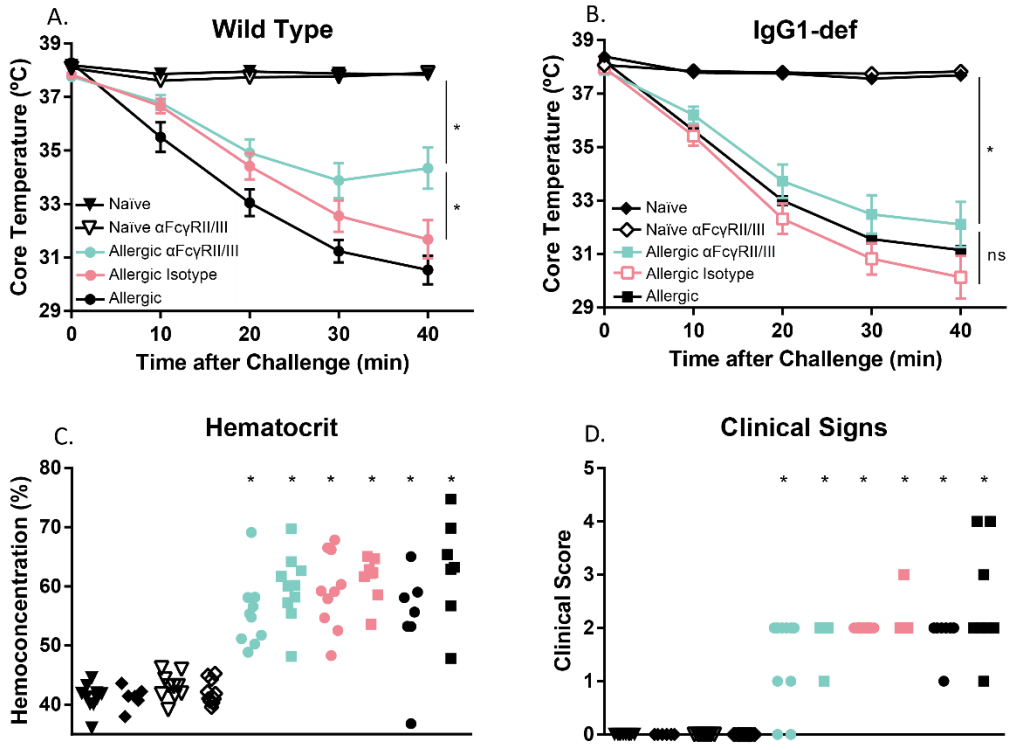


Figure 13: Direct switching is sufficient for anaphylactic IgE production. Sensitized IgG1-def and WT mice were treated with anti-FcγRII/III i.p. 24 hours prior to challenge. A-B) Rectal measurement of core body temperature, C) blood concentration at 40 minutes after challenge, D) clinical signs of anaphylaxis. N=7-10, per group. * $p<0.05$ measured by one- or two-way ANOVA. Unless specified, indicated statistical significance is compared to naïve.

The role of sequential switching does not depend on route of allergic sensitization.

Our observation that intragastrically sensitized IgG1-def mice generated anaphylactic IgE antibodies by direct switching contrasts with a previous report that sequential switching was required for high affinity IgE production in the context of systemic sensitization with the hapten antigen NP[99]. We questioned whether intragastric sensitization preferentially utilized direct switching to generate anaphylactic IgE, while systemic sensitization required sequential switching. We therefore intragastrically sensitized IgG1-def mice and WT controls using the hapten NP conjugated to OVA (NP-OVA) to determine if anaphylactic anti-NP IgE would be produced. We observed a similar absence of NP-IgG1, decrease in total NP-specific antibodies, and equal production of NP-IgE in IgG1-def mice compared to WT mice (Figure 14A-C) as was observed when mice were sensitized with PN or OVA (Figure 11, 12). We evaluated the affinity of NP-IgE by taking a ratio of binding to lowly conjugated (NP_8) vs highly conjugated (NP_{30}) NP-BSA[165,166]. The affinity of NP-IgE generated by intragastrically sensitized IgG1-def mice had a significantly lower ratio of NP8:NP30 binding than NP-IgE generated by WT mice (Figure 14D), consistent with observations of reduced NP-IgE affinity in IgG1-def mice following systemic immunization[99]. Upon challenge, IgG1-def mice were partially protected from hypothermia and vascular leakage and exhibited less severe signs of anaphylaxis compared to WT mice (Figure 14E-G). Sequential switching is, therefore, required for high-affinity NP-IgE and severe NP-

mediated anaphylaxis. The difference in route of antigen administration does not explain the sufficiency of direct switching to generate anaphylactic IgE against food allergens.

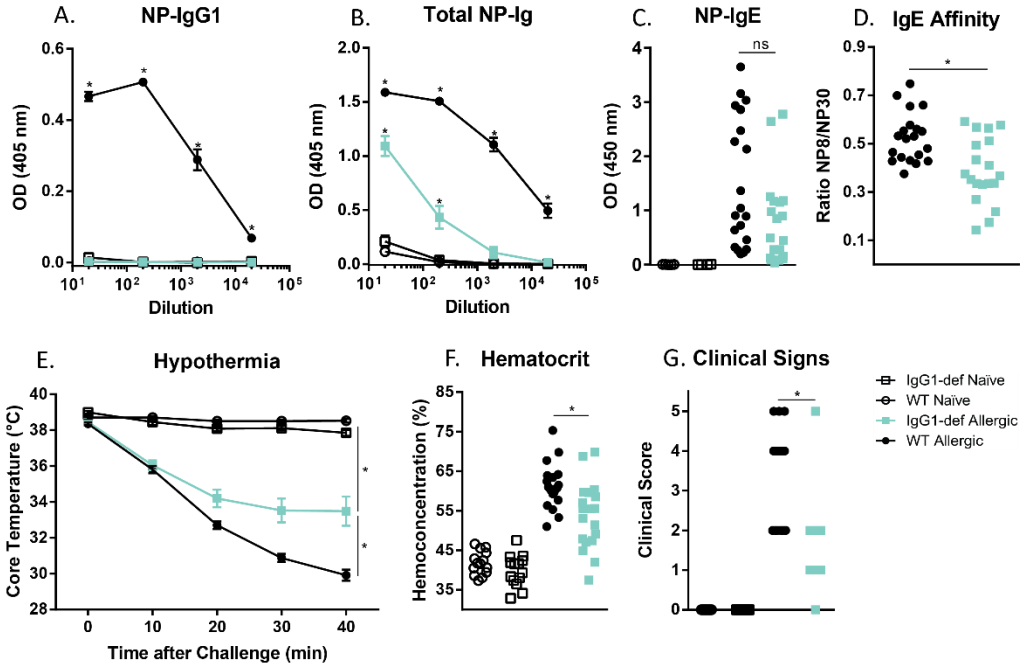


Figure 14: Impaired NP-IgE affinity and function in the absence of sequential switching. IgG1-def and WT mice were sensitized with NP_{19}OVA+CT and challenged two weeks later with NP_{30}BSA. A-D) Analysis of serum immunoglobulin production by ELISA. A) NP-IgG1, B) total NP-specific immunoglobulin, C) NP-IgE. D) Affinity of NP-IgE measured as a ratio of OD from NP_8/NP_{30}. E-G) Clinical assessment of anaphylaxis. E) Rectal measurement of core body temperature, C) blood concentration at 40 minutes after challenge, D) clinical signs of anaphylaxis. n=15-20 per group. * $p<0.05$ measured by one- or two-way ANOVA. Unless specified, indicated statistical significance is compared to naïve.

Direct and sequential switching generate IgE of similar functional affinity against food allergens.

We next assessed the affinity of food allergen-specific IgE derived from direct switching compared to sequential switching. First, we sought methods to address

functional differences between IgE generated in the presence or absence of sequential switching. We reasoned that high affinity antibodies would be required to elicit potent clinical reactivity to low doses of allergen challenge. Indeed, decreasing the dose of allergen challenge from 2.5 mg (Figure 11D) to 0.25 mg (Figure 15A-D) resulted in a dose dependent decline in reactivity in WT mice. Across all doses of challenge, IgG1-def and WT mice experienced similar clinical reactivity (Figure 11D, 15A-D). Furthermore, rat basophil leukemia (RBL) cells, which are a surrogate for mast cells and basophils, degranulated to a similar extent when sensitized with either IgG1-def or WT serum and challenged with allergen (Figure 15E). Importantly, when the serum was heat-inactivated at 56°C prior to sensitization, RBL cells did not degranulate when sensitized with either IgG1-def or WT serum. IgE is uniquely inactivated at 56°C due to a heat-labile domain which is not present on other isotypes of antibody, and therefore the reactivity seen in this system was IgE-mediated[167]. Lastly, we directly compared the allergen-binding capacity of allergen-specific IgE from IgG1-def mice and WT mice by eluting the allergen-IgE bond using the chaotrope thiocyanate in an allergen-specific IgE ELISA. IgE derived in the presence or absence of sequential switching withstood elution to a similar extent across all concentrations of thiocyanate (Figure 15F). Taken together, these data demonstrate that sequential and direct switching result in IgE of similar polyclonal affinity for food allergens.

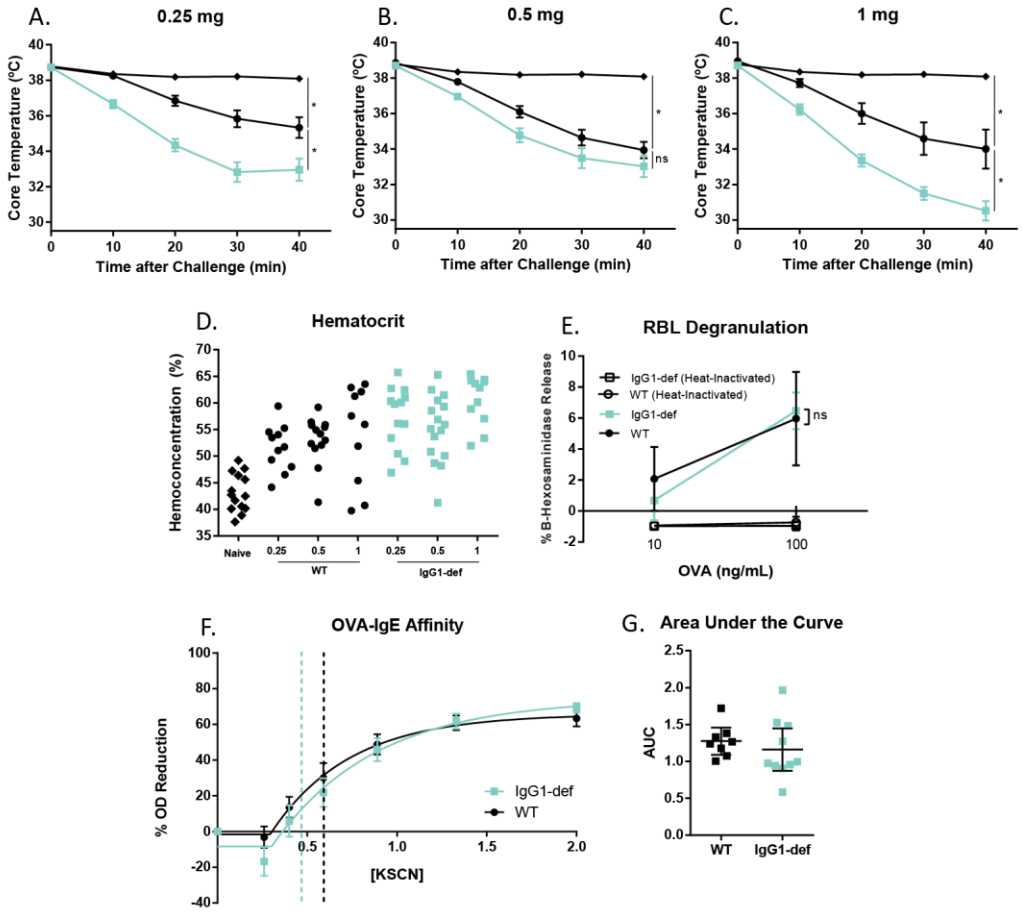


Figure 15: Direct and sequential switching produce IgE of similar affinity for complex allergens. A-D) Clinical evaluation of PN-sensitized IgG1-def mice challenged with varying doses of PN. A-C) Rectal measurement of core body temperature, D) blood concentration at 40 minutes after challenge. n= 10-15 per group. E) β-Hexosaminidase release from rat basophil leukemia cells sensitized with serum from OVA-allergic IgG1-def or WT mice following stimulation with OVA. IgE was denatured by heat-inactivation at 56°C. F) OVA-IgE affinity measurement by thiocyanate elution during ELISA. G) Quantification of area under the elution curve for each biological replicate. * $p<0.05$ measured by one- or two-way ANOVA. Unless specified, indicated statistical significance is compared to naïve.

Compensation between isotypes in the germinal center.

B cells acquire high affinity mutations in the GC, where iterative cycles between mutation in the dark zone and positive selection of high affinity clones in the light zone result in improved BCR affinity[168]. To understand the maturation of the antibody repertoire in the presence and absence of sequential switching, we analyzed the OVA-specific GC B cell repertoire in the mesenteric lymph nodes 6 days post-sensitization, which is the previously reported peak of GC activity in this model[18]. $CD19^+$ $GL\text{-}7^+$ $CD95^+$ B cells were probed for antigen specificity using monomeric OVA-FITC (Figure 1A). IgE^+ GC B cells were identified by first blocking IgE bound to Fc receptors with an unlabeled antibody and then staining intracellularly with a fluorescently-labeled antibody of the same clone, as previously reported[41]. IgG1-def mice had a lower proportion and number of OVA^+ GC B cells (Figure 16B). $IgG1^+$ cells were dominant within the GC; OVA^+ $IgG1^+$ GC B cells were present at a 10-times greater frequency than OVA^+ IgM^+ GC B cells in WT mice (Figure 16C-D). Sensitized IgG1-def mice had an increased frequency of OVA^+ IgM^+ and OVA^+ IgE^+ GC B cells compared to WT mice. This translated to IgG1-def mice having a significantly higher number of IgM^+ and IgE^+ OVA^+ GC B cells compared to WT mice, despite the difference in total OVA^+ GC B cells (Figure 16E-F). These data are consistent with a model where IgM^+ and IgE^+ GC B cells are outcompeted by $IgG1^+$ GC B cells, but in their absence are similarly capable of affinity maturation to yield high-affinity IgE.

