MEMORY $CD4^+$ T CELLS IN FOOD ALLERGY

THE CRITICAL ROLE OF MEMORY $CD4^+$ T CELLS IN THE INAUGURAL GENERATION OF IgE

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LAY ABSTRACT

Food allergies are lifelong in many allergic patients, but mechanisms underlying food allergy development remain unclear. Allergic reactions are mediated by immunoglobulin E (IgE), which are food-specific antibodies. My thesis focuses on studying the incipient events leading up to the generation of food-specific IgE using mouse models. In mice, a single exposure to allergen alongside adjuvant did not generate IgE but did activate immune cells called $CD4^+$ T cells. These $CD4^+$ T cells did not resemble typical allergy-associated T cell subsets but were found to persist at multiple tissue sites. When mice received allergen again months later, the $CD4^+$ T cells evolved into a different subset, called T follicular helper cells, which are known to lead to IgE generation, and this promoted severe allergic reactions. Collectively, my work found that long-lived $CD4^+$ T cells are critical players in the early stages of food allergy development and drive inaugural IgE generation.

ABSTRACT

The global prevalence of food allergies is estimated at 10%, and approximately 80% of food allergies are lifelong. Food allergies are primarily mediated by allergen-specific IgE, which drives allergic reactions and is the hallmark of allergic sensitization. However, the initial generation of IgE is clinically silent and many patients present to the emergency department or clinic upon their first allergic reaction, whereupon they are already sensitized. This feature of allergic disease renders it difficult to study the incipient cellular events leading to inaugural IgE generation in humans, which may be important for generating lifelong allergic memory.

Using a mouse model of intragastric sensitization, we demonstrated that a single priming exposure to allergen alongside cholera toxin (CT) generates allergen-specific memory $CD4^+$ T cells which reside across multiple tissue sites, yet fails to induce B cell activation, antibody production, or clinical reactivity. These allergen-specific $CD4^+$ T cells resemble Teff cells but remain undifferentiated to typical allergy-associated T helper cell lineages, such as Th2 and Tfh cells, and IL-4Rα-mediated signaling is dispensable for their generation. Up to 14 months after their initial generation, primed memory $CD4^+$ T cells potently expanded and displayed plasticity upon allergen re-exposures. They differentiated into IL-4-transcribing Tfh cells and drove *de novo* IgE class-switching in naïve B cells, leading to allergen-specific IgE generation and clinical reactivity. This recall response was

dependent on IL-4 signaling and CD40/CD40L interactions and occurred across multiple tissue sites independent of S1PR-mediated cell migration, suggesting the potency of memory $CD4^+$ T cells in maintaining allergic memory. Altogether, our findings suggest that these plastic memory $CD4^+$ T cells are capable of independently perpetuating allergic disease. Our work positions memory $CD4^+$ T cells as a potentially crucial therapeutic target, which may aid in the design of disease-modifying therapeutics which induce long-term remission of food allergy in patients.

DEDICATION

This thesis is dedicated to my parents, Fiona and Tomasz Grydziuszko.

Mom and Dad, thank you for your constant support and encouragement. You have watched me succeed and fail throughout my undergraduate and graduate degrees and through all the highs and lows I've experienced, your belief in me has never wavered. I never have to wonder whether you're proud of me because you tell me constantly and I am so grateful to have such openly loving parents. I love you!

Mom, you taught me what it means to be kind and how to treat everyone with love. The patience you consistently demonstrate with everyone taught me to rely on my internal peace when everything around me seemed to be chaotic. You've shaped who I am and how I view the world in a way I can't begin to describe. Thank you for always being a phone call away.

Dad, from a young age, I learned the value of hard work and perseverance from you as you provided for a family of 8. I would not have made it through the grueling, long hours in lab without your example in the back of my head. You also passed down to me your scientific curiosity, which I'm convinced is why I view the immune system as a series of gears that are interconnected and why I'm forever interested in learning how it all works. But I'll still leave the work with the physical gears, nuts, and bolts to you…

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I'll leave whoever is reading this with a quote from my high school biology teacher, Peter Vlahovic. He had the best catchphrases, one of which became my all-time favourite phrase about education:

"You're all sponges right now! You just soak up all the information around you. Learn as much as you can while you're young."

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LIST OF ABBREVIATIONS

AF	Alexa Fluor
Alum	Aluminum Hydroxide (Alhydrogel)
ANOVA	Analysis of variance
APC	Allophycocyanin
BSA	Bovine Serum Albumin
CD	Cluster of differentiation
CpG	Cytosine phosphoguanine
cRPMI	RPMI containing 10% FBS
CSR	Class switch recombination
CT	Cholera toxin
cTfh	Circulating T follicular helper
CTV	CellTrace Violet
DC	Dendritic cell
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting/flow cytometry
FBS	Fetal bovine serum
FcεRI	High-affinity IgE receptor
FITC	Fluorescein Isothiocyanate
FPIES	Food protein-induced enterocolitis syndrome
GC	Germinal centre
H+L	Heavy and light chain, gamma immunoglobulins
HBSS	Hank's balanced salt solution
HDM	House dust mite
IC	Isotype control
ICOS	Inducible T cell co-stimulator
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneal
MBC	Memory B cell
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
mLNs	Mesenteric lymph nodes
NP	4-hydroxy-3-nitrophenyl
OD	Optical density
ODN	Oligodeoxynucleotide
OIT	Oral immunotherapy
OVA	Ovalbumin
PBS	Phosphate-buffered saline
PC	Plasma cell
PE	Phycoerythrin

RPMI	Roswell Park Memorial Institute medium
SEB	Staphylococcal enterotoxin B
ST2	IL-33 receptor
TCR	T cell receptor
Teff cell	Effector T cell
Tfh cell	T follicular helper cell
Th2 cell	T helper 2 cell
TMB	Tetramethylbenzidine
WT	Wild-type

DECLARATION OF ACADEMIC ACHIEVEMENT

Comprehensive Examination May 2023: **Pass**

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Declaration

This thesis document is an accurate summary of the research that I have conducted during my time as a Master's student. This thesis was written entirely by me with editing input provided by Dr. Manel Jordana.

I was the principal contributor to the work presented herein and was directly involved in performing, analyzing, and interpreting all experiments discussed below. This thesis work is in preparation for submission for publication and I share the co-first author position with Dr. Joshua Koenig. In addition to shaping the overall narrative and contributing throughout, Dr. Koenig contributed in large part to the OVA-specific B cell analyses herein. Dr. Roopali Chaudhary was responsible for the initial observations discussed in Figure 1 and performed the preliminary characterization of this model, with input from Dr. Rodrigo Jiménez-Saiz. Allyssa Phelps optimized the priming phenomenology with CpG in Figure 5, performed the preliminary allergen-specific B cell flows with CpG, and aided in experimental design. Dr. Justin J. Taylor from Fred Hutchinson Cancer Research Center generously provided OVA B cell tetramers as well as the tetramer construction

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INTRODUCTION

Introduction to Food Allergy

Food allergy is characterized by a type 2 immunopathogenic response to innocuous food antigens. Food allergies are primarily mediated by allergen-specific immunoglobulin E (IgE), the principal effector molecule in allergic responses and the hallmark of allergic sensitization.[1] Allergen-specific IgE binds to the surface of mast cells and basophils through the high-affinity IgE receptor (FcεRI).[2] Cross-linking of bound IgE to the offending allergen causes immediate degranulation of these cells and the release of pre-formed granules containing vasoactive mediators and rapidly synthesized inflammatory mediators which mediate clinical reactivity.[2] The gold standard for food allergy diagnosis is an oral food challenge, though the risk of allergic reactions has resulted in the frequent use of surrogate tests for diagnosis, such as determining allergen-specific IgE through skin prick tests and serum IgE levels.[1]

The Burden of Food Allergy

The prevalence of food allergy has been steadily increasing over the past few decades, with the global prevalence estimated at 10% as of 2018.[3] Currently, over 3 million Canadians self-report food allergies, almost 500,000 of which are children under 18 years of age.[4] In the United States, the total economic costs of food allergies is approximately $25 billion, which amounts to $4000 per person per year.[5] Indeed, food allergies are the leading cause of anaphylaxis and a staggering

124% increase in emergency department visits for food allergy-related anaphylaxis was observed between 2005 and 2014.[6] Furthermore, food allergies to peanut, tree nuts, fish, and shellfish persist for a lifetime in approximately 80% of patients.[7,8]

Current clinical recommendations include strict allergen avoidance and administration of emergency epinephrine upon accidental exposure.[1] Oral immunotherapy (OIT) has emerged as a treatment option, with OIT for peanut approved by the US Food and Drug Administration and with OIT using off-the-shelf foods recommended by the Canadian Society for Allergy and Clinical Immunology.[9,10] The majority of patients undergoing OIT achieve transient desensitization, such that their threshold for allergen consumption without clinical reactivity is increased while consuming a maintenance dose, followed by a relapse after cessation of therapy.[10–12] Remission of food allergy following the cessation of OIT is rare, and is thought to be maintained by frequent allergen consumption.[11] Therefore, no curative, disease-modifying treatments for food allergy exist for the majority of patients.

Food Allergy as a Phasic Disease

The development of food allergic sensitization in humans is associated with various genetic, dietary, and environmental factors; however, the complex interactions between these factors and underlying mechanisms remain poorly understood.[1] Many patients visit a physician or emergency department during or following their first allergic reaction, often upon their first *known* exposure to the offending food

allergen.[13–15] Indeed, 25% of food-induced anaphylactic reactions in Canadian children occurred in patients not previously diagnosed with food allergy.[16] This implies that elevated allergen-specific IgE already existed in the serum of these patients and, therefore, that they had been silently sensitized. Thus, the investigation of events leading to sensitization in humans is challenging because it is difficult to predict which patients will develop food allergy.

The available evidence regarding the development of food allergy suggests that it is a phasic disease. In patients allergic to aeroallergens, the generation of allergen-specific IgG predates the emergence of allergen-specific IgE.[17] Additionally, our lab has shown that, in an epicutaneous mouse model of food allergy, allergic sensitization also begins with an IgG1-dominant phase which progresses to IgE production following subsequent subclinical allergen exposures.[18] Furthermore, the mechanisms underlying non-IgE-mediated food allergies may point to a pre-humoral phase of atopy. For example, infants and young children with food protein-induced enterocolitis syndrome (FPIES) present with symptoms of delayed profuse vomiting manifesting within 1 to 4 hours of allergen ingestion that is thought to be mediated by $CD4^+$ T cells and innate immune cells.[3,19] Allergen-specific IgE is not initially present in the majority of these patients, but one study found that ~40% transitioned from milk FPIES to an IgE-mediated phenotype characterized by typical allergic reactions.[20] Furthermore, in a murine model of sensitization to the aeroallergen *B. tropicalis*, initial exposure to a low dose of allergen led to the generation of Th2 cells and group 2 innate lymphoid cells (ILC2s) but not IgE and

IgG1 production, which later emerged upon subsequent allergen re-exposures.[21] Collectively, this evidence suggests that allergic sensitization has a prolonged trajectory with successive phases, with the production of allergen-specific IgE representing the "final" phase. However, the immunological mechanisms underlying the incipient events leading to food allergy sensitization remain to be elucidated.

The Generation of Food Allergy

In the context of allergy, migratory dendritic cells (DCs) capture food allergens in the mucosa or skin and become activated, expressing costimulatory molecules such as CD80, CD86, CD40, and OX40L based on environmental cues.[2,22] Activated DCs begin expressing peptide-MHC II complexes and migrate to the draining lymph nodes where they activate naïve $CD4^+$ T cells.[2] These interactions lead to Th2 cell differentiation, with a particular role for OX40/OX40L interactions, IL-2 signaling, and autocrine/paracrine IL-4 signaling in driving fate decisions.[2,23–29] Th2 cells are characterized by elevated expression of transcription factor GATA3, the IL-33 receptor (ST2), and production of Th2 cytokines, particularly IL-4, IL-5, and IL-13, and have long been implicated in allergic disease as drivers of inflammation.[2,30,31] Particular subsets of Th2 cells such as the Th2A cell subset have been identified in patients allergic to multiple allergens as well as in other atopic disorders such as atopic dermatitis, asthma, and eosinophilic esophagitis.[31,32] These cells are highly differentiated, as evidenced by their lack of CD27 and CD45RB expression, and are characterized by co-expression of CRTh2, CD49d, and

CD161.[32] Th2A cells are considered clinically relevant, as their numbers are reduced following successful immunotherapy, though the mechanisms underlying their role in allergic pathogenesis remain unclear.[31,32]