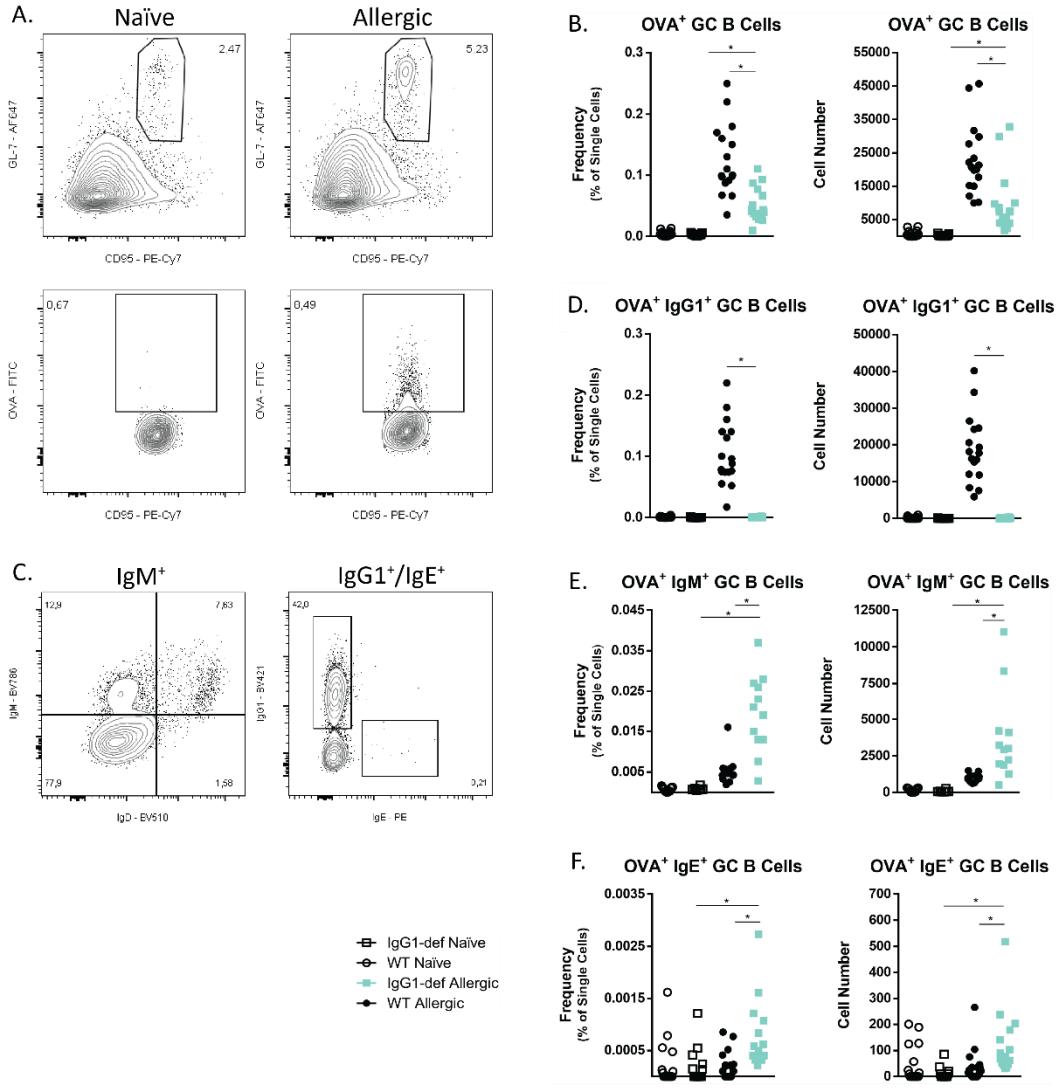


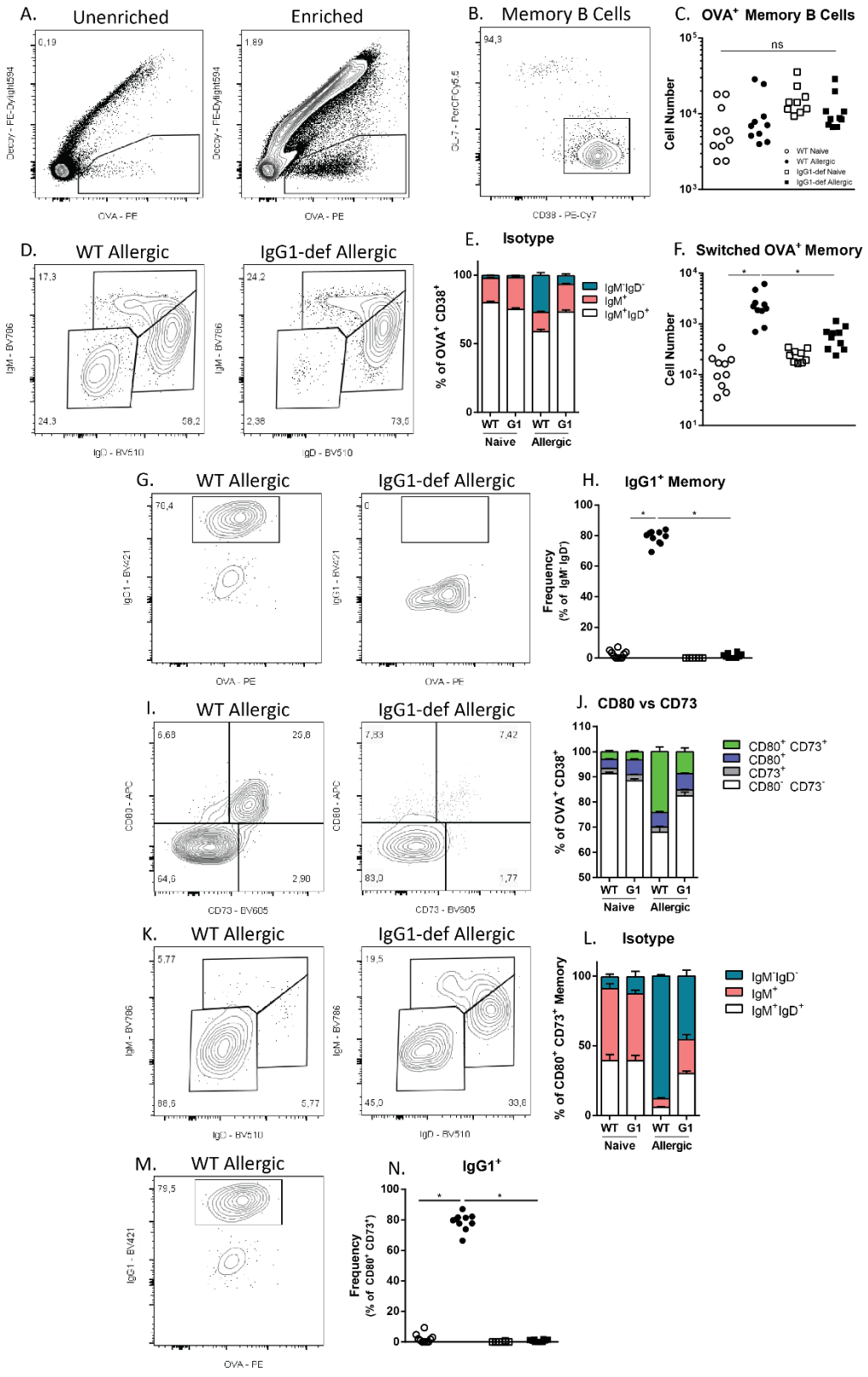
Figure 16: Compensation within the GC in the absence of IgG1. IgG1-def and WT mice were sensitized with OVA+CT and GC activity was assessed in the mesenteric lymph nodes at day 6 by flow cytometry. A) Representative plots of GC B cells (GL-7$^+$ CD95$^+$) and OVA-specificity among GC B cells in WT mice. B) Frequency and number of OVA$^+$ GC B cells. C) Isotype staining of GC B cells. D-F) Frequency and number of D) IgG1$^+$ GC B cells, D) IgM$^+$ GC B cells, F) IgE$^+$ GC B cells. N=12-15 per group. * P<0.05 one-way ANOVA.

Direct switching is sufficient to hold the memory of IgE responses.

Sequential switching of IgG1$^+$ MBCs to IgE during a memory response has been previously reported[127], though it remains unknown whether this mechanism is required for secondary IgE responses. We first analyzed the OVA$^+$ MBC repertoire 1 month following the last OVA+CT intragastric sensitization to observe the mature MBC repertoire in the presence or absence of IgG1. We enriched for antigen-specific B cells using OVA-PE tetramers to greatly increase the number of cells available for analysis (Chapter 3, Figure 17A). MBCs were gated based on expression of CD38 and lack of expression of the GC B cell marker GL-7 (Figure 17B). There were no significant differences between allergic and naïve mice in terms of the number of OVA$^+$ CD38$^+$ B cells, indicating that at 1-month post-sensitization the MBC repertoire had contracted back to baseline (Figure 17C). IgG1-def mice had a lower proportion and number of switched MBCs compared to WT mice which was compensated for by a slight increase in the frequency of IgM$^+$ MBCs (Figure 17D-F). In WT mice, the switched MBC compartment was dominated by IgG1$^+$ MBCs (~ 80%) consistent with a principal role for these cells in secondary responses (Figure 17G-H). As CD80$^+$ CD73$^+$ MBCs have been previously reported to be rapid responders which quickly differentiate into IgE$^+$ PCs[127], we examined CD80 and CD73 expression on OVA$^+$ MBCs in IgG1-def and WT mice. IgG1-def mice had a >50% reduction in the frequency of CD80$^+$ CD73$^+$ compared to WT mice (Figure 17I-J). The majority of MBCs in IgG1-def mice were CD80$^-$ CD73$^-$, an immature phenotype associated with poor secondary antibody

secretion (Figure 17I-J). In WT mice, ~90% of $CD80^+$ $CD73^+$ MBCs were class switched, most of which expressed IgG1(Figure 17K-N). Among the remaining $CD80^+$ $CD73^+$ MBCs in IgG1-def mice, IgM^+ and IgM^+IgD^+ MBCs made up a much greater proportion (~ 50% vs ~10%) of mature MBCs, virtually equal to the number of switched MBCs (Figure 17K-L).

Figure 17: Compensation of memory B cell maturity in the absence of IgG1. IgG1-def and WT mice were sensitized with OVA+CT. OVA-specific MBCs were detected using OVA-tetramer enrichment from the spleen and mesenteric lymph nodes at 1-month post-sensitization. A) Representative plot of OVA^+ $Decoy^-$ cells in the unenriched and enriched fractions. B) Gating for MBCs ($CD38^+$ $GL\text{-}7^-$) from OVA^+ B cells. C) Quantification of the number of OVA^+ MBCs. D) Representative plots of the OVA^+ MBC isotype. E-F) Quantification of E) proportion of isotypes of OVA^+ MBCs and F) number of switched OVA^+ MBCs. G-H) Representative plots and quantification of $IgG1^+$ frequency among switched OVA^+ MBCs. I-J) Evaluation of MBC maturity based on CD80 and CD73 expression. I) Representative plots, J) quantification of $CD80^{+/-}$ $CD73^{+/-}$ populations among OVA^+ MBCs. K-L) Evaluation of mature ($CD80^+$ $CD73^+$) MBC isotype. K) representative plots, L) quantification of $IgM^{+/-}$ $IgD^{+/-}$ populations among OVA^+ $CD80^+$ $CD37^+$ MBCs. K) representative plot of IgG1-expression among OVA^+ $CD80^+$ $CD73^+$ MBCs L) quantification of frequency of $IgG1^+$ among OVA^+ $CD80^+$ $CD73^+$ MBCs. *(Figure on next page)*


A.
Unenriched
Enriched
0,19
1,89
Decoy - FE-Dylight594
OVA - PE
B.
Memory B Cells
94,3
CD38 - PE-Cy7
C.
OVA+ Memory B Cells
ns
Cell Number
WT Naive
WT Allergic
IgG1-def Naive
IgG1-def Allergic
D.
WT Allergic
IgG1-def Allergic
17,3
24,3
58,2
24,2
2,36
73,5
IgM - BV786
IgD - BV510
E.
Isotype
% of OVA+ CD38+
IgM-IgD-
IgM+
IgM+IgD+
WT G1 WT G1
Naive Allergic
F.
Switched OVA+ Memory
Cell Number
G.
WT Allergic
IgG1-def Allergic
76,4
0
IgG1 - BV421
OVA - PE
H.
IgG1+ Memory
Frequency
(% of IgM- IgD-)
I.
WT Allergic
IgG1-def Allergic
6,69
26,8
64,6
2,90
7,83
7,42
83,0
1,77
CD80 - APC
CD73 - BV605
J.
CD80 vs CD73
% of OVA+ CD38+
CD80+ CD73+
CD80+
CD73+
CD80- CD73-
WT G1 WT G1
Naive Allergic
K.
WT Allergic
IgG1-def Allergic
5,77
86,6
5,77
19,5
45,0
33,6
IgM - BV786
IgD - BV510
L.
Isotype
% of CD80+ CD73+ Memory
IgM-IgD-
IgM+
IgM+IgD+
WT G1 WT G1
Naive Allergic
M.
WT Allergic
79,5
IgG1 - BV421
OVA - PE
N.
IgG1+
Frequency
(% of CD80+ CD73+)

The relative paucity of switched and mature MBCs led us to question whether IgG1-def mice could generate secondary IgE responses. We waited 10 months for OVA-specific IgE production and clinical reactivity to wane in sensitized IgG1-def and WT mice. We have previously shown that allergen-alone re-exposures at this timepoint result in a robust secondary response which replenishes allergen-specific IgE titres[18]. In IgG1-def mice, the production of allergen-specific IgE was no-longer statistically increased compared to naïve mice around 20 weeks post-sensitization, while allergen-specific IgE titres returned to baseline in most WT mice around 45 weeks post-sensitization (Figure 18A). IgG1-def and WT mice which had been re-exposed intragastrically with OVA alone produced similar amounts of secondary allergen-specific IgE, while unexposed mice experienced no change (Figure 18B). IgG1-def and WT mice which had been waited for 10 months without allergen re-exposure lost the majority of their clinical reactivity upon OVA challenge, consistent our previous report[18]. In contrast, clinical reactivity returned in both IgG1-def and WT mice following re-exposures with allergen alone as evidenced by their similarly robust decrease in core body temperature, increase in hematocrit, and the severity of their observable clinical signs (Figure 18C-E). Taken together, these data demonstrate that direct switching is sufficient to establish memory and elicit secondary IgE responses.

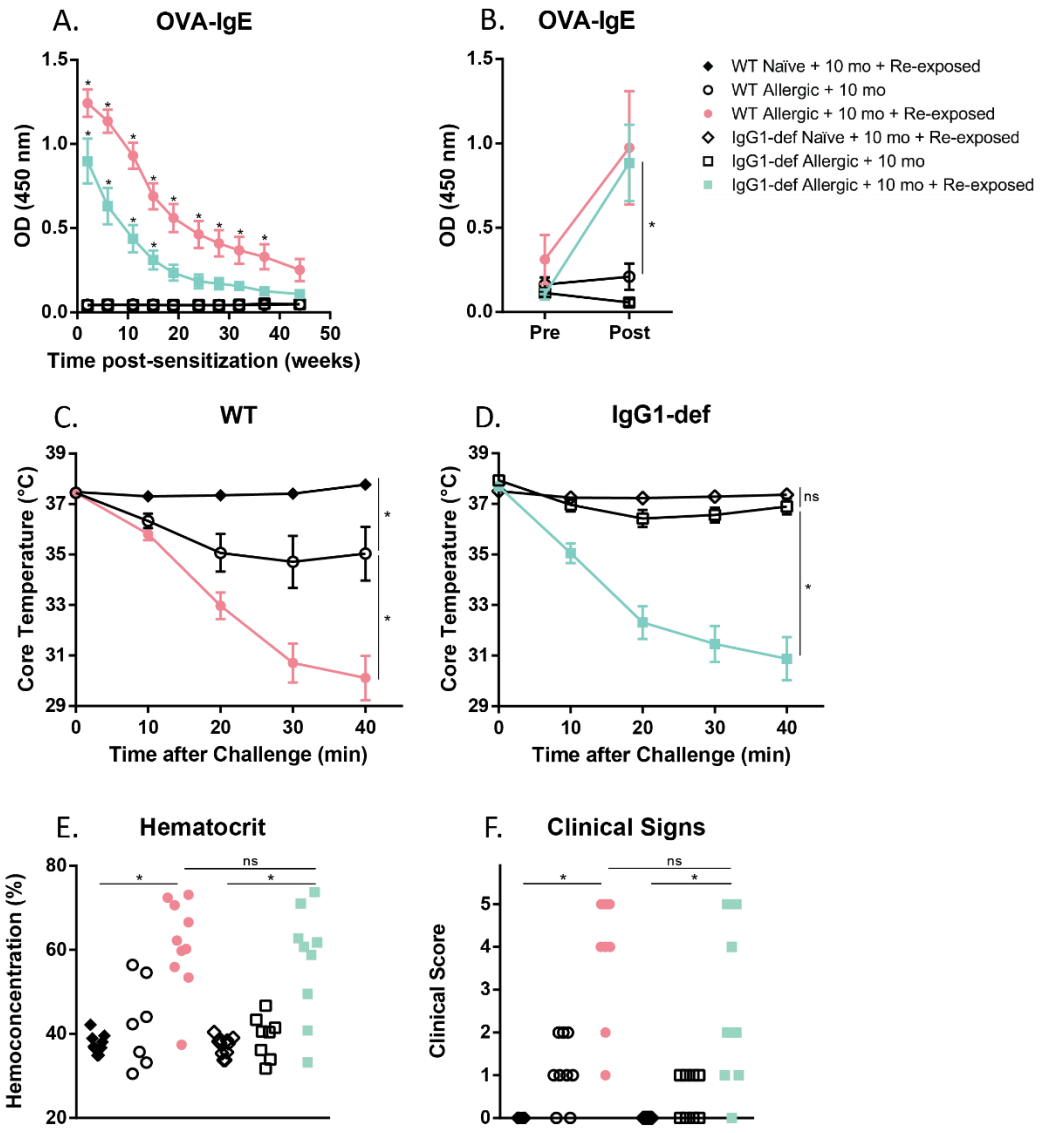


Figure 18: Direct switching is sufficient for memory IgE responses. A-B) Serum OVA-specific IgE measured by ELISA. A) Desensitization over 45 weeks, B) change in serum OVA-IgE pre-and post OVA-alone re-exposure. C-F) Clinical assessment of anaphylaxis. C-D) Rectal measurement of core body temperature, E) blood concentration at 40 minutes after challenge, F) clinical signs of anaphylaxis. n=8-10 per group. * $p<0.05$ measured by one- or two-way ANOVA. Unless specified, indicated statistical significance is compared to naïve.

Discussion

The first observations of sequential switching were made >30 years ago, but our understanding of this pathway of IgE generation has only modestly improved since. The majority of IgE-expressing cells are derived from sequential switching in numerous settings in both humans and in mice[100,169]. Considerable importance has been placed on this mechanism in the pathophysiology of allergic disease. Until the presented work, there has not yet been a direct evaluation of the requirement of sequential switching in allergic sensitization against food allergens.

We evaluated the importance of sequential switching in the formation of anaphylactic IgE using mice which cannot switch to IgG1. We used a well-established model of intragastric sensitization to food antigens to evaluate the requirement of sequential switching in both a primary and secondary immune response. Consistent with previous reports, sequential switching was not required to generate allergen-specific IgE during a primary response[99]. However, despite the reported prevalence of sequential switching, particularly in systems which provide several antigen exposures[99,126], the absence of sequential switching did not impact the clinical severity of IgE-mediated anaphylaxis. Similar observations were made using both PN and OVA (egg), indicating that IgE response against food allergens can be elicited through direct switching alone.

Our work is consistent some of the findings made by Xiong *et al.* (2012), who reported the critical importance of sequential switching for high affinity IgE production against NP[99]. We observed that IgG1-def mice had impaired NP-IgE

affinity compared to WT, but to a lesser extent than was reported. However, upon challenge with the hapten NP, IgG1-def mice were only partially protected from anaphylaxis, indicating that the impairment in NP-IgE affinity may not be clinically relevant. Further, we did not observe a similar decrease in the functional or measured affinity of IgE against food allergens. Since our data recapitulate the difference in affinity in NP-immunization, it is clear that differences in the route of sensitization do not explain the retained high affinity of allergen-specific IgE against food allergens in the absence of sequential switching. Instead, it appears that the observation by Xiong *et al.* of impairment in affinity in the absence of sequential switching may be a feature of the NP-specific response or the response against haptens[99]. Indeed, affinity maturation against complex antigens is known to be permissive of B cell clones with a wider range of affinities, prioritizing a response against multiple epitopes which ultimately has higher polyclonal affinity[142]. The intensely competitive and purifying selection observed against single epitope haptens may rely heavily on sequential switching because of our observation of dominance of IgG1 within the Th2 GC. By comparison, the multi-epitope response against complex antigens may be more permissive of lowly competitive IgM^+ and IgE^+ clones, thereby allowing them to be retained in the GC longer and ultimately result in high affinity IgE production.

The dominance of $IgG1^+$ cells is a crucial finding of this report that may explain why the primary path of IgE generation is sequential switching, despite it not being a critical path. Using a highly sensitive antigen-enrichment approach to study

allergen-specific B cells, we found that the vast majority of GC B cells and mature ($CD80^+CD73^+$) MBCs in WT mice were $IgG1^+$, while IgM^+ and IgD^+ cells made up only a minority of these two populations. In contrast, IgG-def mice had a greater number of IgM^+ and IgD^+ GC B cells, and a much larger fraction of mature MBCs were IgM^+ or IgD^+. We observed a dramatically reduced proportion of mature MBCs in IgG1-def mice, but which ultimately had no effect on the secondary, anaphylactic IgE response. On the surface, this observation is perplexing given that mature MBCs are thought to differentiate rapidly into IgE-secreting PCs and yield the majority of secondary IgE[127]. However, our observations are in line with very recent work which, together, positions interclonal competition as the defining feature of GC and MBC response rather than phenotype or isotype alone[121,124]. IgG^+ cells are reported to have a competitive advantage compared to IgM^+ cells in the GC, even when they have similar affinity for antigen[170]. We propose that this advantage allows $IgG1^+$ cells to near-exclusively populate late GCs to gain affinity enhancing mutations, which are thought to be the main source of mature MBCs[171]. During a recall response, the highest affinity B cell clones are reported to be selected into the secondary PC fate[124]. We further propose that, as $IgG1^+$ cells are so dominant in the late GC, these would be the highest affinity MBC clones which are competitively selected for entry into the secondary PC repertoire. As a result, several immunizations would result in a progressively increased proportion of sequentially switched IgE^+ PCs. In the absence of $IgG1^+$ cells, IgM^+ and IgD^+ cells are able to persist in the GC and gain greater affinity. As these are the most mature

and highest affinity MBCs, these are selected during the secondary response, yielding high affinity IgE. We therefore surmise that direct switching is not a causal mechanism with any biologically relevance *per se*, but rather that the observed prevalence of sequential switching is primarily a feature of the competitive fitness of $IgG1^+$ cells compared to IgM^+ and IgD^+ cells.

Acknowledgement

I directly performed >90% of the work in this chapter. The RBL degranulation data in Figure 15E was generated by Kelly Bruton. The thiocyanate elution data in Figure 15F-G was generated by Adam Wade-Vallance.

CHAPTER 5

EARLY MEMORY CD4$^+$ T CELLS ARE GENERATED DURING THE CLINICALLY SILENT PHASE OF FOOD ALLERGY AND HOLD THE MEMORY OF IgE RESPONSES.

Introduction

Food allergy is characterized by a dominant type 2 adaptive immune activation resulting in the production of allergen-specific IgE[172]. Following accidental allergen ingestion, crosslinking of IgE bound on the surface of mast cells and basophils causes degranulation, resulting in the signs and symptoms of an allergic reaction[164]. Allergic patients present in the clinic during their first allergic reaction to a food allergen, often during the first known exposure to the offending food allergen[46,47]. As these patients already have circulating allergen-specific IgE, the mechanisms that result in allergic sensitization occur in a clinically silent manner, and therefore are difficult to study.

B cell activation, CSR, and differentiation into antibody secreting cells require previous antigen exposure. The context of these exposures in allergic patients is poorly understood. In *Chapter 1: Introduction*, we introduced the concept of food allergy as a phasic disease. The adverse event which results in allergic sensitization, *i.e.* generation of allergen-specific IgE, likely happens in early life as multiple

contextual factors in the first year of life are associated with allergic sensitization (e.g. microbial dysbiosis, allergen avoidance, infections, skin rashes)[50–52]. In mouse models of food allergy and in birch allergic patients, allergen-specific IgG is first produced which later progresses to allergen-specific IgE production[53,54]. The progression of adaptive immune activation prior to the generation of allergen-specific IgG has not been studied.

In this Chapter, we established a novel model of incipient allergic sensitization to investigate the clinically silent nature of allergic disease. We found that a single oral administration of allergen and adjuvant results in allergic $CD4^+$ T cell memory in the absence of B cell activation and clinical reactivity to allergen. This memory was retained indefinitely and, following allergen-only re-exposures, $CD4^+$ T cells induced IgE production from naïve B cells. Our research identifies a previously unknown role for memory T cells as keepers of IgE memory and suggests that identification of early allergic T cells may be a prognostic marker of clinical allergy.

Results

A single mucosal allergen-exposure generates type 2 immune memory in the absence of humoral immunity.

A well-characterized model of allergic sensitization to food allergens involves intragastric administration of allergen in the presence of the Th2 adjuvant CT. Typically, four to six administrations result in the generation of allergen-specific IgE which induces the clinical signs of anaphylaxis following intraperitoneal

challenge with allergen[26]. Since these mice are clinically reactive, we reasoned that the observable phases of allergic sensitization would occur during the repeated administrations. Repeated administration is a common feature of most allergic models, but the immunological trajectory during these re-exposures is not well characterized. We found that a single administration of allergen+CT caused allergic sensitization, defined as the generation of allergen-specific IgE or reactivity upon challenge, in only a very small fraction of mice (<1 in 20, data not shown). For the vast majority of mice, a single allergen+CT administration did not result in the generation of allergen-specific IgG1, allergen-specific IgE, nor clinical reactivity upon allergen challenge (Figure 19B-E). We sought to understand whether any immunity was generated following the exposure, or if it was simply ignored by the immune system. We therefore opted to re-expose the mice with 3 oral gavages of allergen in the absence of adjuvant, a regimen that would typically induce oral tolerance in a naïve mouse (Figure 19A). Mice that were primed with allergen+CT and re-exposed with OVA alone produced similar amounts of allergen-specific IgG1 and IgE to allergic mice which were sensitized with 4 allergen+CT administrations (Figure 19B-C). Upon intraperitoneal challenge with allergen, these mice experienced a drastic (~9°C) drop in core body temperature, and an increase in vascular leakage (measured via blood concentration); which are classical signs of allergic anaphylaxis in mice (Figure 19D-E). Thus, our data demonstrate that a single adverse allergen exposure is not enough to sensitize mice

but is sufficient to prime allergic immunity. We will therefore refer to mice receiving a single allergen+CT administration as “primed.”

Given the long temporal gap that appears to exist between the early life events which trigger sensitization (e.g. early feeding, microbial dysbiosis) and the time of diagnosis, we sought to determine whether primed mice retained long-term allergic memory. Mice were primed with a single OVA+CT administration, and different groups were re-exposed with OVA-alone following waits of 1, 3 or 9 months (Figure 19F). Levels of allergen-specific IgE remained undetectable up to 9 months post-priming, suggesting that the immunity initiated following 1 OVA+CT was quiescent and did not mature to clinical allergy over the waiting period (Figure 19G). However, oral re-exposure with OVA alone resulted in the robust generation of allergen-specific IgE consistently in all mice, regardless of the length of wait (Figure 19G-H). Re-exposure after 1, 3 or 9 months resulted in a similar drop in core body temperature and increase in hematocrit following re-exposure (Figure 19I-J). Altogether, these data suggest that allergic memory can be primed in a clinically silent manner and the capacity to generate allergen specific IgE is retained indefinitely; a model which resembles the phasic disease progression experienced by allergic patients.