In addition, DC-T cell interactions involving ICOS-ICOSL interactions, low IL-2Rα signaling, and robust IL-21 signaling lead to T follicular helper (Tfh) cell differentiation.[2,33] Tfh cells are classically defined by their expression of CXCR5, PD-1, and the transcription factor Bcl6, though there is extensive heterogeneity within the Tfh cell family with distinct polarizations present within different disease states (e.g. allergy, infection, autoimmunity).[33] During sensitization, naïve B cells capture allergen from subcapsular macrophages, internalize the antigen, and present it as peptide-MHC-II complexes before migrating to the T-B border.[2,33] Activated Tfh cells also migrate to the T-B border, mediated by their expression of CXCR5 which binds CXCL13 produced in the B cell follicle, and here they provide help to antigen-stimulated B cells.[2,33] Class switch recombination (CSR) is thought to be instructed at the T-B border through cognate interactions involving costimulation (e.g. CD40/CD40L, ICOS-ICOSL) and cytokine signals.[33] The generation of allergen-specific B cells is T cell-dependent and murine studies indicate that Tfh cells are critical for IgE CSR as producers of IL-4.[2,33–37] The genetic depletion of Tfh cells completely abrogated allergen-specific IgE and protected against anaphylaxis following attempted sensitization to peanut and aeroallergens.[34,36,38] Derived from human blood, $CXCR5^+$ circulating Tfh (cTfh) cells demonstrate

functional capabilities of Tfh cells and in culture, Th2-skewed cTfh cells induced naïve B cells to produce IgE and IgG through IL-4 and IL-21 secretion.[39]

Following interactions and CSR at the T-B border, the cognate Tfh cell-B cell pair migrate together to the B cell follicle and seed germinal centres (GCs).[2,33] A subset of IL-13^{hi} IL-4^{hi} IL-5^{hi} IL-21^{lo} GATA3^{hi} Tfh cells termed Tfh13 cells found in patients and mice sensitized to peanut or aeroallergens are required for high-affinity IgE production, potentially driven by their GC localization and promotion of IgE^{+} and IgG1^{+} GC B cell survival.[40] Allergen-specific memory B cells (MBCs) are thought to be primarily GC-derived, as this is the major site of affinity maturation.[2] MBCs are long-lived cells generated following the primary response and are the only previously identified reservoir of allergen-specific IgE memory.[18,41–44] IgE^{+} MBCs are extremely rare in mice and humans and thus are not considered clinically relevant reservoirs of memory.[2,45,46] Additionally, evidence has shown that IgE^{+} plasma cells (PCs) are short-lived, and thus long-lived IgE^{+} PCs are unlikely contributors to the maintenance of food allergy.[2,45] In contrast, IgG1^{+} MBCs have been shown to be long lived in a mouse model of food allergy and, as such, are thought to be the primary reservoir of allergen-specific IgE memory as, during recall responses, they rapidly undergo sequential CSR to produce high-affinity allergen-specific IgG1 and IgE.[18,41–44] CD4^{+} T cells and IL-4 are considered fundamentally required to initiate secondary IgE responses,[47–49] but whether naïve or memory CD4^{+} T cells are required in this process remains unclear.

Recall Responses of Memory CD4$^+$ T cells in Allergy

Following antigen clearance and GC collapse, the effector Tfh cell population contracts and memory Tfh cells persist, which downregulate expression of Bcl6, CXCR5, and PD-1 and express folate receptor 4.[50] The critical role of Tfh cells in IgE CSR during primary responses in mice may suggest a role for memory Tfh cells in secondary IgE responses and the perpetuation of allergic disease, though conflicting reports exist on Th2-polarized Tfh cell recall responses in mice.[33–35,51,52] Ballesteros-Tato *et al.* demonstrated that IL-4$^+$ Tfh cells generated following house dust mite (HDM) sensitization displayed plasticity upon recall and could differentiate into IL-4$^+$ IL-13$^+$ Th2 cells which drove allergic airway disease.[51] In contrast, Coquet *et al.* demonstrated that IL-21$^+$ Tfh cells generated following HDM sensitization did not differentiate into Th2 cells upon recall, but the Tfh cells did contribute to airway eosinophilia.[52] While the plasticity of allergic Tfh cells remains to be fully elucidated, HDM sensitization models also can not sufficiently address the role of Tfh cells in driving anaphylaxis-inducing IgE CSR upon recall, as asthmatic pathology is not primarily driven by IgE. Furthermore, work on recall responses of Th2-polarized memory Tfh cells in humans is limited. Human cTfh cells were capable of driving *de novo* IgE production in staphylococcal enterotoxin B (SEB)-pulsed naïve B cells in culture whereas naïve CD4$^+$ T cells did not induce antibody production, though these data may not be representative of food allergen-specific memory responses.[39]

In addition, the potential role of memory Th2 cells in perpetuating allergic disease remains largely unaddressed. Th2 cells are thought to primarily contribute to allergic inflammation in food allergy through recruitment and activation of eosinophils, mast cells, and basophils, rather than through driving IgE CSR.[31,33] For example, lung-resident HDM-specific memory Th2 cells were sufficient in driving airway hyperresponsiveness in an allergic asthma model, indicating the role of these cells in perpetuating certain allergic diseases.[53] However, the role of Th2 cells in holding memory of IgE in food allergy remains unclear. Th2A cells persist in food allergic patients and expand upon allergen exposure, suggesting their implication in allergic pathology.[32] The evidence that Th2 cell generation can occur prior to IgE production in response to aeroallergen exposure also suggests a potential role for these cells in holding allergic memory.[20,21]

Overall, MBCs are likely not the only cells required to trigger a recall response in food allergy and in this context, the role of T cells requires further investigation. Evidence relating to allergen-specific memory $CD4^+$ T cell responses upon recall is conflicting and has not sufficiently interrogated the role of memory $CD4^+$ T cells in driving *de novo* B cell responses versus reactivating MBCs to perpetuate and maintain allergen-specific IgE. This thesis aims to address the role of memory $CD4^+$ T cells in driving *de novo* B cell activation and inaugural allergen-specific IgE generation during the incipient events of food allergic sensitization. This work also investigates the mechanistic requirements for the generation of allergen-specific memory $CD4^+$ T cells and for the performance of their functions upon recall.

MATERIALS AND METHODS

Ethics Statement

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

Mice

Age and sex-matched controls were used for all experiments. Typically, female or male mice aged 6-8 weeks old were used. Wild-type (WT) C57Bl/6 mice were purchased from Charles River Laboratories (Wilmington, Mass.). Female CD45.1 mice (B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ) and male TCRβKO mice (B6.129P2-*Tcrb*^{tm1Mom}/J) were purchased from the Jackson Laboratory (Bar Harbor, Maine). CD90.1^{+} OT-II mice were a generous gift from Dr. Christopher D.C. Allen (University of California, San Francisco). Homozygous CD90.1^{+} OT-II mice and 4get (*Il4*^{tm1Lky}) mice on C57Bl/6 backgrounds were crossbred to generate OT-II/4get mice. Male OT-II mice and OT-II/4get mice were used in experiments within Figure 6 and 9.

Conventional model of intragastric sensitization

Mice were exposed via intragastric gavage to 1 mg ovalbumin (OVA) (Millipore Sigma, Cat: A5378) alongside 5 µg cholera toxin (CT) (List Biological Labs, Cat: 100B) suspended in 0.5 mL PBS. Intragastric gavages were performed using a 12-gauge metal gavage needle once per week for 4 weeks or twice per week for 2 weeks, for a total of 4 gavages. Two weeks after the final gavage, the mice were

challenged through intraperitoneal (i.p.) injection with 5 mg OVA. Core body temperature was recorded with a rectal thermometer every 10 mins for 40 mins post-injection. Retroorbital bleeds were performed the day before challenge to collect serum for OVA-specific antibody analysis by ELISA. In flow cytometry experiments, mice were sacrificed 7 days following the final gavage unless otherwise specified.

Adapted model of intragastric sensitization

Mice were exposed via intragastric gavage to either 1 mg OVA or 1 mg 4-hydroxy-3-nitrophenyl (NP)-OVA (LGC Biosearch Technologies, Cat: N-5051-100) alongside 5 µg CT suspended in 0.5 mL PBS using a 12-gauge metal gavage needle. Alternatively, mice were exposed via intragastric gavage to 1 mg OVA alongside 40 µg CpG ODN 1826 VacciGrade (InvivoGen, Cat: VAC-1826-1) suspended in 0.5 mL PBS using a 12-gauge metal gavage needle. After resting for a period from 1 week to 14 months, they were administered three intragastric gavages with either 1 mg OVA, 1 mg NP-OVA, or 1 mg NP-BSA without CT, either once weekly or every other day. Two weeks after the final gavage, the mice were challenged through i.p. injection with 5 mg OVA or 5 mg NP-BSA. Core body temperature was recorded with a rectal thermometer every 10 mins for 40 mins post-injection. Retroorbital bleeds were performed the day before challenge to collect serum for OVA-specific antibody analysis by ELISA. In flow cytometry experiments, mice were sacrificed 7 days following the final gavage unless otherwise specified.

ELISA Detection of Allergen-specific Antibodies

ELISA protocols used 96-well flat bottom absorptive plates. Serum dilutions were prepared in 1% skim milk in PBS using 96-well U bottom plates before being added to the ELISA plate. Washes were performed with PBS containing 0.05% TWEEN 20 using a Tecan Hydroflex. ELISAs were read using a ThermoScientific Multiskan FC plate reader read at 405 nm or 450 nm.

Allergen-specific IgG1

Plates were coated with 4 µg/mL of OVA (Sigma, Cat: A5378) or NP-BSA (LGC Biosearch Technologies, Cat: N-5050H-100) in 100 µL of carbonate bicarbonate buffer (Sigma, Cat: C-3041) and incubated overnight in the fridge. The following day, plates were aspirated through flicking upside down into the sink. Plates were blocked with 100 µL of 5% skim milk in PBS for 2 hours at room temperature. After 3 washes, serum dilutions which were prepared at 1:20, 1:200, 1:2000, and 1:20000 were added to the plate and incubated overnight in the fridge. The following day and after 3 washes, 50µL of 0.25 µg/mL biotinylated anti-mouse IgG1 (Southern Biotech, Cat: 1070-08) in 1% skim milk was added to the plate and incubated for 2 hours at room temperature. After 3 washes, 50 µL of 1:1000 streptavidin alkaline phosphatase in 0.3% BSA (Sigma, Cat: A4503) in PBS was added to the plate and incubated for 1 hour at room temperature protected from light. After 3 washes, the plates were developed through the addition of 50 µL of substrate, which was a 4-nitrophenyl phosphate tablet (Sigma, Cat: N-9389)

dissolved in 1x diethanolamine substrate buffer (ThermoFisher, Cat: 34064). Plates were stopped through addition of 25 µL of 1N NaOH and were read at 405 nm.

Allergen-specific IgE, IgG2b, IgG2c, or IgA

Plates were coated with 50 µL of 2 µg/mL anti-mouse IgE (R35-72 clone, BD Biosciences, Cat: 553413), 2 µg/mL goat anti-mouse IgG2b (Southern Biotech, Cat: 1091-01), 2 µg/mL goat anti-mouse IgG2c (Southern Biotech, Cat: 1078-01), or 2 µg/mL goat anti-mouse IgA (Southern Biotech, Cat: 1040-01) in PBS overnight in the fridge. The following day, plates were washed 3 times and blocked with 100 µL of 5% skim milk in PBS for 1 hour at 37°C. After 3 washes, serum dilutions which were prepared at 1:2 and 1:8 were added to the plate and incubated overnight in the fridge. The following day and after 5 washes, 50 µL of 300 ng/mL OVA or 300 ng/mL NP_{27}BSA conjugated to digoxigenin following manufacturer instructions (ANP Technologies, Cat: 90-1023-1KT) in 1% skim milk was added to the plate and incubated for 90 minutes at room temperature. After 5 washes, 50 µL of 1:5000 anti-digoxigenin POD fragments (Roche, Cat: 11 633 716 001) in 0.3% BSA in PBS was added to the plate and incubated for 1 hour at room temperature protected from light. After 5 washes, the plates were developed through the addition of 50 µL of substrate, which was TMB (Sigma, Cat: T0440). Plates were stopped through addition of 25 µL of 2N H_2SO_4 and read at 450 nm.

Fecal Pellet Processing

Upon collection, fecal pellets were stored at -80°C if they were not immediately used. To homogenize fecal pellets, the cOmplete Protease Inhibitor Cocktail (Sigma, Cat: 11697498001) was first prepared by dissolving a tablet in PBS according to manufacturer instructions. Fecal pellets from each mouse were weighed and placed into 5 mL round-bottom polystyrene tubes containing 500 µL of homogenizing buffer. Each sample was then homogenized for approximately 10 seconds using a POLYTRON PT 2100 Homogenizer (Kinematica). In between each sample, the probe was cleaned by pulsing in 70% ethanol and then by pulsing in PBS. Fecal homogenates were spun down at 13000 RPM for 10 minutes at 4°C. Supernatants were collected into new tubes and, prior to performing OVA-specific IgA ELISAs, supernatants were normalized based on the initial weight of the fecal pellets through dilution with PBS. Supernatants were stored at -80°C until use.