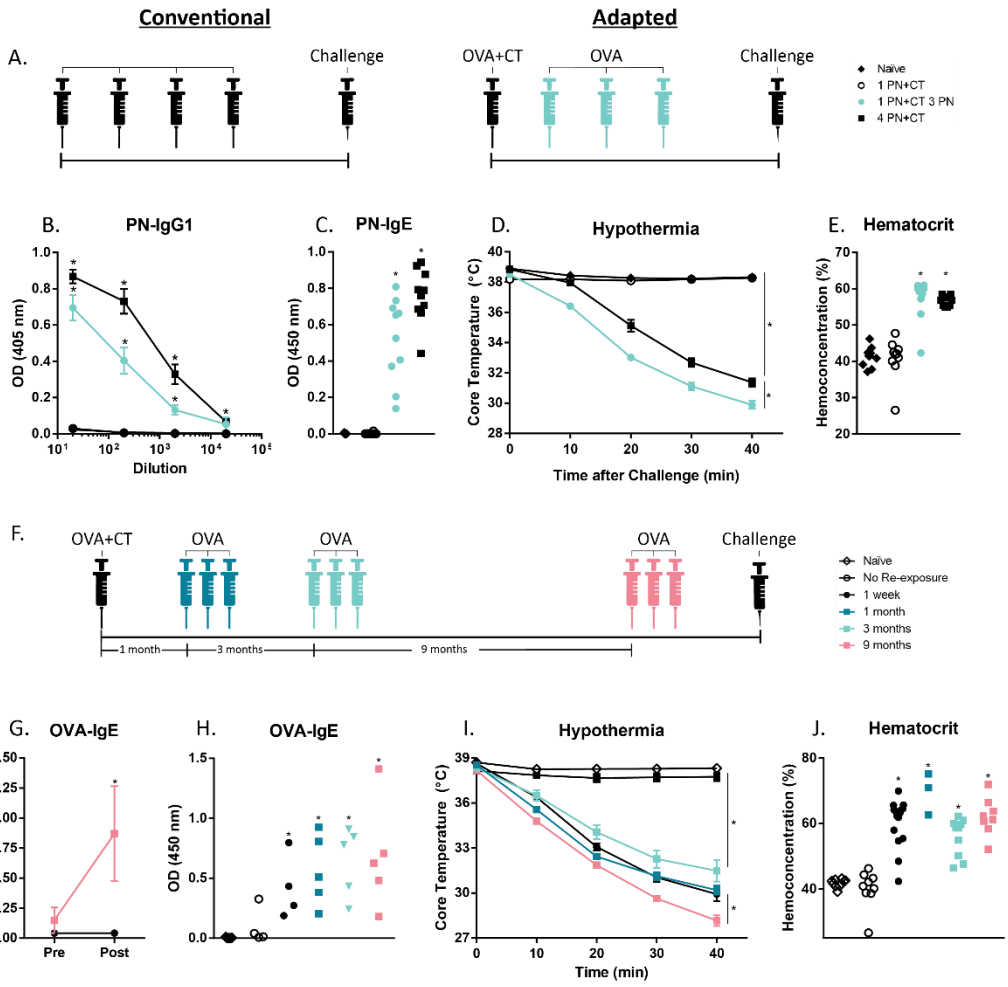


Figure 19: A single OVA+CT exposure is non-sensitizing but generates long-lived memory. A-E) Mice were administered either 1 PN+CT, 1 PN+CT, 3 PN alone, or 4 PN+CT exposures and challenged intraperitoneally 2 weeks later. A) Schematic of conventional (4 PN+CT) and adapted (1 PN+CT, 3 PN) models. B-C) Evaluation of serum PN-IgG1 and PN-IgE by ELISA prior to challenge. D-E) Clinical assessment of anaphylaxis. D) Rectal measurement of core body temperature at 10-minute intervals over 40 minutes of challenge. E) Blood concentration at 40 minutes. n=8-10 per group. F-J) Mice were administered 1 OVA+CT and rested for 1, 3 or 9 months prior to 3 OVA-alone re-exposures. F) Schematic of experiment. G) Assessment of OVA-IgE by ELISA prior to re-exposures and post 3 OVA alone re-exposures in the 9-month group. H) Representative assessment of OVA-IgE by ELISA after 3 OVA alone re-exposures. I-J) Clinical assessment of anaphylaxis. I) Rectal measurement of core body temperature at 10-minute intervals over 40 minutes of challenge. J) Blood concentration at 40 minutes. n=8-10 per group. * $p<0.05$ measured by one- or two-way ANOVA. Unless specified, indicated statistical significance is compared to naïve.

Allergic patients often report that an allergic reaction occurred on the first known consumption of the offending allergen[46,47]. One potential explanation is that any prior exposure was likely in trace amounts. We therefore questioned whether administering greater amounts of antigen during a single gavage with allergen would cause allergic sensitization rather than silent memory. Mice were administered a single gavage of either 1 mg (same as Figure 19), 10 mg, or 100 mg of OVA intragastrically, all in the presence of the same amount of CT. One administration of OVA+CT induced IgG1 and IgE in a dose-dependent manner (Figure 20A-B). Interestingly, while the levels of OVA-IgG1 increased between 2- and 4-weeks post-challenge, the levels of OVA-specific IgE declined sharply (Figure 20C-D). Despite this decline, when challenged intraperitoneally with OVA, mice which were sensitized with 100 mg of OVA experienced hypothermia, vascular leakage, and clinical signs of anaphylaxis upon challenge, while mice primed with 10 mg were mostly unreactive, and those which were primed with 1 mg were, as observed previously, completely unreactive (Figure 20E-G). Arguably, the initiation of silent allergic memory is prioritized when allergen is in limiting supply, which may provide further insight into how sensitization progresses in humans.

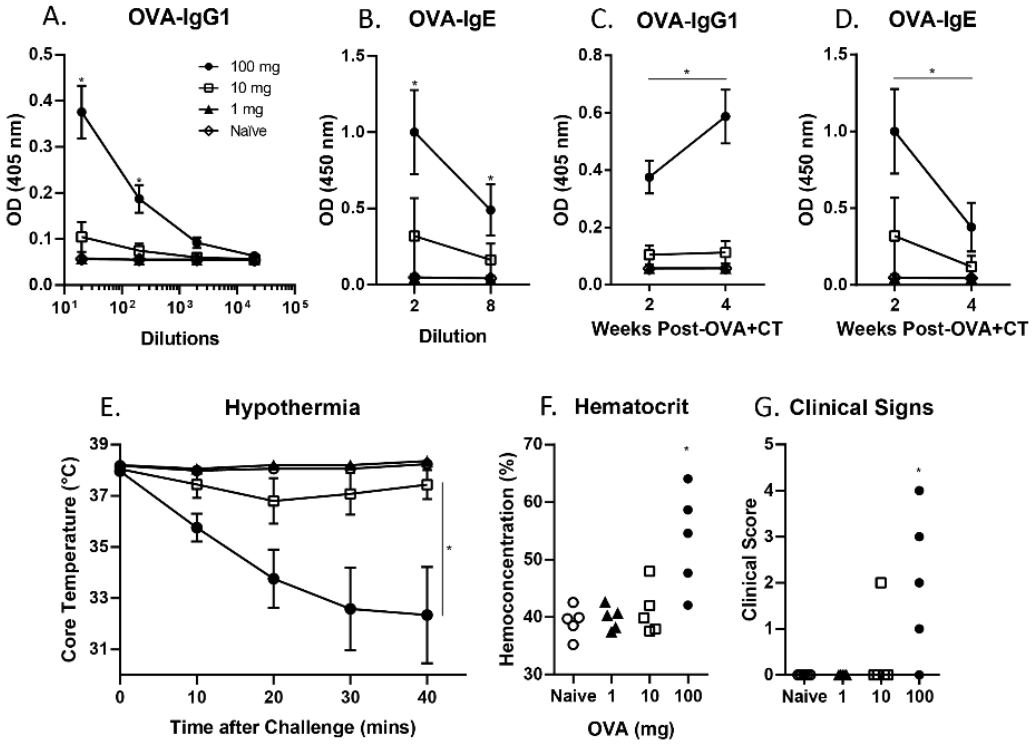


Figure 20: High dose of OVA+CT drives allergic sensitization after a single dose. Mice were administered a single gavage of either 1, 10, or 100 mg of OVA with 5 μg CT and challenged 4 weeks later. A-D) ELISA assessment of serum OVA-IgG1 and IgE production at A-B) 2 weeks post-gavage or B) change between 2- and 4-weeks post-gavage. E-G) Clinical assessment of anaphylaxis upon challenge. E) Rectal measurement of core body temperature at 10-minute intervals over 40 minutes following challenge. F) Blood concentration at 40 minutes. G) Clinical signs of anaphylaxis observed over 40 minutes. n =5 per group. * $p<0.05$ measured by one- or two-way ANOVA. Unless specified, indicated statistical significance is compared to naïve.

Persistent, clinically silent allergic priming occurs in the absence of B cell activation.

$IgG1^+$ MBCs are thought to be the primary reservoir for memory IgE responses[173]. To understand how silent primed memory which maintains IgE responses persists for >9 months, we investigated the phenotype of allergen-specific B cells following allergic priming with 1 mg of OVA and CT. Attempts to

study the allergen-specific B cell repertoire using OVA-monomer staining did not yield enough cells for analysis (data not shown), so we opted to use the tetramer enrichment technology described in Chapter 3 of this thesis. Tetramer enrichment provided an otherwise unattainable resolution of allergen-specific B cells for analysis (Figure 21A). The number of OVA-specific B cells in the mesenteric lymph nodes and spleen peaked following the second OVA-alone re-exposure, and slightly contracted following the third re-exposure (Figure 21B). The number of unswitched B cells remained relatively consistent over time, with the majority of additional cells above baseline being class switched. Contrary to our expectations, primed mice had a similar number of switched and unswitched B cells as naïve mice or tolerant mice which were only administered OVA-alone exposures (Figure 21B). Primed mice had an absence of OVA-specific GC B cells and $IgG1^+$ B cells, both of which emerged following additional re-exposures with OVA (Figure 21C-D). We sacrificed mice at varying timepoints up to 14 days following priming with OVA+CT and found that across all timepoints the OVA-specific repertoire consisted near-exclusively of unswitched B cells (Figure 21E). These data are consistent with our observation that OVA-IgE did not emerge when primed mice were waited for 9 months (Figure 19G-H). These data suggests that B cells at the known inductive sites of intragastric sensitization remained naïve following priming with a single OVA+CT exposure.

We next sought evidence of B cell activation following priming. B cells upregulate CD86 rapidly (<12 hours) following interaction with their cognate

antigen and expression does not return to baseline until >3 days following activation[86]. We therefore compared surface CD86 expression on OVA-specific B cells from naïve, primed (1 OVA+CT), and allergic (4 OVA+CT) mice. The majority of OVA-specific B cells from allergic mice had upregulated CD86 compared to those from naïve mice (Figure 21F). Both GC B cells and switch MBCs in this system expressed CD86, indicative of an active immune response and a lack of quiescent cells (Figure 21F). OVA-specific cells from mice which received a single exposure of OVA+CT had identical CD86 surface expression to naïve mice, indicating that the cells were not activated and therefore had likely remained in a naïve state. As a final measure, we sought to determine whether OVA-specific B cells proliferated in response to priming and could, therefore, create a larger pool of OVA-specific B cells for a secondary response; potentially adopting an unswitched MBC phenotype. We labeled OVA-enriched cells from naïve CD45.1 mice with the proliferation-reporting dye CellTrace Violet (CTV) and adoptively transferred them into B cell-deficient (μMT) mice prior to priming with 1 OVA+CT (Figure 21G). OVA-specific cells from positive control mice, which were immunized with OVA-Alum to induce robust proliferation, had no detectable CTV signal- indicating that the cells had extensively divided and diluted CTV (Figure 21H). OVA-specific B cells from both naïve mice and mice receiving 1 OVA+CT administration retained bright, undiluted CTV labelling in the majority of cells, demonstrating that these cells had not divided (Figure 21H). Taken together, these data demonstrate that OVA-specific B cells remain naïve following allergic priming

and provide a strong argument that early memory of IgE responses is maintained in a compartment other than B cell memory.