Tissue Processing

The mice were humanely killed under inhaled isoflurane anesthetic by cervical dislocation, and their mesenteric lymph nodes (mLNs), spleens, and/or small intestines were collected immediately thereafter. Mesenteric lymph nodes and spleens were collected directly into HBSS. The lymph nodes were crushed between frosted slides into a single-cell suspension with HBSS. The spleens were crushed through a 40-µm filter using a 3-mL syringe plunger with HBSS. Unless otherwise specified, mesenteric lymph nodes and spleens were resuspended in FACS buffer (2% FBS, 1 mM EDTA) and pooled. Following cuts at the pylorus and ileocecal

junction, the contents of the small intestines were flushed out with PBS using a 12-gauge gavage needle and then collected into complete RPMI containing 10% FBS (cRPMI). After fat and mesentery were removed manually using forceps, the intestines were opened longitudinally, and mucus was manually scraped away. Intestines were cut into approximately 3- to 5-mm pieces and excess mucus was eliminated through a 15-minute incubation at 37°C while shaking in PBS containing 10 mM HEPES and 1 mM DTT. Following the incubation, the tubes were vortexed and poured out over a strainer to collect the pieces of small intestine. Epithelial cells were then removed from the small intestine pieces by 3 rounds of 10-minute incubations at 37°C while shaking in PBS containing 10 mM HEPES, 5 mM EDTA, and 10% FBS. Tubes were again vortexed and the solution was poured out over a strainer. Intestine pieces were then digested through a 60-minute incubation at 37°C while shaking in RPMI containing 0.5 mg/mL collagenase A, 0.1 mg/mL DNase I, and 10% FBS. The tubes containing sample were then vortexed and pressed through a 70-µm cell strainer using a plunger from a 3-mL syringe, using fresh RPMI to aid in crushing. Following a wash, immune cells were purified using a 40%/70% Percoll gradient and centrifugation for 30 minutes. After the immune cell interface was collected into RPMI using a Pasteur pipette, the cells were washed and underwent the OVA-specific B cell tetramer enrichment protocol.

OVA-specific B cell enrichment

PE-conjugated or APC-conjugated OVA B cell tetramers were initially generously provided by Dr. Justin J. Taylor (Fred Hutchinson Cancer Research Center) and

then made in-house, as previously described.[54] The decoy tetramer was constructed as previously described[54] to gate out non-OVA binding B cells that bind to the backbone of the tetramer. Spleens and mLNs were crushed and pooled. Cells are resuspended in 20 µL FACS buffer with 1 µL of 1 µM PE-Dylight594-conjugated or 1 µL of 3 µM APC-Dylight755-conjugated decoy and incubated for 5 mins at room temperature. Next, 1 µL of the 1 µM PE-conjugated or 1 µL of 1 µM APC-conjugated OVA B cell tetramer was added without washing and incubated for 25 mins on ice. Cells were washed with 10-15 mL FACS buffer. Pellets were resuspended and incubated with 25 µL of anti-PE or anti-APC microbeads (Miltenyi Biotec) for 15 mins on ice. Two rounds of 5 mL of FACS buffer were added to cells and samples were run through LS columns (Miltenyi Biotec) on a magnetic stand. Columns were removed and two rounds of 5 mL of FACS buffer were plunged through the column to collect tetramer-bound cells (enriched fraction). Pellets were resuspended to a concentration of $\leq 1.0 \times 10^7$ cells per mL and $\leq 3.0 \times 10^6$ cells were plated in a 96 well U-bottom plate for subsequent flow cytometry staining and analysis.

OVA-specific T cell enrichment

APC-conjugated OVA2C-I-A^b-4E and OVA3C-I-A^b-4E MHC class II tetramers were generously provided by Drs. Marc Jenkins and Thamotharampillai Dileepan (University of Minnesota). Spleens and mLNs were crushed and pooled in 196 µL in cRPMI containing 50 nM dasatinib and incubated for 20 minutes at 37°C. 4 µL of the tetramers were added to the samples to a final concentration of 10 nM and

incubated for 45 mins at 37°C protected from light. Cells were washed with 10-15 mL FACS buffer to remove unbound tetramer. Pellets were resuspended and incubated with 50 µL of anti-APC microbeads for 30 mins on ice protected from light. 5 mL of FACS buffer was added to cells and samples were run through LS columns on a magnetic stand, including a second wash of 5 mL of FACS. Columns were removed and two rounds of 5 mL of FACS was plunged through the column to collect tetramer-bound cells (enriched fraction). Pellets were resuspended to a concentration of $\leq 1.0 \times 10^7$ cells per mL and $\leq 3.0 \times 10^6$ cells were plated in a 96 well U-bottom plate for subsequent flow cytometry staining and analysis.

CD90.1$^+$ donor T cell enrichment

Tissue processing was performed as usual with Hank's buffer and a subsequent wash step with FACS buffer. Cell pellets were then resuspended in 98 µL of FACS buffer and 1 µL of Fc block, purified anti-mouse CD16/32 (93 clone, Biolegend, Cat: 101302), and incubated for 10 mins on ice. 1 µL of APC-conjugated anti-mouse CD90.1 antibody was added to the samples without washing to a final dilution of 1:100. Cells were mixed thoroughly and subsequently incubated for 30 mins on ice protected from light. Cells were washed with 10-15 mL FACS buffer to remove unbound antibody. Pellets were resuspended and incubated with 25 µL of anti-APC microbeads for 15-30 mins on ice protected from light. 5 mL of FACS buffer was added to cells and samples were run through LS columns on a magnetic stand, including a second wash of 5 mL of FACS. Columns were removed and two rounds of 5 mL of FACS was plunged through the column to collect CD90.1

antibody-bound cells (enriched fraction). Pellets were resuspended to a concentration of ≤ 1.0 x 10^7 cells per mL and ≤ 3.0 x 10^6 cells were plated in a 96 well U-bottom plate for subsequent flow cytometry staining and analysis.

Flow Cytometry

Flow staining cells from tissues

Each sample was plated at a concentration of ≤ 1.0 x 10^7 cells per mL in a 96 well U-bottom plate. After spinning and decanting the supernatant, pellets were resuspended in Fc block, purified anti-mouse CD16/32 (93 clone, Biolegend, Cat: 101302), at a concentration of 1:50 in 25 µL FACS buffer and incubated for ≥ 10 mins on ice. For CD90.1^+ enriched fractions, this step was skipped as samples had already been incubated with Fc block. For samples which would later be stained intracellularly to detect IgG1 or IgE in B cells, purified anti-mouse IgE antibody (RME-1 clone, Biolegend, Cat: 406902) and/or purified anti-mouse IgG1 antibody (RMG1-1 clone, Biolegend, Cat: 406602) were included in the Fc block to avoid binding to IgE bound by FcεRI/II and IgG1 bound by FcγRs and thus ensure BCR isotypes could be identified.

Extracellular antibodies were added to samples without washing to final concentrations listed in **Appendices 1 and 2** based on a 50 µL staining volume and incubated for 30 mins on ice protected from light. If staining for CXCR5, the CXCR5 antibody was added to samples immediately following the Fc block and incubated for 30 mins at room temperature protected from light, followed by a wash

step with FACS buffer and staining with all other extracellular antibodies. If staining for GL7, the GL7 antibody was added to samples after the other extracellular antibodies, following a wash step with FACS buffer to avoid interactions between the IgM antibody and the GL7 antibody (rat IgM isotype), and incubated for 30 mins on ice protected from light. Following all extracellular antibody incubations, cells were washed twice with FACS buffer. If intracellular staining was required, the cells underwent fixation as described below. Otherwise, they were resuspended in 150 µL FACS buffer for flow cytometry analysis.

If staining for antibody isotypes such as IgE, IgG1, IgG2c, and IgG2b or for gamma immunoglobulins heavy and light chains (H+L), the BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences, Cat: 554714) was utilized and manufacturer instructions were followed. Cells were resuspended with 100 µL Fixation/Permeabilization solution and incubated for 20 mins on ice protected from light. Cells were then washed twice with BD Perm/Wash buffer. Intracellular antibodies were added to samples to final concentrations listed in **Appendix 1** based on a 50 µL staining volume and incubated for 30 mins on ice protected from light. Cells were washed twice with BD Perm/Wash buffer and subsequently resuspended in 150 µL FACS buffer for flow cytometry analysis.

If staining intracellularly for transcription factors such as GATA3, Bcl6, and FoxP3, the eBioscience Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher, Cat: 00-5523-00) was utilized and manufacturer instructions for

intracellular (nuclear) proteins were followed. Cells were resuspended with 200 µL FoxP3 Fixation/Permeabilization working solution and incubated for 30 mins on ice protected from light. Cells were then washed twice with Permeabilization Buffer. Intracellular antibodies were added to samples to final concentrations listed in **Appendix 2** based on a 50 µL staining volume and incubated for 45 mins on ice protected from light. Cells were washed twice with Permeabilization Buffer and subsequently resuspended in 150 µL FACS buffer for flow cytometry analysis.

Data were acquired with a BD LSRFortessa flow cytometer or with a BD FACS Aria II flow sorter. Data were analyzed with FlowJo version 10.8 software.

Flow staining cells from blood

Mice were anesthetized using isoflurane and bled retro-orbitally using heparinized capillary tubes. Blood was collected into K2EDTA-lined Microcontainer blood collection tubes (BD Biosciences, Cat: 365974) and immediately placed on ice. From collection tubes, 99 µL of blood from each sample were transferred into corresponding 1.7 mL Eppendorf tubes. 1 µL of Fc block was added to each sample, tubes were inverted several times to mix, and incubated for ≥ 20 minutes on ice. Extracellular antibodies were then added to samples without washing to final concentrations listed in **Appendix 3** based on a 150 µL staining volume and incubated for 30 mins on ice protected from light. Next, tubes were filled with 1.5 mL of eBioscience 1-step Fix/Lyse Solution (ThermoFisher, Cat: 00-5333-54), inverted several times to mix, and incubated for 15 mins at room temperature

protected from light. Blood was then spun down, supernatant was aspirated, and pellets were washed with 1.5 mL FACS buffer. The cells were spun down again, supernatant was aspirated, and pellets were resuspended in 200 µL FACS buffer for flow cytometry analysis.

Data were acquired with a BD LSRFortessa flow cytometer. Data were analyzed with FlowJo version 10.8 software.

Adoptive Transfers

B cell transfers

For transfers of OVA-enriched B cells in **Fig. 3E-F,** mLNs and spleens were collected from naïve CD45.1^{+} WT mice aged 6-8 weeks old. After processing, tissues were pooled, and cells underwent a magnetic bead-based enrichment for OVA-specific B cells using OVA B cell tetramers. The enriched fraction of B cells was stained with CellTrace Violet (CTV) (ThermoFisher, Cat: C34557) according to manufacturer recommendations. CTV-stained OVA-enriched B cells were then resuspended in 200 µL PBS and injected i.v. through the tail vein into age-matched C57Bl/6 recipients. The following day, WT recipients were gavaged with 1 mg OVA + CT or i.p. injected with 200 µg OVA + 2 mg Alhydrogel adjuvant 2% (Alum) (InvivoGen, Cat: VAC-ALU-250). Eight days later, mLNs and spleens from WT recipients were collected, pooled, and underwent a magnetic bead-based enrichment for OVA-specific B cells using OVA B cell tetramers prior to flow cytometry analysis.

$CD4^+$ T cell transfers

For transfers of naïve $CD4^+$ T cells in **Fig. 6E-K**, mLNs and spleens were collected from naïve $CD90.1^+$ OT-II or $CD90.1^+$ OT-II/4get mice aged 6-10 weeks old. After processing, tissues were pooled and $CD4^+$ T cells were isolated with the EasySep Mouse $CD4^+$ T cell Isolation Kit (StemCell, Cat: 19852) while following manufacturer instructions for the EasyEights EasySep Magnet (StemCell, Cat: 18103). Cells were stained for flow and $CD44^{lo}$ $CD4^+$ T cells were flow sorted into polypropylene round-bottom tubes which had been coated overnight with cRPMI. 1.0×10^5 flow-sorted $CD44^{lo}$ $CD4^+$ T cells were resuspended in 200 µL PBS and injected i.v. through the tail vein into age-matched C57Bl/6 recipients. Recipients were gavaged the next day with 1 mg OVA + CT or 100 mg OVA + CT or remained untreated as a control. Seven days later, mLNs and spleens from WT recipient mice were collected, pooled, and underwent a magnetic bead-based enrichment for $CD90.1^+$ T cells prior to flow cytometry analysis.

For transfers of naïve $CD4^+$ T cells in **Fig. 9**, mLNs and spleens were collected from naïve $CD90.1^+$ OT-II or $CD90.1^+$ OT-II/4get mice aged 6-10 weeks old. After processing, tissues were pooled and $CD4^+$ T cells were isolated with the EasySep Mouse $CD4^+$ T cell Isolation Kit. Cells were stained for flow and $CD44^{lo}$ $CD4^+$ T cells were flow sorted into polypropylene round-bottom tubes which had been coated overnight with cRPMI. Between 7.5×10^5 and 1.0×10^6 flow-sorted $CD44^-$ $CD4^+$ T cells were resuspended in 200 µL PBS and i.v. injected into age-matched C57Bl/6 recipients. Recipients were gavaged the next day with 1 mg OVA + CT.

Seven days later, mLNs and spleens were collected from the primed WT recipient mice and $CD4^+$ T cells were isolated as before with the EasySep Kit. Cells were stained for flow and $CD90.1^+$ $CD44^{hi}$ $CXCR5^-$ $CD4^+$ T cells (primed non-Tfh) were flow sorted into polypropylene round-bottom tubes precoated with cRPMI. Between 1.0×10^3 and 3.0×10^3 flow-sorted primed non-Tfh cells were resuspended in 200 µL PBS and i.v. injected into age-matched TCRβKO recipients. As a control group, a matched cell number of flow-sorted $CD44^{lo}$ $CD4^+$ T cells (naïve) derived from naïve $CD90.1^+$ OT-II or OT-II/4get mice were i.v. injected into a separate group of age-matched TCRβKO recipients. The following day, the TCRβKO recipients were i.p. injected with 200 µg OVA and 2 mg alum. Nine days later, mLNs and spleens from TCRβKO recipient mice were collected, pooled, and underwent a magnetic bead-based enrichment for $CD90.1^+$ T cells prior to flow cytometry analysis. Additionally, blood was collected from TCRβKO recipient mice for flow cytometry analysis.

Antibody Blockades and Depletions

IL-4Rα Blockade

A hybridoma secreting monoclonal rat IgG2a anti-mouse IL-4Rα (M1) was generously provided by Dr. Fred Finkelman (University of Cincinnati). Hybridomas were expanded and antibodies were purified as previously described.[47] Mice received a 250 µL i.p. injection of 1 mg of anti-IL-4Rα or 1 mg of rat IgG2a isotype control (2A3 clone, BioXCell, Cat: BE0089) the day before receiving allergen by intragastric gavage.

CD4 Depletion

Mice received an i.p. injection of 200 µg of anti-mouse CD4 monoclonal antibody (GK1.5 clone, BioXCell, Cat: BE0003-1) in 500 µL PBS three times every other day prior to priming or prior to OVA re-exposures, as previously described.[55] Administration of 100 µg anti-mouse CD4 antibody in 500 µL PBS once weekly maintained the depletion throughout the re-exposures.

CD40L Blockade

Mice received an i.p. injection of 350 µg of anti-mouse CD40L monoclonal antibody (MR-1 clone, BioXCell, Cat: BP0017-1) or 350 µg of polyclonal Armenian hamster IgG isotype control (BioXCell, Cat: BP0091) in 500 µL PBS three times every day prior to receiving allergen by intragastric gavage. Administration of 125 µg anti-mouse CD40L antibody in 500 µL PBS once weekly maintained the blockade throughout the re-exposures.

OX40L Blockade

Mice received an i.p. injection of 250 µg of anti-mouse OX40L monoclonal antibody (RM134L clone, BioXCell, Cat: BE0033-1) or 250 µg of rat IgG2b isotype control (LTF-2 clone, BioXCell, Cat: BE0090) in 500 µL PBS three times every other day prior to receiving allergen by intragastric gavage. When administered during priming, one additional injection of 250 µg antibody 2 days following the priming gavage maintained the blockade. When administered during

the re-exposures, injections of 250 µg antibody continued every other day throughout the gavage re-exposures until challenge to maintain the blockade.

FTY720 Treatment

Fingolimod (FTY720) HCl (SelleckChem, Cat: S5002) stock solution was prepared by dissolving in MilliQ water to a concentration of 69 mg/mL and stored at -80°C. For treatment, FTY720 was resuspended to 1.75 mg/L in autoclaved tap water. To deplete circulating lymphocytes by >90%, mice received 1.75 mg/L FTY720 as their drinking water ad libitum for 5 days prior to allergen exposure.

Data Analysis

Data were analyzed using Prism 9 software. Raw flow data were analyzed with FlowJo version 10.8 software and exported for Prism 9 analyses. Statistical analyses were performed using Mann-Whitney tests or one- or two-way ANOVAs. Data were considered significant if $p < 0.05$.

RESULTS

Priming with allergen and adjuvant establishes long-lived memory responses which are independent of humoral immunity.

A single intragastric exposure with adjuvant establishes long-lived memory in the absence of antibody production.

We have adapted a conventional murine model of intragastric sensitization to mimic the clinically silent phase of allergic sensitization. The well-characterized conventional model involves intragastric administration of an allergen, such as ovalbumin (OVA), alongside the Th2-polarizing mucosal adjuvant, cholera toxin (CT) to wild-type (WT) C57Bl/6 mice. Typically, four gavages of 1 mg OVA + 5 µg CT are administered, resulting in the consistent and robust generation of allergen-specific IgE. When these mice later receive an i.p. allergen challenge with 5 mg OVA, they undergo anaphylaxis due to the presence of high-affinity allergen-specific IgE. The multiple administrations of allergen in a sensitizing context allows for the study of active, clinical food allergy. However, the trajectory of sensitization throughout these administrations remains elusive and is difficult to assess due to the repeated exposure to the adjuvant.

To assess the immunological changes that may occur upon experiencing the allergen in a sensitizing context *for the first time*, we adapted that model of intragastric sensitization (**Fig. 1A)**. We found that a single administration of 1 mg OVA + 5 µg CT to WT mice did *not* result in the generation of allergen IgG1,

allergen-specific IgE, nor clinical reactivity upon i.p. allergen challenge (**Fig. 1B-F**). However, upon re-exposing the mice with three intragastric administrations of 1 mg OVA in the absence of adjuvant (which alone would typically induce tolerance in a naïve mouse), we found that the mice progressed to active allergy characterized by the production of allergen-specific IgG1 and IgE, which was comparable to the antibody production of allergic mice sensitized with four allergen + CT administrations (**Fig. 1B-C**). Upon challenge with the allergen, the mice experienced a drop in core body temperature, clinical signs of an allergic reaction, and an increase in hemoconcentration, which is the result of an increase in vascular leakage, collectively indicating that they had undergone an anaphylactic reaction (**Fig. 1D-F**). Thus, a single allergen exposure in the presence of a Th2 adjuvant is insufficient to sensitize mice, understood as the generation of allergen-specific IgG1 and IgE, but is sufficient to prime allergic immunity.

Next, we investigated whether the "priming" event in mice following a single 1 mg OVA + 5 µg CT intragastric exposure induced a memory response. At 1 week, 1, 3, 9, and 14 months following a priming exposure with 1 mg OVA + 5 µg CT, mice were exposed to three 1 mg OVA-alone administrations (**Fig. 1G**). In the absence of allergen re-exposures, levels of allergen-specific IgE remained undetectable up to 9 months post-priming, indicating that this response did not progress to clinical reactivity following priming (**Fig. 1H**). However, upon non-sensitizing OVA re-exposures, mice generated allergen-specific IgE which resulted in a drop in core body temperature and an increase in hemoconcentration upon challenge (**Fig. 1H-**

J). Collectively, these data suggest that allergic memory was established in a clinically silent manner, and that the capacity to generate allergen-specific IgE is retained for virtually the lifetime of the mouse. These findings further suggest that this model closely resembles the phasic progression of food allergy observed in humans compared to conventional food allergy models. Thus, we have used this mouse model to investigate the role of $CD4^+$ T cells in the early sensitization events of food allergy.

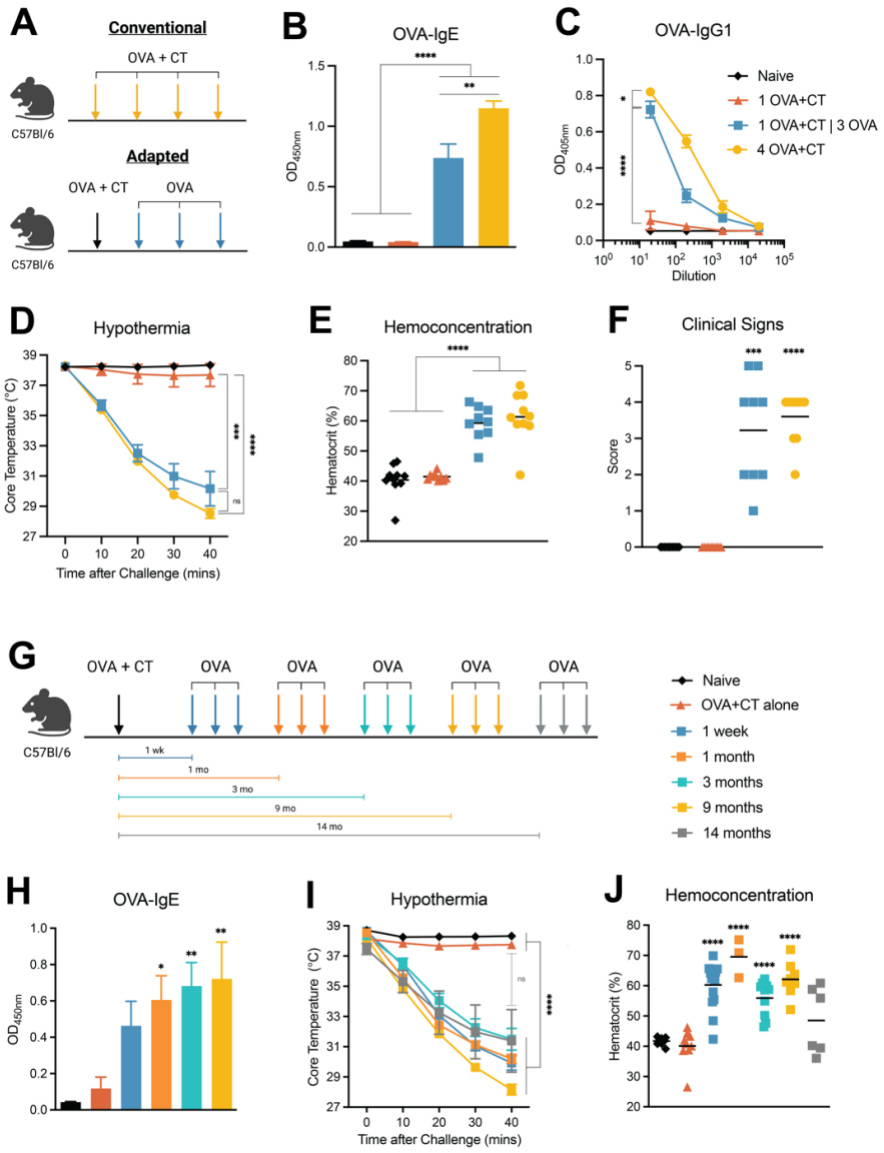

Fig. 1: A single intragastric exposure with adjuvant establishes long-lived memory in the absence of antibody production.

(A) Schematic of experimentation for panels B-F. Conventional intragastric sensitization models use multiple adjuvanted allergen exposures. The adapted model uses a single adjuvanted allergen exposure followed by multiple allergen-alone re-exposures. **(B-C, H)** Serum OVA-specific IgE and IgG1. **(D, I)** Core body temperature at 10-minute intervals during systemic challenge with OVA. **(E, J)** Hemoconcentration at 40 minutes after challenge. **(F)** Scoring of clinical signs during systemic allergen challenge. **(G)** Schematic of experimentation for panels H-J. Allergen-alone re-exposures were provided at various intervals of time following an initial adjuvanted allergen exposure. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$ via one-way or two-way ANOVA. n=3-6 per group, two (A-F) or three (G-J) independent experiments, plotted as means ± SEMs or medians (F).

Allergen-specific antibody production following priming is dependent on the allergen dose provided.

We posited that the lack of antibody production upon a single gavage exposure of 1 mg OVA + 5 µg CT was related to limited antigen availability. Thus, to test the dose dependency of this phenomenon, we provided a single gavage exposure to WT mice with either 1 mg, 10 mg, or 100 mg OVA alongside 5 µg CT **(Fig. 2A)**. No allergen-alone re-exposures were provided in these experiments **(Fig. 2A).** We then assessed the antibody response and clinical reactivity of the mice 2 weeks later by conducting ELISAs on serum and performing an i.p. allergen challenge. As before, we found that priming the mice with 1 mg OVA + CT did not induce OVA-specific IgE or IgG1 production **(Fig. 2B-C).** However, when the dose of allergen was increased to 10 mg OVA + CT, some mice exhibited limited antibody production **(Fig. 2B-C).** When the dose of allergen was further increased to 100 mg OVA + CT, all mice produced OVA-specific IgE and IgG1 **(Fig. 2B-C).** Additionally, all mice which received the 100 mg OVA + CT gavage exhibited clinical reactivity to the allergen, as indicated by their drop in core body temperature, elevated hemoconcentration, and high clinical score upon allergen challenge **(Fig. 2D-F)**. Thus, a single gavage with a high dose of OVA alongside CT drives antibody production. Conversely, a low dose of OVA alongside CT does not result in antibody production but does establish allergic memory **(Fig. 1)**. Therefore, this low dose gavage uncouples the imprinting of immunological memory from the generation of a humoral response, thus providing a useful mouse model to

interrogate the incipient events leading to food allergy that precede antibody production and clinical reactivity.