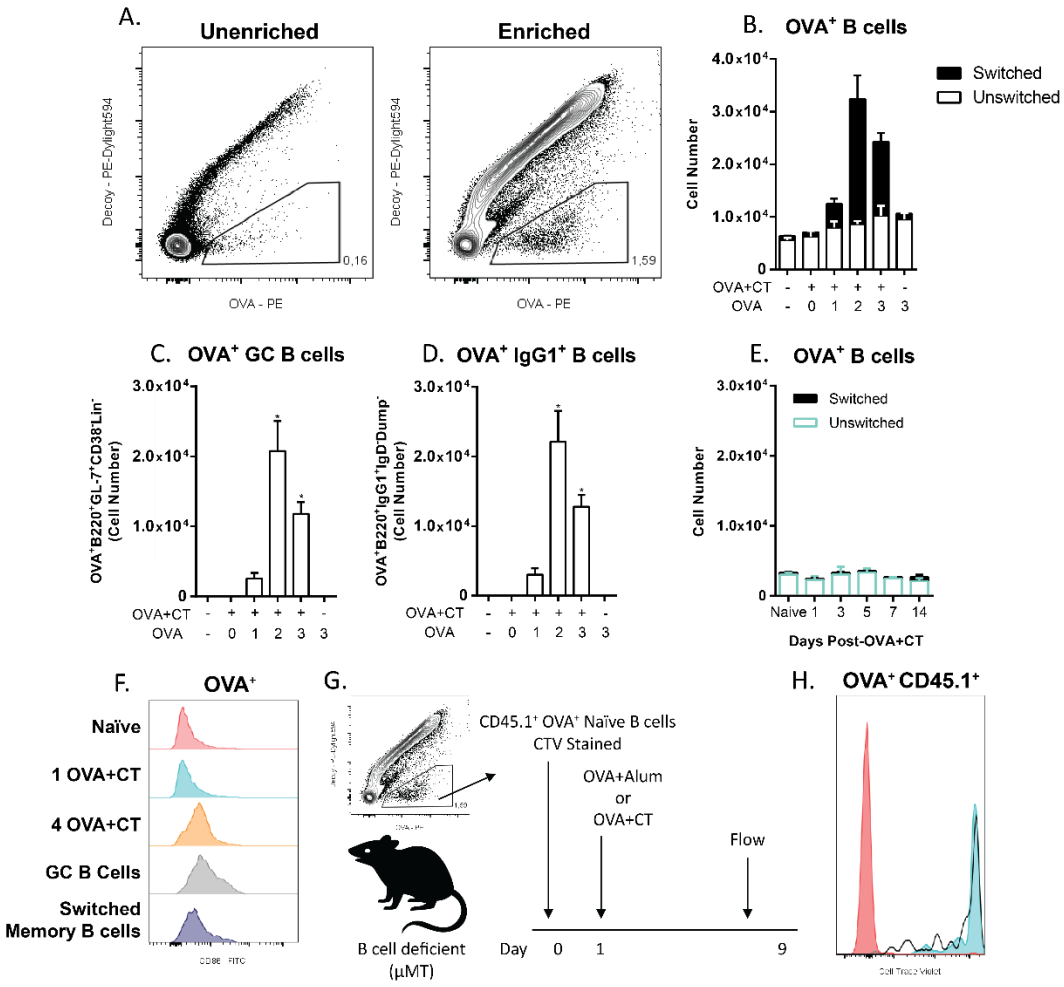


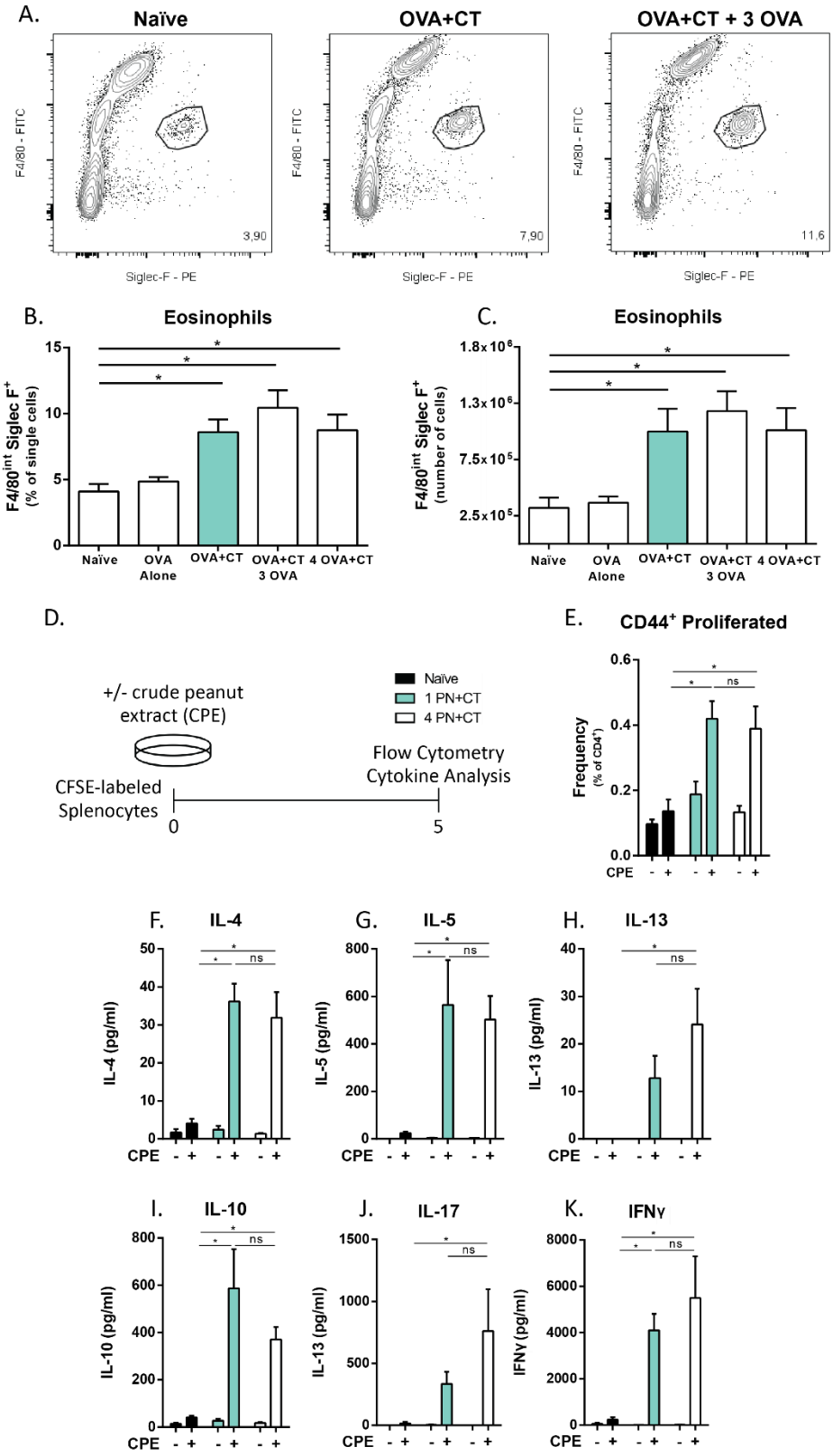
Figure 21: B cells remain naïve following a single exposure to OVA+CT. A) Representative plot of OVA-specific B cells in unenriched and enriched fractions. B-D) Mice were administered 1 mg OVA+CT and the B cell repertoire was evaluated 7 days following the indicated number of re-exposures. B) Number of OVA^+ unswitched and switched B cells. C) Number of OVA^+ GC B cells. D) Number of OVA^+ $IgG1^+$ B cells. E) Number of OVA^+ switched and unswitched B cells at the indicated days following a single OVA+CT exposure. F) Expression of CD86 on OVA^+ cells. G-H) Naïve OVA^+ cells from CD45.1 cells were stained with CellTrace Violet and transferred to CD45.2 µMT mice prior to OVA+CT or OVA-Alum exposure. H) Proliferation of OVA^+ donor-derived cells.

T cells are activated following allergic priming and are required maintain silent allergic memory.

Long-term immune memory is typically attributed to adaptive immune cells. In the absence of B cell activation, we next asked whether T cells were activated following priming. Allergen challenge results in two phases of reactivity, an immediate phase characterized by anaphylactic symptoms and a late phase response characterized by eosinophilic inflammation at the site of challenge[26]. Late phase responses are mediated by IL-5 secretion from local Th2 cells. We sought to determine whether primed mice retained late phase inflammation despite their lack of immediate phase reactivity. Peritoneal lavage of primed mice revealed an increase in proportion and number of F4/80^{int} Siglec-F^{+} cells, a phenotype characteristic of eosinophils[174], 3 days post-challenge compared to naïve mice (Figure 22A-C). The extent of peritoneal eosinophilia was similar to that observed in clinically reactive mice which received either 1 OVA+CT and 3 OVA, or 4 OVA+CT, suggesting that primed mice generated functional Th2 cells. Further, we cultured CFSE labeled cells from mice primed with 1 PN+CT exposure with crude PN extract for 5 days and observed a similar proportion of proliferating activated CD4^{+} T cells compared to mice sensitized with 4 PN+CT (Figure 22D-E). CD4^{+} T cells from primed mice secreted a complex signature of cytokines consistent with allergic sensitization: canonical Th2 cytokines like IL-4, IL-5, and IL-13, and the generation of IL-10, IL-17, and IFN-γ (Figure 22F-K)[26]. These data provide

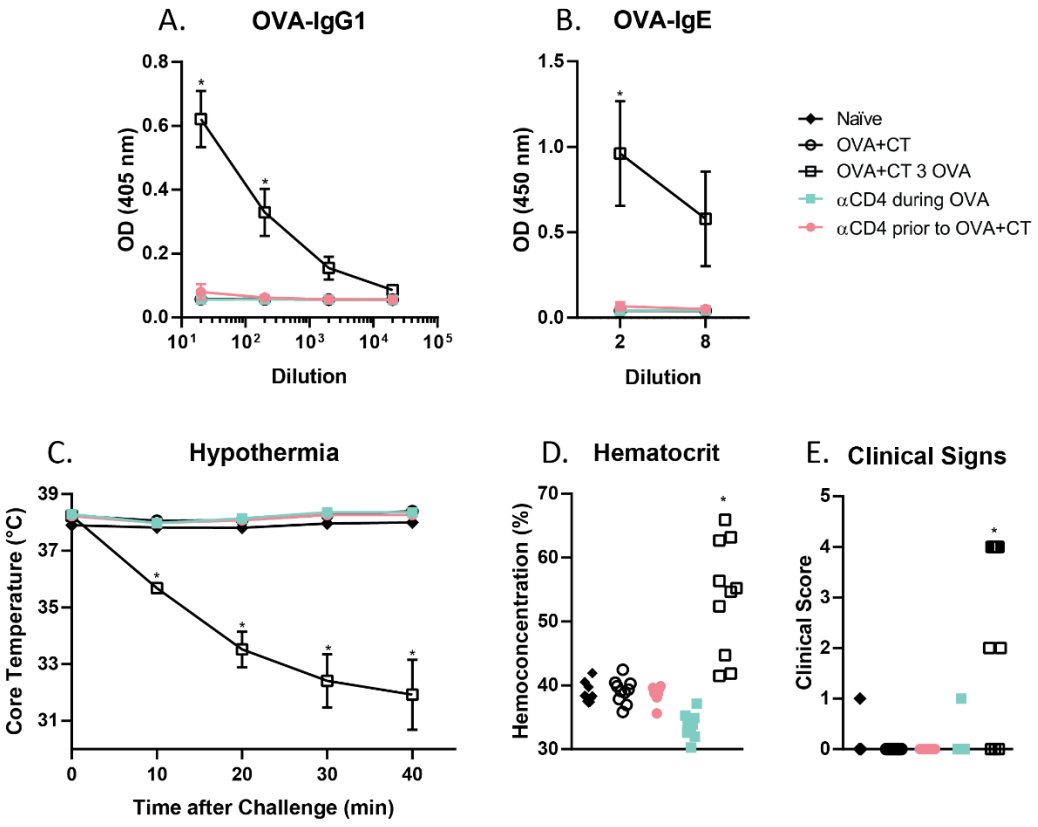
evidence that Th2 polarized $CD4^+$ T cells are generated following silent allergic priming.

Figure 22: Evidence of T cell activation following a single OVA+CT administration. A-C) Late phase inflammation of the peritoneal cavity 3 days post challenge. A) Representative flow cytometry plots of peritoneal eosinophils. B-C) Proportion and cell number of peritoneal eosinophils. n=10 per group. D-K) Culture of CFSE splenocytes with PN. E) Frequency of proliferating $CD44^+$ T cells. F-K) Magpix measurement of cytokines in culture supernatants. Three repeats, n=5. * $p<0.05$ one-way ANOVA. (*Figure on next page).*


A.
Naïve
OVA+CT
OVA+CT + 3 OVA
F4/80 - FITC
Siglec-F - PE
3.90
7.90
11.6
B.
Eosinophils
F4/80int Siglec F+ (% of single cells)
C.
Eosinophils
F4/80int Siglec F+ (number of cells)
Naïve
OVA Alone
OVA+CT
OVA+CT 3 OVA
4 OVA+CT
D.
+/- crude peanut extract (CPE)
CFSE-labeled Splenocytes
Naïve
1 PN+CT
4 PN+CT
Flow Cytometry Cytokine Analysis
0
5
E.
CD44+ Proliferated
Frequency (% of CD4+)
CPE
F.
IL-4
IL-4 (pg/ml)
G.
IL-5
IL-5 (pg/ml)
H.
IL-13
IL-13 (pg/ml)
I.
IL-10
IL-10 (pg/ml)
J.
IL-17
IL-13 (pg/ml)
K.
IFNγ
IFNγ (pg/ml)
ns

We next asked whether $CD4^+$ T cells are required to seed silent allergic memory, for the emergence of IgE following re-exposure, or both. We depleted $CD4^+$ T cells using anti-CD4 (GK1.5) either prior to allergic priming or prior to and during the OVA-alone re-exposures. It was important to continue the treatment during re-exposure as allergen-specific $CD4^+$ T cells which remained following depletion rapidly expanded upon allergen exposure (data not shown). Mice primed or re-exposed in the absence of $CD4^+$ T cells did not generate OVA-specific IgG1 nor IgE, and were not clinically reactive upon challenge (Figure 23A-E). The lack of OVA-IgE following re-exposure in anti-CD4-treated mice may be expected as T cell help is required for $IgG1^+$ MBCs to sequentially switch to IgE upon recall. However, the lack of OVA-IgG1 in these mice corresponds with our observed absence of $IgG1^+$ MBCs following priming, and strongly suggests that $CD4^+$ T, rather than MBCs, hold the memory of IgE responses following silent allergic priming.

Figure 23: $CD4^+$ T cells are required for primed allergic memory. $CD4^+$ T cells were depleted by administration of anti-CD4 (GK1.5) either prior to OVA+CT or during OVA alone re-exposures. A-B) Evaluation of OVA-specific immunoglobulins by ELISA. A) OVA-IgG1, B) OVA-IgE. C-E) Clinical assessment of anaphylaxis. C) Rectal measurement of core body temperature at 10-minute intervals over 40 minutes following challenge. D) Blood concentration at 40 minutes. E) Clinical signs of anaphylaxis measured over 40 minutes. N=10 per group. * $p<0.05$ measured by one- or two-way ANOVA, significance compared to naïve. (*Figure on next page.)*

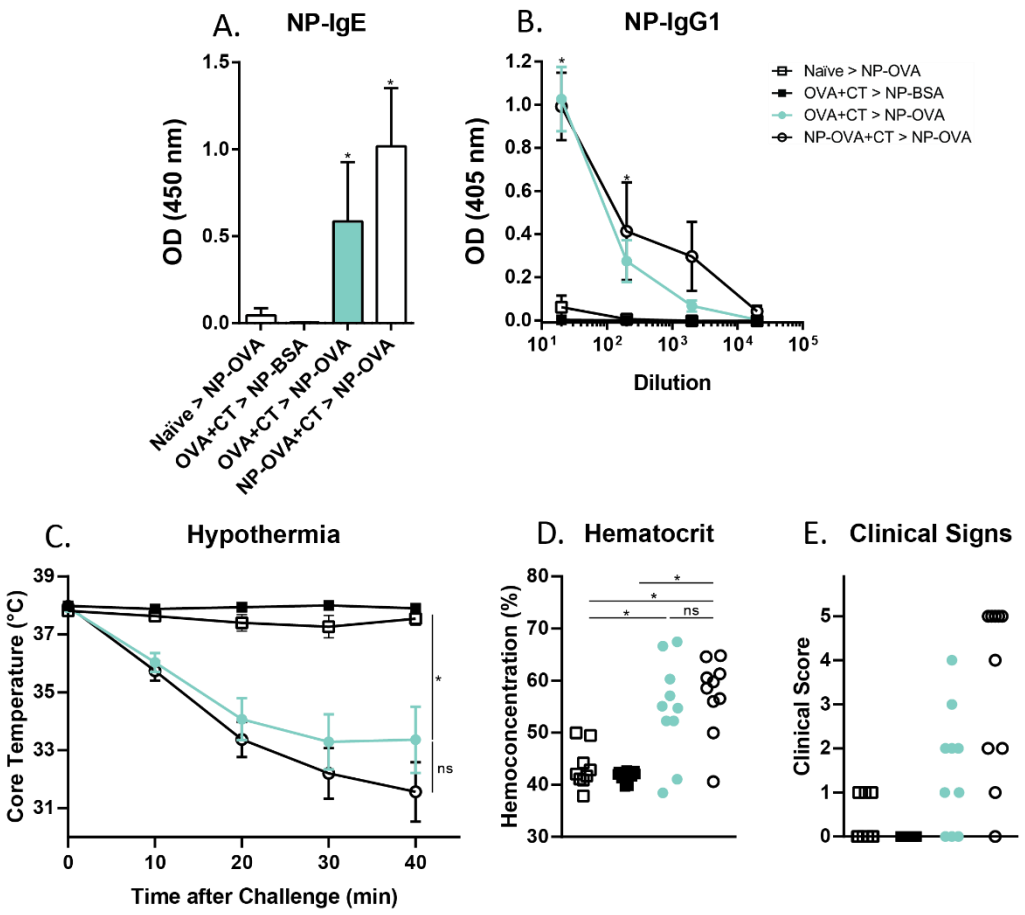


Primed CD4⁺ T cells hold the memory of IgE responses in an IL-4Rα and CD40L-dependent manner.