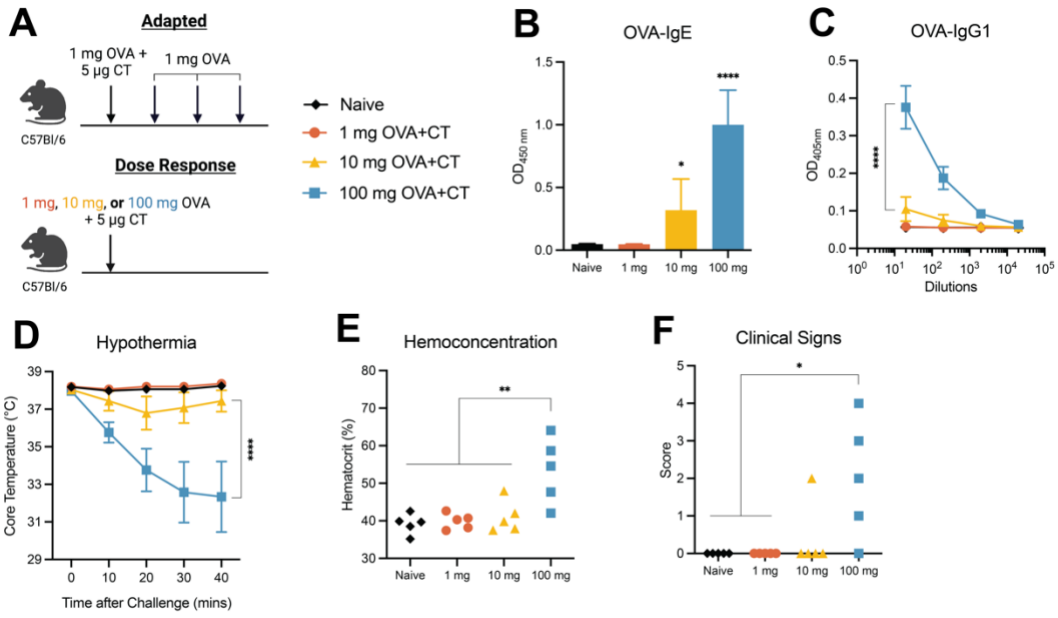

Fig. 2: Antibody generation following priming is dependent on allergen dose.
(A) Schematic of experimentation. **(B-C)** Serum OVA-specific IgE and IgG1 at 2 weeks post-gavage. **(D)** Core body temperature at 10-minute intervals during systemic challenge with OVA. **(E)** Hemoconcentration at 40 minutes after challenge. **(F)** Scoring of clinical signs during systemic allergen challenge. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$ via one-way or two-way ANOVA. n=5 per group, one independent experiment, plotted as means ± SEMs or medians (F).

Long-lived, clinically silent allergic priming occurs in the absence of B cell activation.

$IgG1^+$ MBCs are thought to be the primary reservoir of memory IgE responses which, upon allergen re-exposures, regenerate serum IgE through sequential CSR.[18,41] Thus, we wanted to determine whether the memory seeded following priming with 1 mg OVA + CT which persists for up to 14 months in mice was held by allergen-specific MBCs. Although no antibody production occurred following priming **(Fig. 1B, C),** it was possible that B cells were activated in a manner that restricted PC differentiation. To detect and characterize the allergen-specific B cell repertoire with a high resolution, we utilized OVA B cell tetramers which were constructed as previously described.[54] We then performed a magnetic bead-based enrichment on OVA-specific B cells from the mesenteric lymph nodes and spleens prior to flow cytometry analysis, which improves the resolution at which OVA-specific B cells can be identified compared to an unenriched sample **(Fig. 3A)**.

First, we interrogated whether B cells demonstrated evidence of activation following priming. We sacrificed WT mice at various timepoints following priming with 1 mg OVA + CT and found that the number of OVA-specific B cells detected was not significantly different compared to naïve mice across all timepoints **(Fig. 3C).** At all timepoints, the OVA-specific B cell repertoire consisted almost exclusively of unswitched B cells (IgD^+), with little to no class-switched B cells (IgD^-) **(Fig. 3B-C).** This suggested a lack of B cell activation following priming

with 1 mg OVA + CT. We also assessed surface CD86 expression on OVA-specific B cells, which is typically upregulated by B cells within 6 hours of antigen-specific BCR stimulation.[56] CD86 expression on OVA-specific B cells 7 days following 1 mg OVA + CT priming was not significantly different in MFI compared to naïve mice or compared to tolerant mice gavaged with OVA-alone **(Fig. 3D).** However, upon OVA-alone re-exposures administered after priming, OVA-specific B cells upregulated CD86 to a statistically significant degree **(Fig. 3D).** Furthermore, within OVA-specific B cells from allergic mice which received 3 OVA-alone re-exposures, both GC B cells ($GL7^+$ $CD38^-$) and MBCs ($GL7^-$ $CD38^+$) upregulated CD86 to a detectable degree, indicating that if either subset had been generated following priming alone, an increase in CD86 would be detectable **(Fig. 3D)**.

Although we had detected no evidence of OVA-specific B cell expansion at any timepoint following priming **(Fig. 3C),** we decided to assess whether the B cells proliferated in response to priming as a final measure. To do so, we performed a tetramer enrichment for OVA-specific B cells from the mLNs and spleens of naïve CD45.1 WT mice and subsequently stained the enriched B cells with CellTrace Violet (CTV), a dye which is diluted following cell proliferation **(Fig. 3E).** We then adoptively transferred the CTV-labelled B cells into naïve CD45.2 WT mice and the following day, we exposed the mice to antigen either by gavage with 1 mg OVA + CT gavage or by i.p. injection with OVA + alum as a positive control **(Fig. 3E).** When we collected mLNs and spleens from the recipient mice 8 days later and enriched for OVA-specific B cells, we found that the positive control group had no

detectable CTV signal in the donor CD45.1$^+$ OVA-specific B cells, indicating that the cells had proliferated extensively and diluted the CTV past the threshold of detection **(Fig. 3F).** However, the donor CD45.1$^+$ OVA-specific B cells from the primed recipients retained a bright CTV median fluorescence intensity (MFI) which was not significantly different from the donor B cells from recipients which had not received an antigen exposure, indicating that OVA-specific B cells did not undergo division following priming with 1 mg OVA + CT **(Fig. 3F).** Altogether, these data demonstrate that OVA-specific B cells remain naïve following priming.

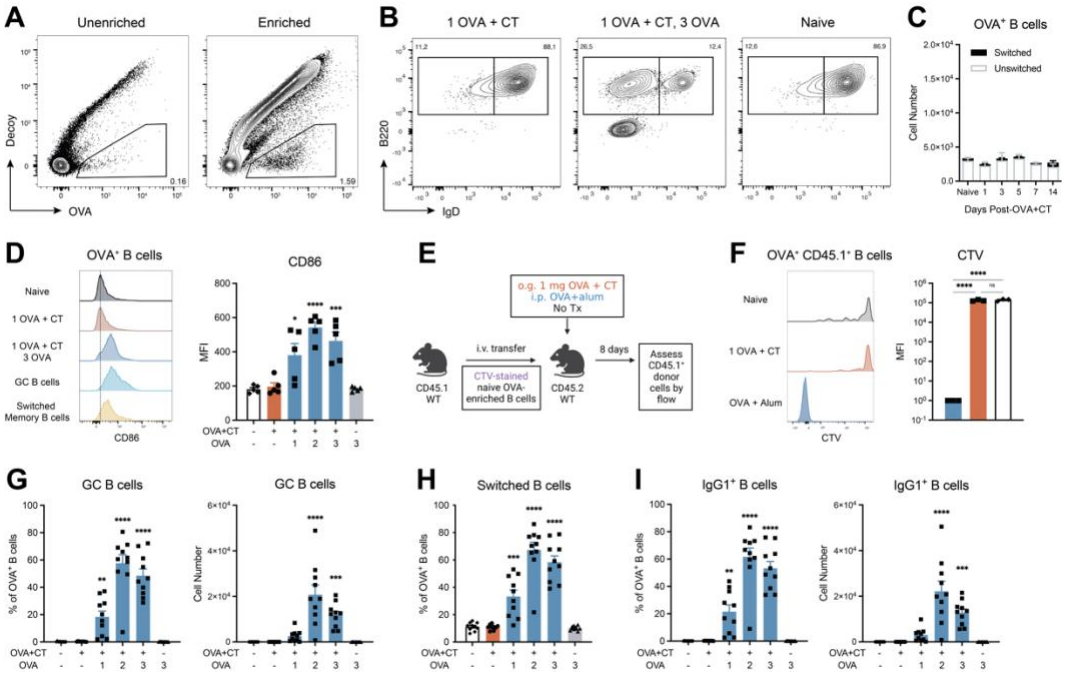

Fig. 3: B cells remain naïve following priming in the known inductive sites of the mLNs and spleen.

(A-I) WT mice received a priming gavage of OVA and CT followed by a varying number of OVA-alone re-exposures administered 7 days apart. Pooled cells from the spleens and mesenteric lymph nodes were analyzed by flow cytometry 7 days after the final gavage. **(A)** Sample flow cytometry plots of tetramer-enriched OVA-specific B cells. **(B)** Representative flow cytometry plots of gating on class-switched OVA-specific B cells in primed, primed + re-exposed, and naïve mice. **(C)** Summary plot of frequency and number of switched IgD^- and unswitched IgD^+ OVA-specific B cells at the indicated days following a single OVA+CT priming exposure. **(D)** Histograms and MFI of CD86 on OVA-specific B cells. (**E)** Schematic of experimentation. **(F)** Histograms and MFI of CellTrace Violet (CTV) dye on donor $CD45.1^+$ OVA-specific B cells. **(G)** Frequency and number of $GL7^+$ $CD38^-$ OVA-specific GC B cells. **(H)** Frequency of switched IgD^- OVA-specific B cells. **(I)** Frequency and number of OVA-specific $IgG1^+$ B cells. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$ via one-way ANOVA, with comparisons to the naive group and primed group plotted. n=5 per group, two independent experiments, plotted as means ± SEMs.

We next assessed the changes to the B cell compartment during the OVA-alone gavage re-exposures after the priming event, whereupon we observe the emergence of OVA-specific IgE and IgG1 and clinical reactivity **(Fig. 1B-F).** Here, we used tetramers to characterize the OVA-specific B cell repertoire in the mLNs and spleens 7 days following each gavage exposure. Following the first OVA-alone re-exposure, the OVA-specific GC B cell response began to emerge, which peaked after the second re-exposure with 21,000 GC B cells that comprised 58% of OVA-specific B cells, and slightly contracted after the third re-exposure **(Fig. 3G).** When we assessed class-switched B cells, we observed a similar pattern where the frequency of class-switched B cells peaked after the second OVA-alone re-exposure at 67% of OVA-specific B cells, though this population was present following each re-exposure **(Fig. 3H).** Most of these OVA-specific class-switched B cells were $IgG1^+$, which is the dominant isotype associated with typical Th2 responses and comprised 62% of OVA-specific B cells at the peak of the response **(Fig. 3I).** In contrast, we found no increase in the frequency or number of OVA-specific GC B cells or class-switched B cells ($IgG1^+$ or otherwise) following priming with 1 mg OVA + CT compared to either naïve mice or tolerant mice gavaged 3 times with OVA-alone **(Fig. 3G-I).** This was consistent with previous evidence demonstrating a lack of B cell activation in response to priming **(Fig. 3B-F).** These data further demonstrate that B cells remain naïve at the known inductive sites of the mLNs and spleens following priming and that OVA-alone re-exposures are sufficient to induce GC responses and CSR. This is notable, as providing 3

OVA-alone re-exposures is typically tolerizing when not preceded by priming with 1 mg OVA + CT, which suggests that immunological memory of IgE responses generated following priming is held by immune cells other than MBCs.

To further confirm that B cells remain naïve following priming, we assessed OVA-specific B cells in the Peyer's patches and lamina propria of the small intestines following a priming gavage with 1 mg OVA + CT or sensitizing gavage with 100 mg OVA + CT. We did not perform a magnetic bead-based enrichment for OVA-specific B cells as in previous experiments, as the yield of cells from the small intestines was lower and thus analysis of the entire unenriched sample was feasible. With the OVA B cell tetramers, we were able to detect an average of 20,000 OVA-specific B cells per mouse in the 100 mg OVA + CT gavage group, which was significantly higher than the number of B cells detected in the primed mice at all timepoints and in the naïve mice **(Fig. 4A).** Within the OVA-specific B cells, 65% were GC B cells following the 100 mg OVA + CT gavage for an average of 13,000 GC B cells, which was significantly greater than the frequency and number of GC B cells detected in the naïve mice and primed mice at all timepoints **(Fig. 4B-C).** Thus, we concluded that GC activity is not present in the small intestines following priming with 1 mg OVA + CT, which is consistent with the lack of GC responses in the mLNs and spleen.

Next, CSR within OVA-specific B cells in the small intestines was assessed. Across all timepoints post-priming with 1 mg OVA + CT, there was no significant increase

in frequency or number of switched B cells compared to naïve mice **(Fig. 4D).** Meanwhile, in mice exposed to 100 mg OVA + CT, approximately 97% of OVA-specific B cells were class-switched (IgM^- IgD^-) **(Fig. 4D).** Furthermore, approximately 72% of OVA-specific B cells were switched to the IgG1 isotype following 100 mg OVA + CT gavage, which was significantly greater than the primed and naïve groups of mice **(Fig. 4E)**. Finally, we decided to determine whether IgA-secreting PCs were generated post-priming using a functional readout to assess IgA secretion into the intestinal lumen. We collected fecal pellets from primed mice at 7-, 14-, 21- and 28-days post-gavage and analyzed OVA-specific IgA from the fecal homogenates **(Fig. 4F).** OVA-specific IgA was not present above naïve levels at any of the assessed timepoints post-priming with 1 mg OVA + CT **(Fig. 4F).** This indicated that CSR to IgA was also not occurring in B cells in the small intestines.

Altogether, these data demonstrate that OVA-specific B cells in the small intestines remain naïve and do not become activated following priming with 1 mg OVA + CT, like the OVA-specific B cells in the mLNs and spleen. However, when the initial sensitizing exposure to allergen involves a high dose of 100 mg OVA alongside 5 µg CT, there is sufficient antigen to drive GC responses and CSR with a single exposure. This suggests that limited antigen availability following 1 mg OVA + CT gavage prevents B cell activation upon a single gavage, yet long-lived immunological memory is generated.