Given that the B cell compartment appears to be naïve following allergic priming, memory CD4⁺ T cells would need to induce B cell activation and IgE isotype switching from naïve B cells during an allergen-alone re-exposure. To directly address this hypothesis, we primed mice with a single gavage of OVA+CT, then re-exposed the mice 1 month later with 3 oral exposures of NP-OVA. NP-specific B cells at the time of re-exposure are naïve in these mice as they have never previously experienced NP. However, NP-specific B cells can uptake the NP-OVA

conjugate and present OVA-peptides to OVA-specific primed T cells to receive T cell help. As there is no adjuvant during the re-exposures, any NP-specific immunity elicited in these mice comes from OVA-specific T cells. Mice which were not primed but received NP-OVA alone re-exposures did not generate NP-specific IgG1 or IgE, demonstrating that the NP-conjugate alone was not sensitizing (Figure 24A-B). Further, mice which were primed with OVA+CT and re-exposed to NP conjugated to a different carrier (NP-BSA) were similarly absent of NP-Ig production, as these mice could not make cognate interactions with OVA-specific T cells (Figure 24A-B). Mice which were primed with OVA+CT and re-exposed with NP-OVA alone produced similar amounts of NP-specific IgE and IgG1 compared to positive control mice that were primed and re-exposed with NP-OVA and were similarly clinically reactive when challenged with NP (Figure 24A-E). These data definitively demonstrate that memory $CD4^+$ T cells generated during early allergic priming hold the memory of humoral IgE responses.

Figure 24: Primed memory $CD4^+$ T cells hold the memory of IgE responses. Mice were primed with OVA+CT then re-exposed with NP-OVA alone. A-B) Evaluation of serum NP-specific immunoglobulins by ELISA. A) NP-IgE, B) NP-IgG1. C-E) Clinical assessment of anaphylaxis. C) Rectal measurement of core body temperature at 10-minute intervals over 40 minutes following challenge. D) Blood concentration at 40 minutes. E) Clinical signs of anaphylaxis measured over 40 minutes. N=10 per group. * $p<0.05$ measured by one- or two-way ANOVA, significance compared to naïve. (*Figure on next page.)*

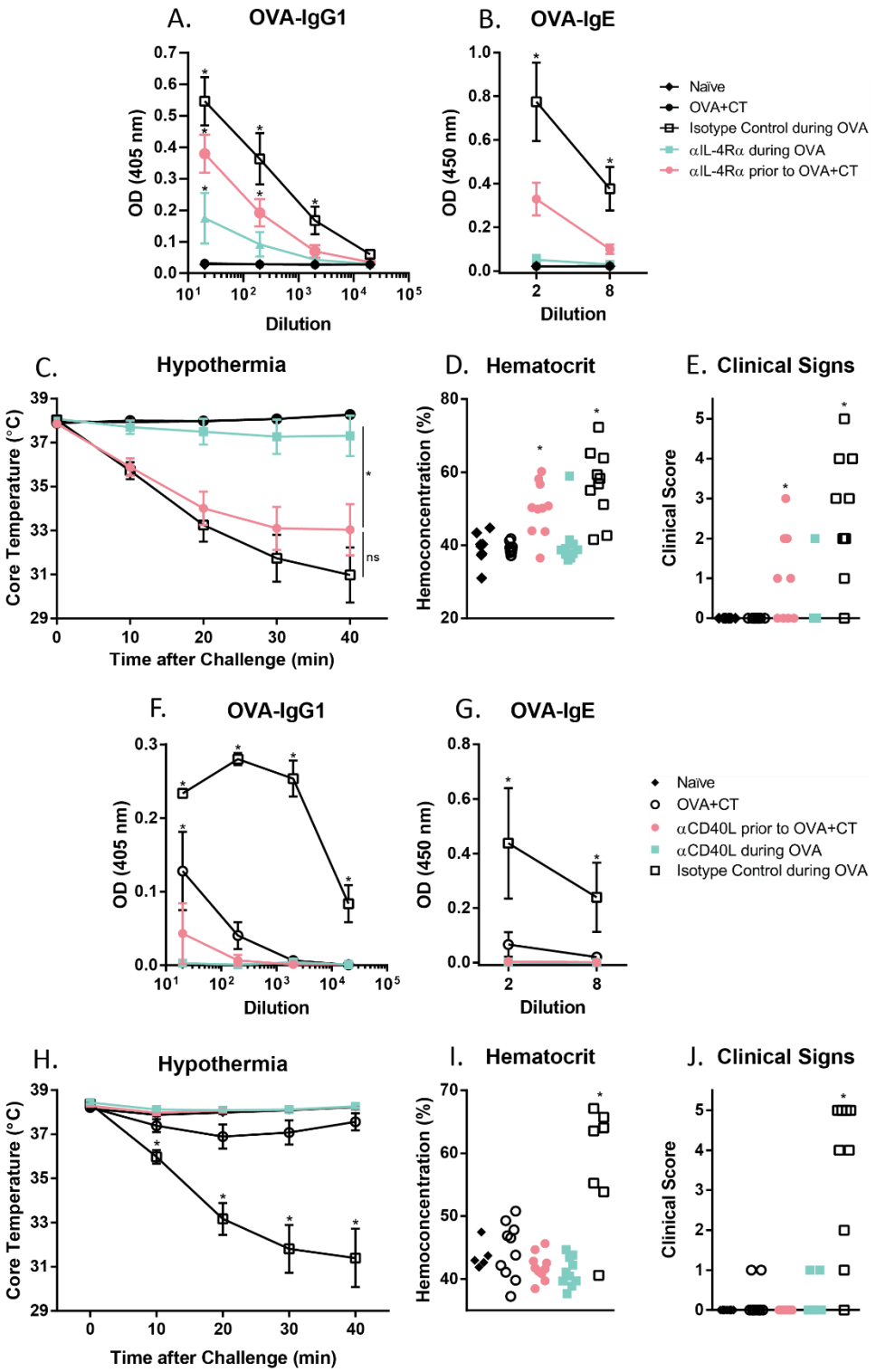


Lastly, we sought to determine the molecular interactions that are required to generate early allergic memory and those which are used by memory CD4$^+$ T cells to induce IgE production in naïve B cells. We treated mice with candidate blocking antibodies either during the priming exposure (to test the requirement during the generation of memory) and during the allergen-alone re-exposures (to test the involvement in inducing IgE upon memory re-activation). We started by blocking IL-4Rα signalling, which is thought to be required for Th2 cell activation and which we have previously reported is required for memory IgE responses[64,131]. Blocking IL-4Rα signalling during the OVA-alone re-exposures completely abrogated the

generation of OVA-IgE and fully protected from anaphylaxis upon allergen-challenge (Figure 25B-E). These mice generated a small amount of OVA-IgG1, indicating that the generation of IgG1 is not strictly dependent on IL-4 signalling (Figure 25A). Intriguingly, despite a reported role for autocrine/paracrine IL-4 signalling during Th2 cell differentiation[64], blocking IL-4Rα signalling during priming had minimal impact on OVA-IgG1 levels, only partially reduced OVA-IgE production, and was not protective against anaphylaxis upon challenge (Figure 25A-E).

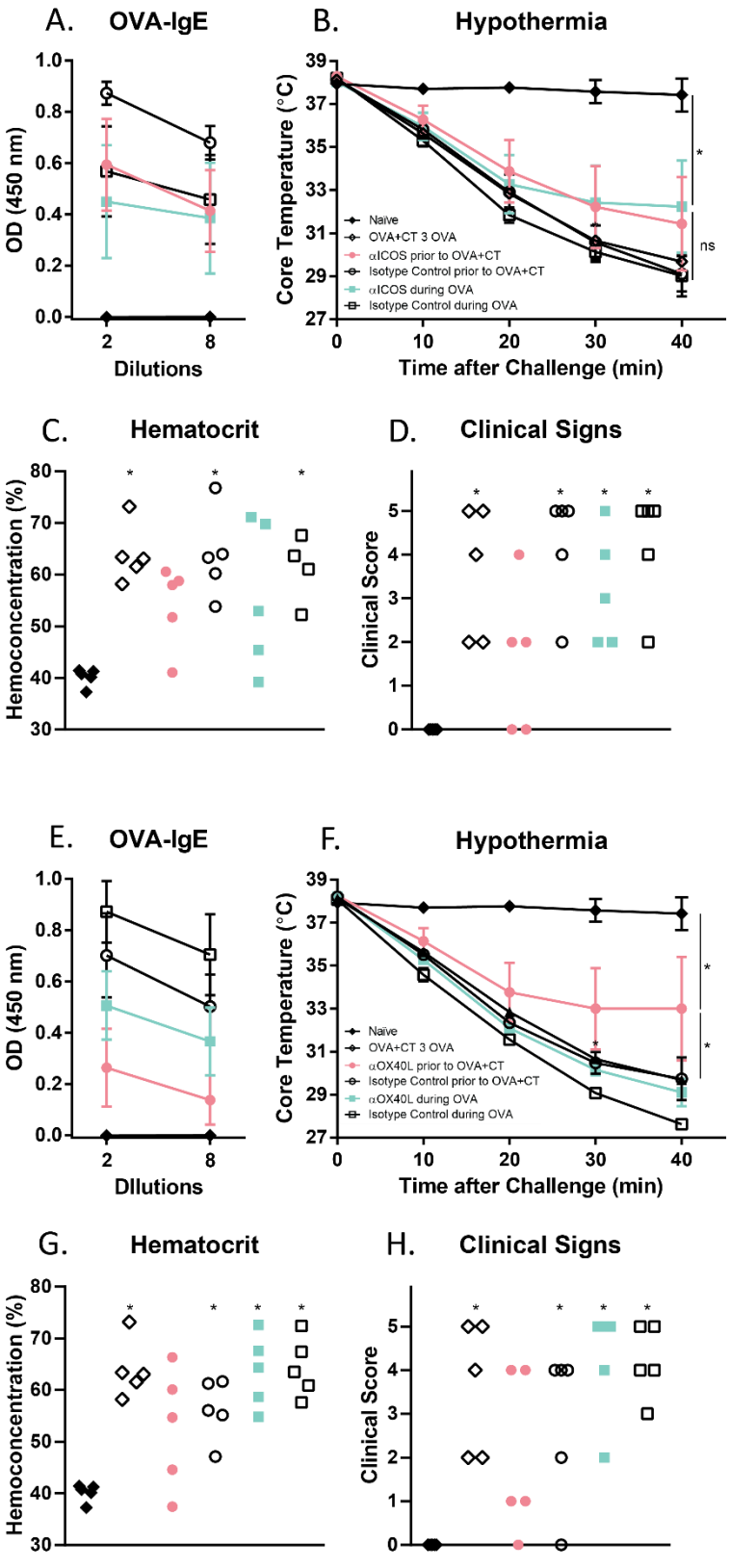
We next blocked CD40/CD40L interactions by treating the mice anti-CD40L. CD40/CD40L interactions are implicated in T cell activation at the DC/T cell interface and are the principal costimulatory interaction between Tfh cells during B cell activation and in the GC[118,175]. Mice treated with anti-CD40L either during priming or during the re-exposures did not generate OVA-IgG1 nor OVA-IgE and were completely protected from anaphylaxis upon allergen challenge (Figure 25F-J). CD40/C40L interactions are therefore required to generate primed memory $CD4^+$ T cells and are utilized by memory T cells to induce IgE production from naïve B cells during a recall response.

Figure 25: $CD4^+$ T cells keep the memory of IgE in a CD40/CD40L, IL-4/IL-4Rα-dependent manner. Mice were treated with anti-IL-4Rα (A-E) or anti-CD40L (F-J) during the priming OVA+CT exposure or during OVA alone re-exposures. A-B, F-G) ELISA measurement of serum A,F) OVA-IgG1, B,G) OVA-IgE. C-E, H-J) Clinical assessment of anaphylaxis. C,H) Rectal measurement of core body temperature at 10-minute intervals over 40 minutes following challenge. D,I) Blood concentration at 40 minutes. E,J) Clinical signs of anaphylaxis measured over 40 minutes. N=8-10 per group. * $p<0.05$ measured by one- or two-way ANOVA, significance compared to naïve. (*Figure on next page.*)


A.
OVA-IgG1
OD (405 nm)
Dilution
B.
OVA-IgE
OD (450 nm)
Dilution
Naïve
OVA+CT
Isotype Control during OVA
αIL-4Rα during OVA
αIL-4Rα prior to OVA+CT
C.
Hypothermia
Core Temperature (°C)
Time after Challenge (min)
ns
D.
Hematocrit
Hemoconcentration (%)
E.
Clinical Signs
Clinical Score
F.
OVA-IgG1
OD (405 nm)
Dilution
G.
OVA-IgE
OD (450 nm)
Dilution
Naïve
OVA+CT
αCD40L prior to OVA+CT
αCD40L during OVA
Isotype Control during OVA
H.
Hypothermia
Core Temperature (°C)
Time after Challenge (min)
I.
Hematocrit
Hemoconcentration (%)
J.
Clinical Signs
Clinical Score

We sought to determine if other costimulatory molecules were critically required for allergic priming or for memory $CD4^+$ T cells to generate secondary IgE from naïve B cells. We selected ICOS/ICOSL interactions based on the expression of ICOSL on Tfh cells, which are thought to be required for IgE production, and OX40/OX40L interactions based on their involvement in Th2 polarization[62,91,176]. Treatment with anti-ICOSL during priming or during re-exposure did not impact levels of OVA-IgE nor provide protection against anaphylaxis (Figure 26A-D). Treatment with anti-OX40L during priming revealed a partial decrease in OVA-IgE levels and partial protection against anaphylaxis (Figure 26E-H). This decrease was consistent with previous reports which demonstrate that OX40/OX40L interactions are involved in allergic sensitization to foods[176]. Treatment with anti-OX40L during re-exposures did not impact OVA-IgE levels nor protected against anaphylaxis. In summary, seeding primed memory requires CD40/CD40L interactions, but only partially requires OX40/OX40L and IL-4Rα signalling. The induction of IgE from naïve B cells by primed $CD4^+$ T cells is critically dependent on both IL-4Rα and CD40/CD40L interactions.