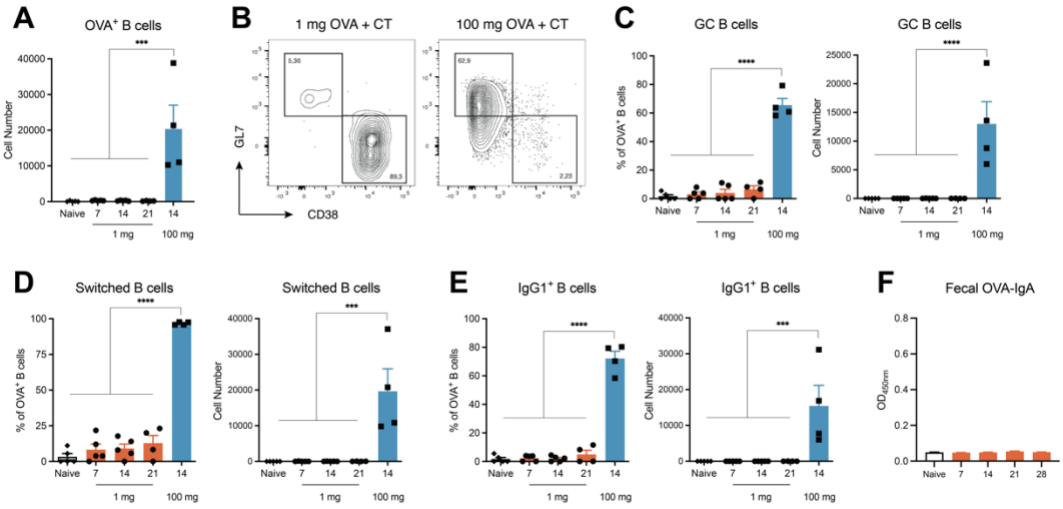

Fig. 4: B cells remain naïve following priming in the small intestines.
WT mice were administered 1 mg OVA + CT or 100 mg OVA + CT. Pooled cells from the Peyer's patches and lamina propria of the small intestines were analyzed by flow cytometry at various timepoints post-gavage. **(A)** Number of OVA-specific B cells. **(B)** Concatenated flow cytometry plots of OVA-specific B cells 14 days after gavage with 1 mg OVA + CT or 100 mg OVA + CT. **(C)** Frequency and number of $GL7^+$ $CD38^-$ OVA-specific GC B cells. **(D)** Frequency and number of switched IgM^- IgD^- OVA-specific B cells. **(E)** Frequency and number of OVA-specific $IgG1^+$ B cells. **(F)** OVA-specific IgA from fecal homogenates at various timepoints following gavage with 1 mg OVA + CT. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$ via one-way ANOVA. n=4-5 per group, one independent experiment, plotted as means ± SEMs.

B cell-independent memory generation occurs following priming under both Th2- and Th1-polarizing conditions, yet divergent antibody isotypes are generated upon allergen re-exposures.

We have demonstrated the utility of the adapted intragastric model for investigating the generation of immunological memory following priming occurring prior to B cell responses and antibody production, which models the natural trajectory observed in patients who ultimately develop allergy. Additionally, we decided to assess whether this priming phenomenology was dependent on the administration of a Th2 adjuvant such as CT or whether a similar phasic progression would occur during a Th1-polarized immune response. CT is a potent mucosal adjuvant derived from *Vibrio cholerae* and is commonly used to induce food allergy in mice.[57] CT is thought to bind mucosal epithelial cells and induce alarmin secretion and, furthermore, CT preferentially engages $CD103^+$ DCs and induces upregulation of costimulatory molecules such as OX40L.[27,28,57,58] These OX40L-expressing DCs drive strong IL-4-dependent Th2-polarized $CD4^+$ T cell responses which in turn drive IgG1, IgE, and IgA antibody responses.[27,28,38,59] The use of cytosine phosphoguanine (CpG) oligodeoxynucleotides (ODNs) as a vaccine adjuvant has emerged in recent years, with CpG 1018 used in the Heplisav-B vaccine for the hepatitis B virus.[60] CpG ODNs act as an agonist for TLR9, which leads to TLR9-mediated MyD88 activation in DCs and potently induces an almost exclusively Th1 response.[59,60]

Thus, to test whether Th1-polarized memory could be also generated following priming, we administered a priming gavage of 1 mg OVA + CpG to WT mice. In one group, 7 days following the priming gavage, we administered three OVA-alone re-exposures every other day. First, we assessed serum antibody responses by ELISA 7 days after the final gavage exposure. Similar to priming with 1 mg OVA + CT, the priming gavage with 1 mg OVA + CpG failed to induce a detectable antibody response, with no elevation in OVA-specific IgG2c, IgG2b, IgE, or IgG1 above naïve levels **(Fig. 5A-D).** However, when OVA-alone re-exposures were administered following priming with OVA + CpG, the mice generated robust OVA-specific IgG2c and IgG2b responses **(Fig. 5A-B).** Importantly, there was no production of OVA-specific IgE or IgG1 in these mice, in contrast to mice primed with OVA + CT and re-exposed with OVA-alone, which demonstrated significant production of IgE and IgG1 and marginal production of IgG2b **(Fig. 5B-D).**

Next, we utilized B cell tetramer enrichments to assess the OVA-specific B cell repertoire within the mLNs and spleens of mice administered a priming gavage of OVA + CpG with or without OVA-alone re-exposures. Within the OVA-specific B cells of OVA + CpG primed mice, we observed a complete lack of GC B cells and class-switched B cells, which were comparable to naïve levels **(Fig. 5E-F).** However, there was an emergence of GC B cells after the OVA re-exposures, which comprised 6.5% of OVA-specific B cells with an average of 560 GC B cells detected per mouse **(Fig. 5E)**. In addition, approximately 1200 class-switched OVA-specific B cells were detected per mouse following the re-exposures, which

comprised 12.6% of the OVA-specific repertoire **(Fig. 5F).** We assessed the isotype of these class-switched B cells and demonstrated that $IgG2b^+$ and $IgG2c^+$ OVA-specific B cells were significantly elevated in frequency and number in the primed and re-exposed group compared to both the naïve mice and the primed mice **(Fig. 5G-H)**. Additionally, we detected a small population of $IgG1^+$ OVA-specific B cells in the primed and re-exposed group, though this was not a significant proportion of the OVA-specific repertoire **(Fig. 5H)**.

Altogether, these data demonstrate that the priming phenomenology is not exclusive to Th2 responses and that priming with both Th2 and Th1 adjuvants can generate immunological memory that is B cell-independent. Furthermore, allergen-alone re-exposures following priming lead to divergent CSR depending on the adjuvant administered during priming, which suggests that polarized memory cells are generated which are programmed to activate B cells through distinctive mechanisms. Since this memory persists for up to 14 months following priming with OVA + CT and can be recalled to initiate IgE production and clinical reactivity, we hypothesized that $CD4^+$ T cells hold this memory following priming.

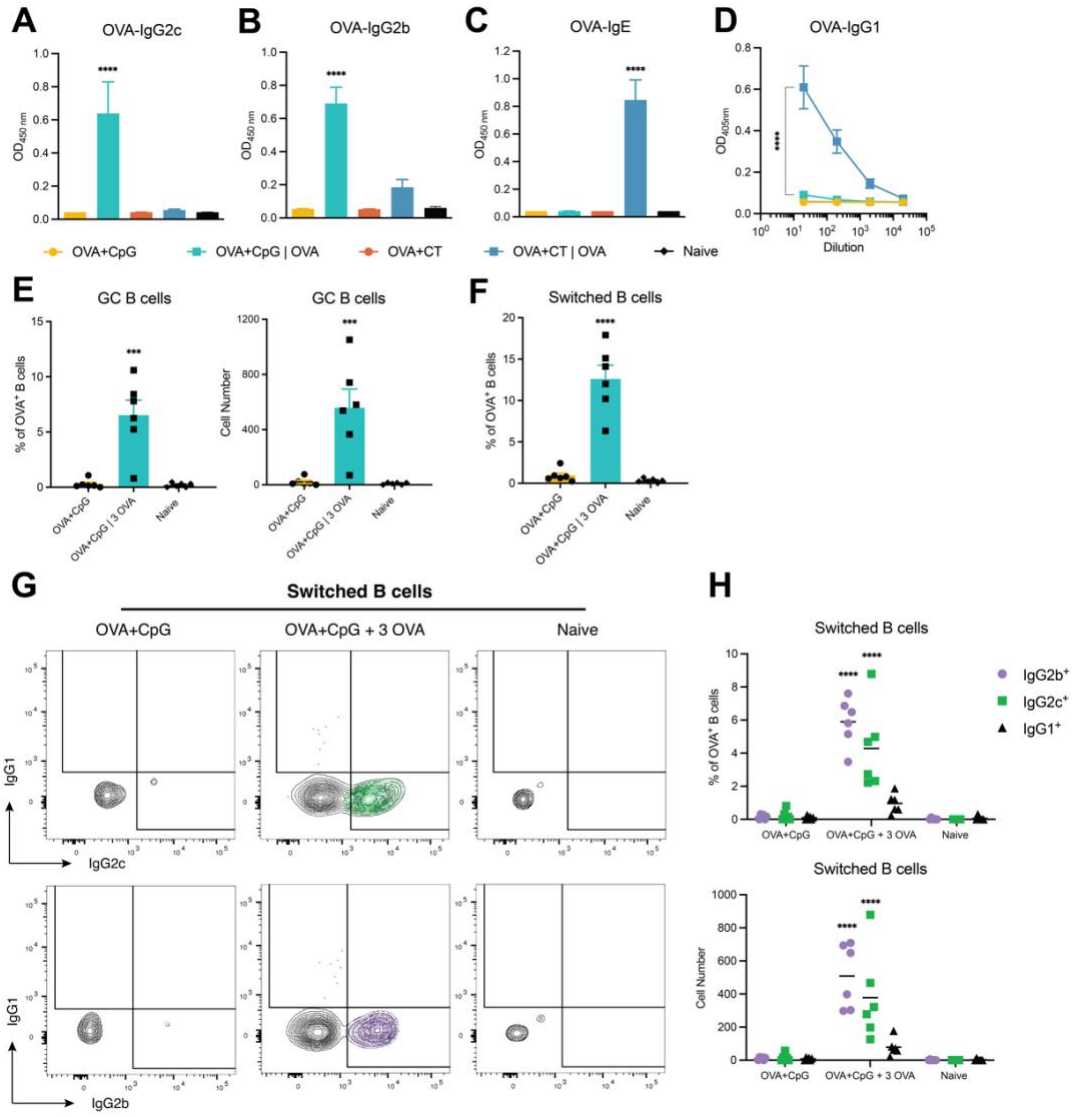

Fig. 5: Priming under Th2- and Th1-polarizing conditions leads to divergent antibody isotype switching upon allergen re-exposures.
WT mice received a priming gavage of 1 mg OVA + CpG or 1 mg OVA + CT. Beginning 7 days after the priming gavage, 3 OVA-alone re-exposures were administered every other day. Seven days after the final gavage, serum was collected and pooled cells from the spleens and mesenteric lymph nodes were analyzed by flow cytometry. **(A-D)** Serum OVA-specific IgG2c, IgG2b, IgE, and IgG1 assessed by ELISA. **(E)** Frequency and number of $GL7^+$ $CD38^-$ OVA-specific GC B cells. **(F)** Frequency of switched IgM^- IgD^- OVA-specific B cells. **(G)** Representative flow cytometry plots of expression of IgG1, IgG2b, and IgG2c on switched IgM^- IgD^- OVA-specific B cells. **(H)** Frequency and number of switched IgM^- IgD^- OVA-specific B cells. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$ via one-way ANOVA or two-way ANOVA (Panel D, H), with comparisons to the naive and primed group plotted. n=3 per group, two independent experiments, plotted as means ± SEMs.

Priming with allergen and adjuvant seeds memory CD4$^+$ T cells which drive the inaugural generation of IgE upon allergen re-exposures.

CD4$^+$ T cells are generated following priming but remain undifferentiated to the allergy-associated Tfh and Th2 lineages.

To investigate whether OVA-specific CD4$^+$ T cells imprint early allergic memory of IgE responses, we first assessed whether these cells are activated and expand following priming. To detect allergen-specific CD4$^+$ T cells, we used affinity-enhanced OVA2C/3C:I-A^b-4E MHC II tetramers to detect these rare cells.[61] We performed a magnetic bead-based enrichment on tetramer-binding CD4$^+$ T cells from the mLNs and spleens of WT mice 7 days following priming with 1 mg OVA + CT for flow cytometry analyses, which enhanced the resolution of the detected OVA-specific CD4$^+$ T cells compared to an unenriched sample **(Fig. 6A)**. Following priming, we observed a small but distinct population of OVA-specific CD4$^+$ T cells with approximately 300 cells detected per mouse, which was 1.6-fold higher than the number of cells detected in naïve mice, suggesting that the CD4$^+$ T cells had expanded in response to antigen stimulation (**Fig. 6B**). As expected, the expansion of OVA-specific CD4$^+$ T cells was greater in allergic mice exposed four times to 1 mg OVA + CT, with approximately 500 cells detected per mouse **(Fig. 6B).** Within the OVA-specific CD4$^+$ T cells, 36% were CD44^{hi} in the primed mice, which suggests an intermediate level of activation compared to CD44^{hi} CD4$^+$ T cells comprising 70% of OVA-specific CD4$^+$ T cells in allergic mice and 24% in

naïve mice **(Fig. 6C)**. We next assessed the expression of CXCR5 and CD62L within OVA-specific $CD44^{hi}$ $CD4^+$ T cells and found that few $CXCR5^+$ $CD62L^-$ Tfh-like cells were present in mice primed with 1 mg OVA + CT, with an average of 18 cells detected per mouse **(Fig. 6D-E)**. Meanwhile, an average of 75 Tfh-like cells per mouse were detected in the allergic group, which was significantly higher than both primed and naïve mice **(Fig. 6D-E).** Instead, the OVA-specific $CD4^+$ T cell response to priming is dominated by $CXCR5^-$ $CD62L^-$ Teff cells, with approximately 80 cells detected per mouse **(Fig. 6D, F)**.