Figure 26: OX40/OX40L and ICOS/ICOSL are dispensable in establishing primed memory and to generate IgE from primed memory. Mice were treated with anti-ICOS (A-E) or anti-OX40L (F-J) during the priming OVA+CT exposure or during OVA alone re-exposures. A,E) ELISA measurement of serum OVA-IgE. B-D,F-H) Clinical assessment of anaphylaxis. B,F) Rectal measurement of core body temperature at 10-minute intervals over 40 minutes following challenge. C,G) Blood concentration at 40 minutes. D,H) Clinical signs of anaphylaxis measured over 40 minutes. N=5 per group. * $p<0.05$ measured by one- or two-way ANOVA, significance compared to naïve. (*Figure on next page.)*


A.
OVA-IgE
OD (450 nm)
Dilutions
B.
Hypothermia
Core Temperature (°C)
Time after Challenge (min)
Naïve
OVA+CT 3 OVA
αICOS prior to OVA+CT
Isotype Control prior to OVA+CT
αICOS during OVA
Isotype Control during OVA
ns
C.
Hematocrit
Hemoconcentration (%)
D.
Clinical Signs
Clinical Score
E.
OVA-IgE
OD (450 nm)
Dilutions
F.
Hypothermia
Core Temperature (°C)
Time after Challenge (min)
Naïve
OVA+CT 3 OVA
αOX40L prior to OVA+CT
Isotype Control prior to OVA+CT
αOX40L during OVA
Isotype Control during OVA
G.
Hematocrit
Hemoconcentration (%)
H.
Clinical Signs
Clinical Score

Discussion

The progression of food allergy from the first adverse exposure to a food allergen to the state of IgE-mediated clinical reactivity has only just begun to be characterized. The inciting events of allergic sensitization likely happen early in life. Prior to birth, maternal transfer of allergen-specific IgG and IgE may polarize immunity prior to exposure to the allergen[177]. Maternal IgE can sensitize progeny mast cells, which can degranulate upon allergen exposure and cause clinical reactivity[177]. After birth, allergic sensitization is associated with microbial dysbiosis in the first 100 days, while early feeding of allergen dramatically decreases the likelihood of sensitization[50,52]. In those who become allergic, evidence from mice and birch allergic humans suggest that allergen-specific IgG production predates allergen-specific IgE production, highlighting the progressive nature of the disease[53,54]. In this work, we sought to determine what disease states exist prior to allergen-specific IgG immunity and to understand what drives the progression of disease towards clinical allergy.

Classical models of allergic sensitization to foods are optimized for robust allergen-specific IgE production and clinical signs of anaphylaxis upon challenge. With very few exceptions, most allergic models require multiple allergen exposures in the presence of some adjuvant to achieve this outcome[59]. Intranasal sensitization can be achieved against soluble allergens like OVA using multiple exposures in the presence of IL-33[178]. Some allergens, like HDM, have inherent adjuvant properties[179]. HDM must be administered 3 times per week for multiple weeks for

clinical allergy- including IgE production[180]. Sensitization to foods through the skin requires multiple weeks of antigen administration to the skin following daily tape stripping as an adjuvant[54]. Intragastric sensitization requires 4-6 gavages of allergen and CT[26]. As these models are driven by an IgE-producing outcome, the trajectory of the allergic diathesis during these exposures has been poorly investigated. We found that a single intragastric exposure of OVA+CT failed to induce OVA-specific B cell activation, and as a result allergen-specific IgE was not generated, and the mice were not clinically reactive upon challenge. Interestingly, these mice also did not produce OVA-specific IgG1, which indicates that a phase prior to B cell activation may exist in allergic patients. The single OVA+CT exposure generated immune memory that, upon future allergen re-exposure at any point up to 9 months later, resulted in IgE production and clinical reactivity challenge. The immunity generated during the first OVA+CT exposure did not mature to IgG1 nor IgE production over these 9 months, suggesting that the seeded memory is dormant and requires some external stimulus to push the transition to clinical allergy. Administering additional allergen in the absence of adjuvant drove allergic sensitization. In allergic patients, accidental consumption of allergen or trace amounts of antigen that exist as contaminants in foods may serve the same function. However, it is possible that other stimuli, like bystander proliferation following immunization or infection, could drive allergic immunity in primed individuals[181].

We have provided indirect evidence of T cell activation through examining their function. The presence of a late phase response in mice primed with one

administration OVA+CT indicates that Th2 immunity was established. Further, $CD4^+$ T cells proliferated in response to allergen stimulation in culture and secreted type 2 cytokines indicative of a Th2 response, as well as type 1 and type 17 cytokines that are frequently observed following intragastric sensitization to foods[26,131]. Future experimentation should focus on phenotyping the allergen-specific T cells that are seeded post-priming using MHC-II tetramers[182]. The phenotype of these early $CD4^+$ T cells may serve as a prognostic marker that could be screened in children at high risk of developing food allergies to determine their likelihood of becoming clinically allergic.

Lastly, we demonstrated a novel role for $CD4^+$ T cells in holding the memory of IgE responses. $CD4^+$ T cells were capable of inducing IgE production from naïve B cells in the absence of adjuvant, indicating that they, alone, are sufficient to hold the memory of IgE responses. This is a non-classical pathway of B cell memory, as typically MBCs are the primary source of secondary antibody production during a recall response[169]. The exact contribution of $CD4^+$ T cells during a memory IgE response in an allergic subject is unclear. This axis is likely more important in the context of allergic priming when B cell immunity is not yet induced. This is a crucial consideration when designing therapeutics for food allergy. Therapeutics designed to target MBCs or the emergence of IgE may not be curative if the allergic $CD4^+$ T cell repertoire remains intact. Indeed, multiple recent publications have identified Th2A cells as a phenotype exclusive to allergic patients which persists following OIT for food allergy or dupilumab therapy in atopic dermatitis[183,184]. The

capacity of this highly pathogenic population to initiate IgE production from naïve B cells has not been tested, but this axis may explain the re-emergence of allergen-specific IgE levels and relapse in disease in those with food allergy and atopic dermatitis when therapy ceases.

In conclusion, the incipient stages of food allergic sensitization are characterized by clinically and humorally silent generation of $CD4^+$ memory T cells which retain the ability to induce IgE production from naïve B cells. This population may be a prognostic biomarker in pre-allergic patients and an important consideration when developing novel therapeutics for food allergy.

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CHAPTER 6

DISCUSSION

The primary objective of this doctoral work was to further our understanding of the trajectory of adaptive immune activation in food allergy murine models. Previous work had uncovered the central role of Th2 cells and IgE in the pathophysiology of allergic diseases[172]. However, the processes which lead to Th2 activation and the generation of allergen specific IgE remain an area of active investigation. Taking the perspective of food allergy as a phasic disease allowed us to study the stepwise maturation of the adaptive immune response from an adverse inciting event to the clinically reactive, IgE-producing state observed when patients first present to the clinic. We have added to the knowledge of these stages and, specifically, analyzed individual pathways that lead to allergic sensitization. These include the evolution of clinically silent sensitization and the involvement of distinct isotype switching pathways in generating anaphylactic IgE. These studies primarily utilized mouse models of food allergy to overcome the major limitations which accompany studying the pre-allergic state in humans. We have identified novel pathways and biomarkers, the study of which will continue into allergic patients.

Summary of Chapters

In Chapter 3, we report the development and extensive validation of B cell tetramers which were subsequently used in research presented in Chapters 4 and 5 to sensitively examine the pathways of allergen-specific B cell activation during the incipient stages of allergic sensitization, and re-activation at memory time points. In both conditions, allergen-specific B cells are exceedingly rare, and sub-phenotyping these cells is limited by the extremely low number available for analysis. Similar to prior reports, allergen-tetramer enrichment greatly increased the frequency of allergen-specific B cells thus allowing for extensive sub-phenotyping[144]. We confirmed that relevant isotypes to the study of food allergy, namely IgG1 and IgE, in both the B cell and antibody secreting cell repertoire are enriched using this strategy. We also applied this strategy to cells from the bone marrow and small intestine lamina propria, which are tissues that have not yet been studied using this technology. Such an analysis in future work will allow for a granular understanding of the B-lineage signatures that may exist uniquely at those tissue locations. In particular, the small intestines are the primary site of allergen exposure in intragastric models of allergic sensitization but remain poorly characterized in terms of IgE-secreting cells or MBCs that may be present at that location. Lastly, we demonstrated the construction of novel antigen-tetramer specificities which will enable future discovery. For example, the SARS-COV-2 RBD-tetramer has been used to study B cell responses in the context of novel

COVID vaccine approaches (unpublished data), and the BLG tetramer which will enable future analysis of B cells from milk allergic patients.

In Chapter 4, we evaluated the importance of direct and sequential isotype switching in the development of anaphylactic IgE against food allergens in primary and secondary responses. Our findings are at variance with the prevailing understanding that sequential switching through IgG1 is required for high affinity, anaphylactic IgE production and memory IgE responses[99]. Instead, IgE derived from direct isotype switching had similar polyclonal binding affinity and functional capacity independent of whether the cells could sequentially switch. Further, we demonstrated that secondary IgE responses are equally potent in the presence or absence of sequential switching. We found that $IgG1^+$ cells were dominant among GC B cells and the mature MBC repertoire of WT mice. In the absence of IgG1, unswitched and IgE^+ B cells gained expanded access to the GC, and unswitched B cells comprised a larger proportion of mature B cells. Our data indicate that sequential switching is not critical to generate anaphylactic IgE in a primary or secondary response but is likely the primary pathway of IgE generation because of the competitive dominance of $IgG1^+$ cells.

In Chapter 5, we studied pathways of adaptive immune activation during the incipient stages of allergic sensitization to foods. We found that a single adverse exposure to allergen did not result in B cell activation, nor the consequent production of allergen-specific IgG1 or IgE, but did generate memory that could drive allergic sensitization when allergen, alone, was next encountered. We

provided evidence that this adverse exposure generated functional T cells, which were critically required to drive B cell activation during allergen re-exposure. We further demonstrated that these memory $CD4^+$ T cells could induce IgE production from naïve B cells in an IL-4Rα and CD40L-dependent manner, a role which has not been previously ascribed to these cells. Ultimately, we uncovered a novel pathway of early allergic priming, demonstrating that T cell immunity can be activated in a manner that is uncoupled from B cell immunity and lay dormant, only to drive sensitization when allergen is encountered at a later time.

Pathway of Adaptive Immune Activation in Food Allergy

The evidence we generated indicates that the pathway of adaptive immune activation during food allergic sensitization can be broken down into two steps. Firstly, T cells are activated in a manner that is uncoupled from B cell activation, and secondly, upon allergen re-exposure, B cells are activated and undergo CSR to IgG1 and IgE. These two steps will be discussed in detail below.

Step 1: T Cell Activation

A single intragastric administration of allergen and CT activated T cells without detectable allergen-specific IgG1 nor IgE production. This single adverse event polarized T cell immunity such that allergen-specific IgG1 and IgE were induced following allergen-alone re-exposures. Our observation is consistent with a recent report which hypothesized that adjuvant would only be required during the first intragastric administration of allergen and CT to induce a meaningful APC-T cell

synapse, resulting in immune activation[185]. The type of APC which induces T cell immunity in this context is not clear, but we have previously demonstrated that $CD103^+$ DCs which migrate from the gut to the secondary lymphoid organs are required for intragastric sensitization[174]. OX40:OX40L interactions at the APC-T cell synapse are thought to induce autocrine IL-4 production which drives Th2 differentiation[64,176]. Paradoxically, we found that blocking either OX40L or IL-4Rα signalling during the single OVA+CT administration had little effect on the generation of humorally silent T cell immunity. These data suggest that a pathway other that Th2 cell differentiation is engaged following a single OVA+CT administration, presumably a less mature phenotype which precedes effector commitment. In this context, additional allergen would drive completion of the effector T cell phenotype, allowing for Th2 and Tfh differentiation and B cell activation. Our data are consistent with this model, as our observations of T cell activation following OVA+CT both involve additional allergen exposure- late phase inflammation following challenge and dominant Th2 cytokine production upon *in vitro* culture with allergen.

A Bottleneck Prior to B Cell Activation

To our knowledge, it has not been previously demonstrated that T cell immunity could be induced uncoupled from B cell activation. Our data demonstrate that B cells at the known inductive sites of allergic sensitization remain naïve, and that transferred naïve B cells do not proliferate upon OVA+CT administration. Contextually, a bottleneck prior to B cell activation makes physiological sense. B

cell responses require immense energy, generating space-intensive GCs which involve many clones rapidly proliferating, only for most to be rejected at selection[118,168]. It was hypothesized that pre-GC selection is very stringent to minimize this energetic cost[121]. Only clones with moderate affinity could gain access to the GC for maturation. However, recent work has demonstrated that this bottleneck in activation is extremely weak, or perhaps non-existent. A large diversity of clones with a very broad range of antigen affinities are recruited into the GC, the vast majority of which are exported early as MBCs[121]. It is advantageous to only induce this taxing B cell response in contexts where it is necessary. Instead of committing to a B cell response, T cell immunity can be activated which retains the ability to activate B cells and seed GCs at any subsequent antigen exposure.

The question remains how the bottleneck between T and B cell immunity is maintained. Here, we present three non-mutually exclusive hypotheses, and which certainly do not encompass all possible explanations.

The first hypothesis is that the B cell repertoire was not activated because there was an insufficient amount of antigen in the system, and therefore none was uptaken by allergen-specific B cells. The B cells would therefore not be able to present peptide on MHC-II to $CD4^+$ T cells and receive T cell help. Indeed, OVA-specific B cells from mice receiving 1mg OVA+CT did not express CD86, a marker upregulated upon antigen encounter. Further, providing a single gavage of 100 mg of OVA+CT induced robust OVA-IgG1 and IgE production, while 1 mg of OVA+CT only induced T cell activation. It is also possible that different antigen

amounts trigger distinct pathways that generate distinct $CD4^+$ T cell phenotypes with different capacities to activate B cells.

Related to the previous point, the second hypothesis is that the priming exposure failed to induce Tfh differentiation. Tfh cells are typically the primary source of help required for B cell activation and are required for IgE isotype switching[62,91]. Failure to induce Tfh differentiation may result in a lack of available T cell help for B cells. B cells which have uptaken antigen but do not receive T cell help are known to return to a naïve state[86], which may explain why B cells remain naïve following a single OVA+CT exposure. As described above, it is possible that OVA+CT priming generates a population of immature memory $CD4^+$ T cells which have not yet committed to a Th2 or Tfh fate. It is also possible that mature effector Th2 cells are generated in the absence of Tfh cells. One study demonstrated that migratory DCs were required for Tfh induction[186]. The single OVA+CT gavage may not be able to drive sufficient DC migration, instead relying solely on resident DC populations which may be predisposed to Th2 polarization. The factors which mediate Th2 vs Tfh differentiation are the focus of active investigation.

The final hypothesis is that specific regulatory mechanisms limit the engagement of an antigen-specific B cell response such that only stimuli with sufficient amplitude to overcome these regulatory mechanisms progress to B cell activation. A candidate population which may enact this regulatory restriction is Tfr cells. Tfr cells are localized at the T/B border and utilize IL-10 and neuritin to restrict PC formation and limit autoimmunity and allergy[125,187]. It is possible that

these cells form synapses with B cells to restrict their activation in contexts where T cell help is limiting. Increasing the strength of that T cell help, for example through providing more antigen, may push the activation signal above the threshold of Tfr restriction, allowing for B cell activation.

Step 2: Memory CD4$^+$ T cells Activate Naïve B Cells

Using a well-controlled hapten-administration system, we demonstrate that memory CD4$^+$ T cells hold the memory of IgE production by activating naïve B cells. CD4$^+$ T cells appear capable of performing this function during a primary response when activating signals are strong, for example when 100 mg of OVA is administered. This can also occur following a prolonged memory phase, in the presence of additional allergen-alone re-exposures. Memory CD4$^+$ T cells provided signals like CD40L and IL-4 which are necessary for IgE isotype switching and B cell differentiation. Future research will investigate which phenotypes of CD4$^+$ T cells can hold IgE memory. As Tfh cells are required for IgE production during a primary response, it is logical to assume that memory Tfh cells hold this memory[61,62]. It is also possible that other memory phenotypes, like central memory T cells or memory Th2 cells, differentiate into Tfh cells upon recall, allowing for B cell activation.

B Cell Activation

Once antigen-activated B cells interact with Tfh cells, they isotype switch and differentiate either extrafollicularly into MBCs or antibody secreting cells, or enter the GC where they affinity mature, and then follow those fates. We have

demonstrated that cells can either directly class switch to IgE or sequentially switch through IgG1 to IgE, both of which yield anaphylactic IgE. In WT mice, early IgE production likely occurs through both routes, but IgE which emerges from late GCs likely exclusively comes from $IgG1^+$ cells which we have found are considerably dominant within the late GC. These late GC $IgG1^+$ cells are exported as high affinity, mature MBCs which are preferentially selected upon a recall response for differentiation into IgE-secreting cells[171]. Because of the dominance of $IgG1^+$ cells, most secondary IgE likely comes from these sources, explaining why the majority of IgE is sequentially switched. However, high affinity IgM cells are equally capable of generating anaphylactic IgE during a recall response, as is observed when they are not outcompeted by $IgG1^+$ cells in IgG1-def mice.

An outstanding question in the pathway of adaptive immune activation delineated here is what stimuli can drive B cell activation by primed memory T cells. We have demonstrated that administering additional allergen effectively drives B cell activation and IgE production. However, most allergic patients experience an allergic reaction on their first known exposure to the offending food[188]. It is plausible that low-dose accidental exposures such as contaminants in food preparation provide sufficient allergen for B cell activation. Alternatively, progression of allergic sensitization may be driven by bystander proliferation of memory cells. A study of patients immunized against tetanus demonstrated that memory for other antigens is boosted at the same time as tetanus specific immunity following immunization[181]. Indeed, in a model of co-sensitization with PN and

OVA, we observed that re-exposure with PN alone resulted in OVA-specific GC B cell activation[54]. Therefore, it is plausible that inflammatory stimuli such as vaccination or infection could drive the progression of pre-clinical adaptive immunity in allergic patients.

Immunological Memory has Multiple Redundancies.

A central concept which emerges from the work in this thesis is the multiple redundant layers that hold the memory of IgE responses. Numerous publications have pursued $IgG1^+$ MBCs as the primary reservoir of IgE memory[18,127,130,173]. Within this subset, the most mature ($CD80^+$ $CD73^+$ $PDL2^+$) cells are those which react immediately[127]. However, it was also observed that $IgG1^+$ MBCs with intermediate and low maturity ($CD80^+$ $CD73^-$, $CD80^-$ $CD73^-$) are also capable of holding IgE memory. A recent publication demonstrated that $CD80^+$ $CD73^+$ MBCs are those which emerge from GCs[171]. This is consistent with observations that the highest affinity MBC clones are selected in a secondary immune response[124]. We demonstrated here that redundancy extends to the isotype of MBC. At a minimum, direct switching of IgM^+ and IgM^+ IgD^+ MBCs was sufficient to elicit a secondary IgE response. Similar to $IgG1^+$ MBCs, varying levels of maturity existed within the IgM^+ MBC population. Given the competitive dominance of $IgG1^+$ cells among the memory repertoire, it is likely that the high affinity, mature $IgG1^+$ MBCs would be selected first for a memory response. In the absence of those cells, less mature $IgG1^+$ MBCs or mature IgM^+ MBCs would be selected. It is not clear which of

those two populations would be selected first. It is likely that the less mature, IgM^+ or IgM^+ IgD^+ MBCs would be the last option among MBCs.

In a scenario in which the allergen-specific MBC compartment has been depleted, the memory of IgE is retained by memory $CD4^+$ T cells, which could induce IgE production from naïve B cells. It has been observed in both mice and humans that the majority of the secondary IgE repertoire overlaps with the IgG repertoire, indicating that most memory IgE is likely derived from MBCs rather than *de novo* activation by memory T cells[161]. However, we have not directly evaluated the contribution of memory T cell/naïve B cell derived IgE to the total secondary IgE pool.

Such a deeply redundant system of humoral immunity is an advantage in infectious contexts. Re-infection with an identical pathogen boosts the most mature, highest affinity B cell population which has already isotype switched to optimize effector function. When re-infected with a mutated pathogen, the broad diversity of MBCs of varying affinity increases the chance that at least a few clones will be able to recognize the mutant with sufficient affinity to boost immunity and to effectively clear the pathogen. In a situation where a pathogen has sufficiently mutated to avoid B cell immunity, a reservoir of $CD4^+$ T cells, which recognize processed antigens rather than native conformations, can induce *de novo* activation of naïve B cells with superior affinity to the memory compartment to accelerate protection. These layers of redundancy ensure that the burden of protection is distributed among multiple cellular phenotypes, ensuring that a pathogen could not mutate

mechanisms to avoid detection by only one cell type and therefore be completely unidentifiable by the immune system.

Some pathogens have arguably evolved to exploit the intricacies of pre-existing immunity. In the context of influenza, infected individuals generate effective anti-influenza antibody responses against the strains that circulated during their first few years of life. This event is referred to as the "original antigenic sin" (OAS)[189]. Subsequent exposures to influenza boost the pre-existing immunity seeded during the OAS, sometimes to the detriment of the host[190,191]. The antibody responses boosted following infection with a mutated influenza strain often have lower affinity for the boosting strain than the OAS strain[189]. In these contexts, we hypothesize that it would be beneficial to induce *de novo* B cell immunity through our uncovered memory $CD4^+$ T cell/naïve B cell axis, which would allow for the most highly effective B cell precursors to be selected for immunity. It is possible that this axis of memory is too far down the hierarchical immune response to be induced when reasonable MBC clones exist. Immunization strategies targeting memory $CD4^+$ T cells may generate more effective antibody responses against some pathogens than those which target MBCs.

Clinical Implications

In the context of allergic disease, multiple layers of memory are a consideration which must be taken when designing novel therapeutics aimed to eliminate allergic immunity and induce tolerance. Existing therapies, like OIT, are relatively successful at altering some aspects of B cell memory. Daily ingestion of food allergens skews the humoral response away from IgE production, driving IgG4 immunity instead[192]. However, OIT does not fully eliminate nor reprogram pro-IgE B cell memory; as allergen-specific IgE returns upon cessation of allergen ingestion in the majority of cases[5]. OIT also dramatically reduces the number of allergen-specific Th2A cells, a population of mature memory $CD4^+$ T cells that are thought to drive allergic pathogenesis[183]. This reduction is not complete, however, as a detectable population of circulating Th2A cells remains following OIT[183]. One possible hypothesis is that these remaining Th2A cells induced *de novo* IgE production from naïve B cells upon cessation of allergen consumption. In summary, to reinstate tolerance in an allergic individual, a successful therapeutic strategy must eliminate or reprogram each layer of memory within highly redundant allergic memory system.

One promising therapeutic strategy is blocking IL-4Rα using a monoclonal antibody. We have recently published that this strategy aborts IgE production in PBMCs from allergic patients[131]. In mice, administering anti-IL-4Rα during allergen exposures blocked the generation of serum allergen-specific IgE during a recall response, and prevented anaphylaxis upon challenge[131]. We further found that

these changes may persist following cessation of anti-IL-4Rα therapy, as treated mice were protected upon subsequent challenges of allergen long after anti-IL-4Rα clearance. This approach makes rational sense. IL-4 signalling is required for IgE isotype switching and drives Th2 cell differentiation. However, Th2A cells remained in patients with atopic dermatitis who underwent anti-IL-4Rα therapy, potentially indicating that this may not fully impact existing terminally differentiated Th2 cells[184]. It is possible that a combination of OIT and anti-IL-4Rα therapy may interrupt or reprogram both the B cell and T cell arms of food allergy. In this context, the capacity of memory B and T cells to differentiate into pro-allergic phenotypes when re-exposed to allergen is blocked by anti-IL-4Rα, which may force the cells into other fates- perhaps Th1, Th17 or Treg fates for T cells, and IgG4 isotype switching for B cells. Should this change persist, allergic immunity could be reprogrammed.

Reprogramming type 2 immunity during the clinically reactive stage of food allergy is complex. Our observations indicate that there may be phases of food allergy that precede IgE production. It may be advantageous to target the early phases before persistent B cell immunity is seeded. There are two issues with this approach. Firstly, we would need to identify patients during these clinically silent phases. Screening for these patients is complex from a public health standpoint, but this is beyond the scope of this PhD thesis. Even if we had the ability to screen, we do not currently have predictive biomarkers that could identify these patients. For this reason, future experimentation in our model of pre-clinical allergic priming

should focus on the phenotype of allergen-specific T cells. These early T cells may be a strong prognostic indicator to identify patients who are likely to become allergic later in life.

Secondly, we would require a therapeutic strategy to interrupt these memory T cells. We identified IL-4Rα and CD40L as potential avenues, but both therapies interrupt many other physiological functions which may be particularly important in infancy. One theoretical approach could be to deplete $CD4^+$ T cells using a monoclonal antibody therapy. Such a measure would eliminate memory against all pathogens and deplete Treg cells which protect against autoimmunity, and therefore is not a feasible approach. A better approach might be to target the differentiation pathways of Th2 polarized effector cells. Aside from IL-4, we do not have a clear enough understanding of Th2 polarization to generate precision therapies to knock down this arm. Our understanding of these pathways is limited by our inability to visualize the critical cues exchanged at the DC/T cell and B/T cell interface *in situ*. Future research should capitalize on advances in massively multiplexed imaging, like mass ion beam imaging and fluorophore bleaching, which allow for many markers to be visualized on a single slide. A discovery approach visualizing many diverse pathways could help advance our understanding of which costimulatory molecules, cytokines, chemokines, and transcription factors are shared during type 2 polarization[193,194]. Further, contextual factors like tertiary immune and stromal cells which support DC/T cell and B/T cell interactions, could be uniquely unveiled using these imaging approaches, as they cannot be studied when cells are crushed

into suspension. A more concrete understanding of type 2 polarization may unveil novel markers against which next-line biologics could be targeted, dismantling $CD4^+$ T cell memory and aborting allergic immunity.

APPENDICES

Appendix 1: Antibodies used in Flow Cytometry Experiments

Marker	Clone	Colour	Step	Dilution	Source	Cat
B220	RA3-6B2	AF700	Extra	100	Biolegend	103232
Bcl6	7D1	APC	Intra	100	Biolegend	358506
Blimp-1	5E7	PE	Intra	100	Biolegend	150006
CD138	281-2	BV605	Extra	100	Biolegend	142516
CD138	281-2	APC	Extra	100	Biolegend	142506
CD19	6D5	PerCPCy-5.5	Extra	100	Biolegend	115534
CD3	17A2	BV711	Extra	100	Biolegend	100241
CD38	90	PECy7	Extra	100	Biolegend	102718
CD45.1	A20	BV605	Extra	100	Biolegend	110738
CD45.2	104	BV421	Extra	100	Biolegend	109832
CD73	TY/11.8	BV605	Extra	100	Biolegend	127215
CD80	16-10A1	APC	Extra	100	Biolegend	104714
CD86	GL-1	FITC	Extra	100	Biolegend	105006
CD95	Jo2	PE-Cy7	Extra	100	BD	557653
Cell Trace Violet			Pre-stain		ThermoFisher	C34571
F4/80	BM8	FITC	Extra	100	Biolegend	123108
F4/80	BM8	BV711	Extra	100-400	Biolegend	123147
GL-7	GL7	PerCPCy-5.5	Extra	100-400	Biolegend	144610
GL-7	GL7	AF647	Extra	100-400	Biolegend	144606
H+L		Pacific Blue	Intra	1000		P31581
IgD	11-26c.2a	BV510	Extra	100	Biolegend	405723
IgD	11-26c.2a	BV650	Extra	100	Biolegend	405721
IgD	11-26c.2a	BV605	Extra	100	Biolegend	405727
IgE	RME1-1	Purified	Extra	20	Biolegend	406902
IgE	RME1-1	PE	Intra	200-800	Biolegend	406908
IgG1	RMG1-1	Unlabeled	Extra	20	Biolegend	406602
IgG1	RMG1-1	BV421	Extra Intra	50 200-400	Biolegend	406616
IgG1	RMG1-1	APC	Extra Intra	50 200-400	BIolegend	406610
IgM	II/41	BV786	Extra	50	BD	743328
OVA		FITC	Extra	100	ThermoFisher	O23020
OVA		AF647	Extra	100	ThermoFisher	O34784
Siglec-F	E50-2440	PE	Extra	100	BD	552126
Fixable Viability Dye		eFluor 780	Extra	600	ThermoFisher	65-0865-18

Appendix 2: Tetramer Specificities used in Flow Cytometry Experiments

Antigen	Colour	Source	Cat
Ara h 1	APC	Recombinant	
BLG	PE	Sigma	L3908
NP-OVA	PE	BioSearch	N-5051
OVA	PE	Sigma	A503
RBD	PE	Recombinant	
Decoy	PE-Dylight594		
Decoy	APC-Dylight755		

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