The MHC II tetramers were not able to detect sufficient OVA-specific $CD4^+$ T cells following priming with 1 mg OVA + CT for in-depth phenotyping of these cells. We assert that this is not due to technical issues with the tetramers themselves, as we were able to detect approximately 9500 OVA-specific $CD4^+$ T cells in mice robustly sensitized with an i.p. injection of OVA + alum (data not shown). Instead, we hypothesize that the low cell yield following priming with 1 mg OVA + CT is due to the phenomenology itself, which is associated with an insufficient number of activated $CD4^+$ T cells and the seeding of memory, which enables the study of immunological memory that is uncoupled from antibody production and B cell activation. To overcome the low number of OVA-specific $CD4^+$ T cells, we used $CD90.1^+$ OT-II transgenic mice or $CD90.1^+$ OT-II mice crossed with IL-4 reporter (4get) mice in an adoptive transfer system. Over 90% of OT-II $CD4^+$ T cells express a transgenic TCR that is specific to OVA; however, OT-II mice cannot be sensitized efficiently and lack the ability to produce OVA-specific antibodies upon intranasal

immunization with OVA and CT.[62,63] Thus, we intravenously transferred 1.0 x 10^5 flow-sorted $CD44^{lo}$ $CD4^+$ T cells from naïve $CD90.1^+$ OT-II mice or OT-II/4get mice into WT recipient mice and exposed them by gavage to 1 mg OVA + CT or 100 mg OVA + CT the following day **(Fig. 6G).** This pre-transfer system allowed us to detect a larger number of allergen-specific $CD4^+$ T cells than through tetramer enrichments by boosting the frequency of naïve OVA-specific $CD4^+$ T cells and tracking the $CD90.1^+$ donor cell population in the WT recipients. Additionally, the use of OT-II/4get mice as recipients allowed us to assess IL-4 transcription within allergen-specific $CD4^+$ T cells through GFP-linked 4get transcription.[47,64] Next, we collected mLNs and spleens from the primed WT recipient mice and performed a magnetic-bead based enrichment on the $CD90.1^+$ donor T cells to assess OVA-specific $CD4^+$ T cell responses via flow cytometry analysis **(Fig. 6G)**.

First, we assessed expression of CXCR5 and PD-1 within the $CD90.1^+$ $CD4^+$ T cells **(Fig. 6H).** Approximately 1.3% of these cells were $CXCR5^{hi}$ $PD\text{-}1^+$ Tfh cells following 1 mg OVA + CT gavage which amounted to less than 60 Tfh cells per mouse, which was not significantly higher than untreated mice **(Fig. 6I).** Meanwhile, the mice which received a 100 mg OVA + CT gavage exhibited an increased frequency of Tfh cells with approximately 875 Tfh cells detected per mouse, which was a statistically significant increase compared to both primed and untreated mice (**Fig. 6I**). Furthermore, among the limited number of Tfh cells generated following priming, the MFI of Bcl6 was 2.2-fold higher than in an oral tolerance group which received three 1 mg OVA-alone gavages yet was 1.4-fold

lower than in allergic mice, suggesting that primed Tfh cells were not strongly committed to the Tfh cell lineage **(Fig. 6J).** Instead, the most prevalent subset in mice primed with 1 mg OVA + CT were non-Tfh cells with a Teff cell phenotype ($CD44^{hi}$ $CD62L^{-}$ $CXCR5^{-}$ $PD\text{-}1^{-}$), with approximately 1520 of these cells detected per mouse **(Fig. 6K)**. These Teff cells comprised 37% of the donor T cells, which was a statistically significant increase in frequency compared to untreated mice at 2.4% **(Fig. 6K)**. The observed phenotype of the primed $CD90.1^{+}$ T cells was consistent with flow analyses assessing the polyclonal OVA-specific $CD4^{+}$ T cell response using MHC II tetramers, which also determined that non-Tfh, Teff cells were the most dominant subset **(Fig. 6D-F)**.

Next, we decided to assess whether these non-Tfh, Teff cells generated following priming with 1 mg OVA + CT exhibited characteristics of Th2 cells, given that Th2 cells have long been implicated in allergic pathology in humans and mice.[2,32,65] We assessed IL-4 transcription via GFP-linked 4get expression and GATA and ST2 expression, which are canonical markers of Th2 cells.[64,66] Following priming with 1 mg OVA + CT, $4get^{+}$ non-Tfh cells and $ST2^{+}$ non-Tfh cells comprised approximately 5% and 0.8% of the donor T cells, respectively, which was not significantly different from untreated mice **(Fig. 6L, M)**. Additionally, the MFI of GATA3 on non-Tfh cells following priming was not significantly different compared to untreated mice or compared to tolerant mice **(Fig. 6N)**. However, the MFI of GATA3 on non-Tfh after the 100 mg OVA + CT gavage was 1.5-fold higher than after the 1 mg OVA + CT gavage, indicating that conventional Th2 cells were

present in the clinically reactive, allergic mice, but not in the primed mice **(Fig. 6N).**

Altogether, these data demonstrate that $CD44^{hi}$ $CD4^{+}$ T cells are generated following priming with 1 mg OVA + CT and that these cells lack features of Tfh-like and Th2-like cells. The lack of a strong Tfh-like phenotypic signature is consistent with the lack of CSR, GC activity, and IgE production we have observed following gavage with 1 mg OVA + CT, as Tfh cells are the source of critical signals for B cell maturation and IgE CSR.[33] Additionally, the lack of a significant Th2-polarized identity within the primed $CD4^{+}$ T cells demonstrates that these T cells remain undifferentiated to T helper lineages associated with allergy. Thus, we hypothesized that non-lineage differentiated primed $CD4^{+}$ T cells were plastic and potent precursor cells that, upon secondary allergen exposures, can differentiate into Tfh cells to drive allergic disease and clinical reactivity.

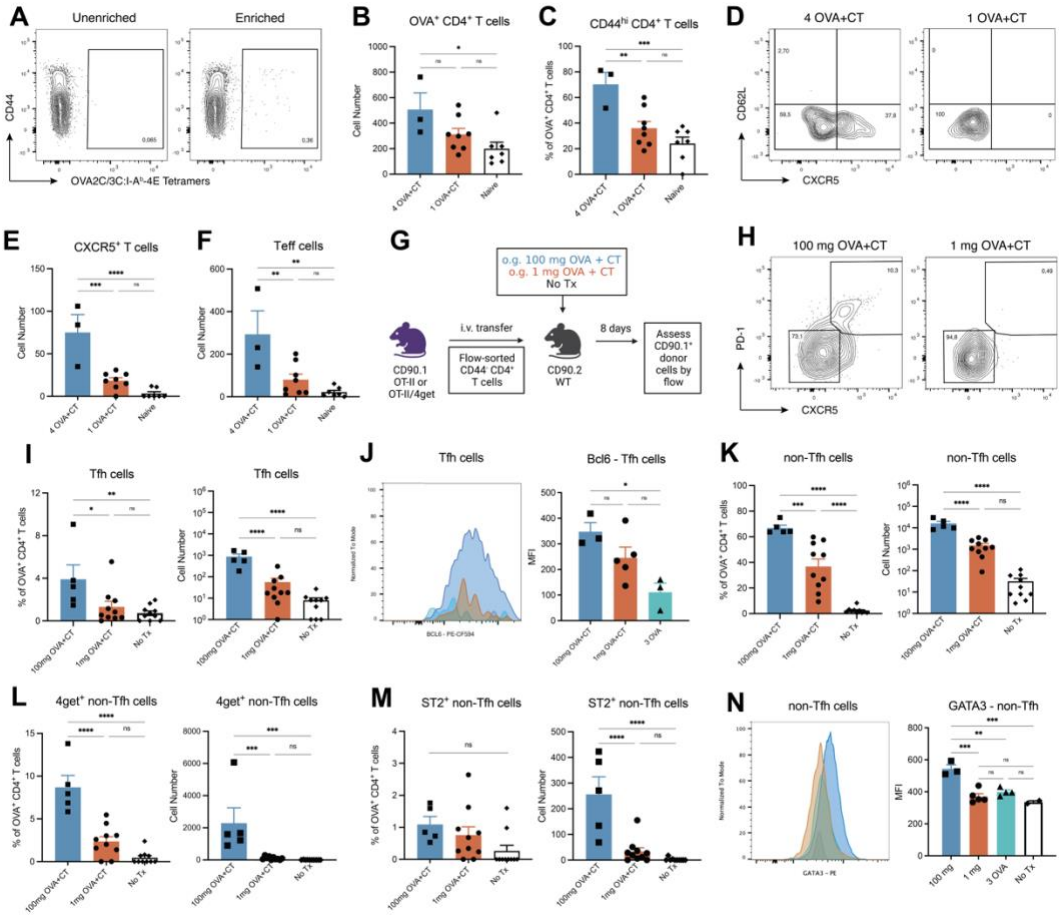

Fig. 6: CD4$^+$ T cells are activated following priming but lack features of Tfh-like and Th2-like cells.

(A-F) WT mice received a priming gavage of OVA + CT or four gavages of OVA + CT administered 4 days apart. Pooled cells from the mLNs and spleen were analyzed by flow cytometry 7 days after the final gavage, with OVA-specific CD4$^+$ T cells identified using OVA2C:I-A^b-4E and OVA3C:I-A^b-4E tetramers. **(A)** Sample flow cytometry plots of tetramer-enriched OVA-specific CD4$^+$ T cells. **(B)** Number of OVA-specific CD4$^+$ T cells. **(C)** Frequency of CD44^{hi} OVA-specific CD4$^+$ T cells. **(D)** Representative flow cytometry plots of CXCR5 and CD62L expression on CD44^{hi} OVA-specific CD4$^+$ T cells. **(E)** Number of CXCR5$^+$ CD62L$^-$ OVA-specific CD4$^+$ T cells. **(F)** Number of CXCR5$^-$ CD62L$^-$ OVA-specific CD4$^+$ T cells. **(G-N)** Donor CD90.1$^+$ OVA-specific CD4$^+$ T cells within pooled cells from the mLNs and spleen were analyzed by flow cytometry 7 days after the final gavage. **(G)** Schematic of experimentation. **(H)** Representative flow cytometry plots of expression of CXCR5 and PD-1 on donor CD90.1$^+$ CD44^{hi} CD62L$^-$ CD4$^+$ T cells. **(I)** Frequency and number of donor CD44^{hi} CD62L$^-$ CXCR5^{hi} PD-1$^+$ Tfh cells. **(J)** Histogram and MFI of Bcl6 on donor CD44^{hi} CD62L$^-$ Tfh cells. **(K)** Frequency and number of donor CD44^{hi} CD62L$^-$ CXCR5$^-$ PD-1$^-$ non-Tfh cells. **(L)** Frequency and number of donor CD44^{hi} CD62L$^-$ 4get$^+$ non-Tfh cells. **(M)** Frequency and number of donor CD44^{hi} CD62L$^-$ ST2$^+$ non-Tfh cells. **(N)** Histogram and MFI of GATA3 on donor CD44^{hi} CD62L$^-$ non-Tfh cells. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$ via one-way ANOVA. n=2-5 per group, two independent experiments, plotted as means ± SEMs.

CD4$^+$ T cells are critically required for seeding primed memory during the inaugural generation of IgE.

We next decided to assess the importance of these non-lineage differentiated CD4$^+$ T cells in the primed memory response through depleting CD4$^+$ T cells via anti-CD4 monoclonal antibody administration. In one group of mice, we depleted CD4$^+$ T cells prior to priming with 1 mg OVA + CT and then waited 1 month to allow for the reconstitution of the naïve T cell repertoire before re-exposing the mice with OVA-alone. In a second group, we primed mice and 1 month later, we depleted CD4$^+$ T cells prior to and during the OVA-alone re-exposures to assess whether they are critical in a recall response for inaugural IgE generation. Optimization experiments determined that it was necessary to continue a maintenance phase of anti-CD4 treatment during the re-exposures to prevent the re-emergence of CD4$^+$ T cells which escaped deletion (data not shown). Two weeks following the final gavage, we assessed serum antibodies and determined clinical reactivity via i.p. challenge.

As expected, we found that mice treated with anti-CD4 during the re-exposures failed to generate OVA-specific IgE and IgG1 upon allergen re-exposures, which resembled the antibody response of primed mice that had not received re-exposures **(Fig. 7A-B).** These results were consistent with literature demonstrating the critical requirement of CD4$^+$ T cells and IL-4 receptor signaling in driving IgE CSR.[47] Notably, mice treated with anti-CD4 during priming who had intact naïve CD4+ T

cells during OVA-alone re-exposures also failed to induce OVA-specific IgE and IgG1 **(Fig. 7A-B).** Furthermore, both anti-CD4 treated groups of mice demonstrated a lack of clinical reactivity following challenge, with no drop in core body temperature and no increase in hemoconcentration or clinical signs in either anti-CD4-treated group **(Fig. 7C-E).** This was in stark contrast to signs of anaphylaxis observed in allergic mice which did not receive anti-CD4 treatment **(Fig. 7C-E).** The complete abrogation of allergic responses due to $CD4^+$ T cell depletion during priming demonstrates that $CD4^+$ T cells are critically required for the seeding of immunological memory during priming. This also suggested that memory $CD4^+$ T cells hold this memory of IgE and are responsible for conferring this memory to naïve B cells to induce *de novo* IgE upon allergen re-exposures.

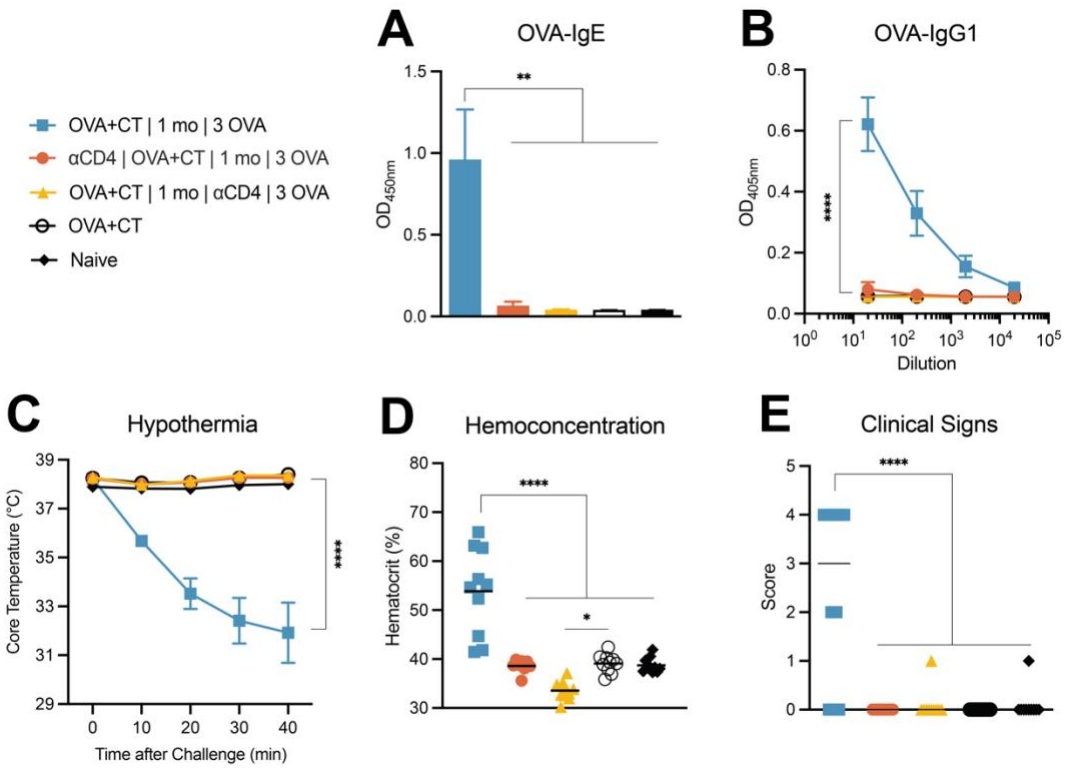

Fig. 7: CD4⁺ T cells are critically required for the seeding of primed memory and the inaugural generation of IgE.
WT mice were depleted of CD4⁺ T cells either during priming or during allergen re-exposures with the monoclonal GK1.5 antibody. **(A, B)** Serum OVA-specific IgE and IgG1 following sensitization with or without CD4⁺ T cell depletion. **(C)** Core body temperature at 10-minute intervals during systemic challenge with OVA. **(D)** Hemoconcentration at 40 minutes after challenge. **(E)** Scoring of clinical signs during systemic allergen challenge. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$ via one-way or two-way ANOVA. n=5 per group, two independent experiments, plotted as means ± SEMs or medians (E).

Memory CD4$^+$ T cells drive de novo IgE CSR in naïve B cells upon secondary allergen re-exposures.

To directly interrogate whether memory CD4$^+$ T cells can induce *de novo* IgE in naïve B cells, we used the hapten, 4-hydroxy-3-nitrophenyl (NP), conjugated to OVA (NP-OVA) to develop a linked recognition system. As antigens, haptens do not have inherent immunogenicity, as they are incapable of independently binding to MHC molecules and must be linked to a carrier molecule to elicit antibody responses.[67] In our system, we primed mice with a single 1 mg OVA + CT gavage, which activates OVA-specific CD4$^+$ T cells in a clinically silent manner **(Fig. 1 and 6),** and then 1 month later we administered three NP-OVA exposures. Two weeks after the final gavage, we assessed NP-specific serum antibodies and NP-mediated clinical reactivity through i.p. injection with NP-BSA (i.e., an irrelevant carrier protein) **(Fig. 8)**. At the time of the NP-OVA exposures, NP-specific B cells are naïve as the mice have not previously been exposed to NP. However, NP-specific B cells can recognize NP-OVA through their BCRs and are subsequently capable of presenting OVA peptides on MHC-II to the primed OVA-specific CD4$^+$ T cells, thus enabling the NP-specific B cells to receive T cell help. Indeed, we found that mice primed with 1 mg OVA + CT and re-exposed with NP-OVA generated robust NP-specific IgE and IgG1 and reacted clinically with symptoms of anaphylaxis **(Fig. 8A-E)**. These results demonstrate that OVA-specific CD4$^+$ T cells hold early allergic memory of IgE after priming.

Importantly, since the NP-OVA re-exposures are not adjuvanted, any NP-specific B cell immunity elicited is a result of help from primed OVA-specific T cells. Veritably, when NP-OVA alone exposures were administered to mice which had not previously been primed with 1 mg OVA + CT, these mice did not generate NP-specific IgE or IgG1 **(Fig. 8A-B)**, which indicates that the primed OVA-specific $CD4^+$ T cells generated during priming are mediating *de novo* antibody production. Additionally, mice which were primed with 1 mg OVA + CT and re-exposed to NP-BSA did not generate NP-specific IgE or IgG1 **(Fig. 8A-B)**, providing further evidence that OVA as a carrier protein provides the source of epitopes to recruit memory $CD4^+$ T cell help to drive CSR to IgE and IgG1. A positive control group was also included wherein mice were primed with 1 mg NP-OVA + CT and then re-exposed with NP-OVA. Mice in this group produced NP-specific antibodies and reacted clinically with symptoms of anaphylaxis, akin to mice primed with 1 mg OVA + CT and re-exposed with NP-OVA **(Fig. 8A-E)**. Collectively, these data demonstrate that memory $CD4^+$ T cells generated during early allergic priming hold the memory of humoral IgE responses and can induce *de novo* IgE generation in naïve B cells.

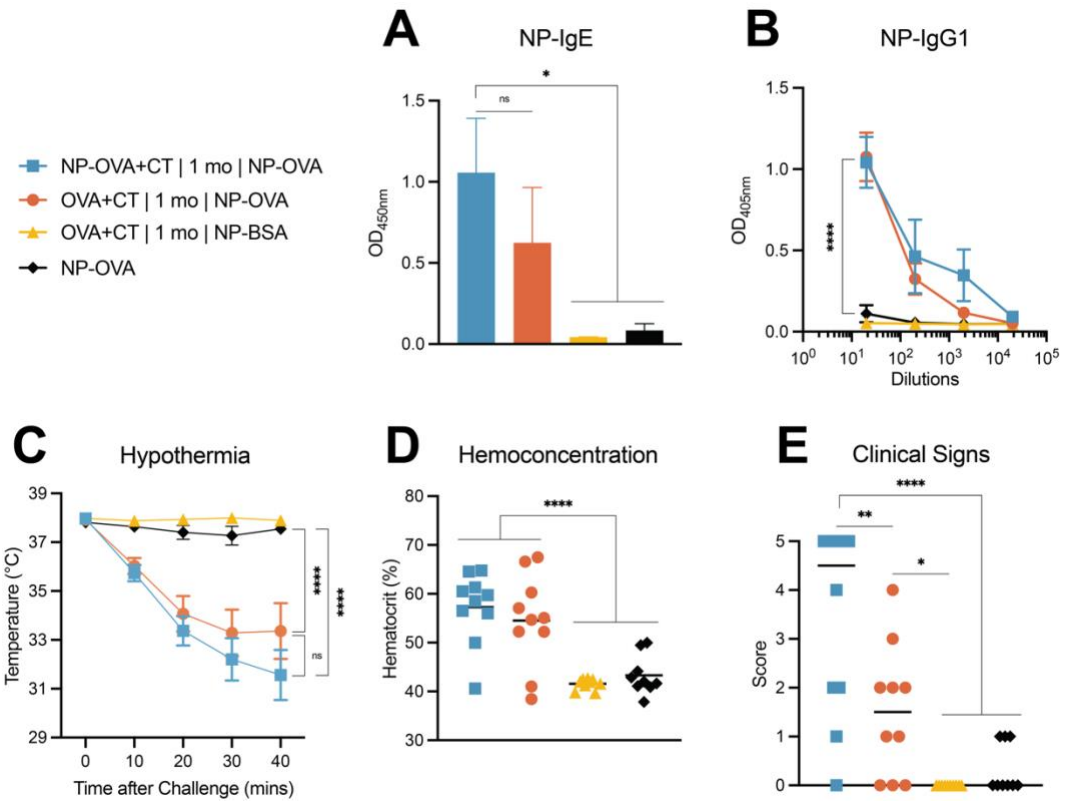

Fig. 8: Memory CD4$^+$ T cells hold early allergic memory and drive inaugural generation of IgE upon allergen re-exposures.
WT mice were primed with OVA + CT and 1 month later received NP-OVA alone re-exposures to assess linked recognition between memory OVA-specific CD4$^+$ T cells and naive NP-specific B cells. **(A, B)** Serum NP-specific IgE and IgG1. **(C)** Core body temperature at 10-minute intervals during systemic challenge with NP-BSA. **(D)** Hemoconcentration at 40 minutes after challenge. **(E)** Scoring of clinical signs during systemic challenge. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$ via one-way or two-way ANOVA. n=5 per group, two independent experiments, plotted as means ± SEMs or medians (E).

Primed CD4$^+$ T cells are plastic and predisposed to upregulate Th2-polarized Tfh signatures and drive IgE CSR upon recall.

Studies in mice and humans demonstrate that Tfh cells and Tfh-derived IL-4 secretion are critical requirements for driving IgE CSR.[33] Given our findings that primed CD4$^+$ T cells hold incipient memory of IgE and drive *de novo* IgE CSR upon recall, we hypothesized that non-lineage differentiated primed CD4$^+$ T cells may be precursors of Tfh cells. Thus, we investigated the functional capacity of primed CD4$^+$ T cells to display plasticity and upregulate Tfh-like phenotypic signatures upon recall.

To address whether primed CD4$^+$ T cells differentiate into Tfh cells upon secondary allergen exposures to drive IgE CSR, we utilized an adoptive transfer system **(Fig. 9A)**. First, we processed spleens and mLNs from naïve CD90.1$^+$ OT-II mice or OT-II/4get mice and flow-sorted CD44^{lo} CD4$^+$ T cells, which were then intravenously transferred into naïve WT recipient mice. The following day, we administered a priming gavage with 1 mg OVA + CT to the WT recipients. The pre-transfer of naïve OT-II cells prior to priming boosted the frequency of naïve OVA-specific CD4$^+$ T cells in the WT mice, allowing for improved detection of primed CD4$^+$ T cells and enabling us to flow-sort this population specifically. Seven days following the OVA + CT gavage, we processed spleens and mLNs from the primed WT recipients and enriched for CD4$^+$ T cells. We then flow-sorted the donor cells and specifically, the CD90.1$^+$ CD44^{hi} CXCR5$^-$ CD4$^+$ T cells from the primed WT mice

(Fig. 9B). Although most primed T cells do not express CXCR5 **(Fig. 6, 9B)**, flow sorting specifically on $CXCR5^-$ non-Tfh cells ensured that any CXCR5 expression observed in the primed T cells was upregulated in response to the secondary allergen exposure. We then intravenously transferred 1.0 – 3.0 x 10^3 flow-sorted primed non-Tfh cells into TCRβ-KO mice, which are deficient in T cells. As a control group, we intravenously transferred a matched number of flow-sorted $CD90.1^+$ $CD4^+$ T cells from naïve OT-II mice into a separate group of TCRβ-KO recipients **(Fig. 9B)**. The following day, the TCRβ-KO recipients received an i.p. injection of OVA + alum, which is a robust, systemic exposure that would ensure the survival and proliferation of the transferred cells. Nine days following the OVA + alum injection, we collected spleens and mLNs from the TCRβ-KO recipients and enriched for the $CD90.1^+$ donor T cells through a magnetic bead-based enrichment. We then assessed the phenotype of the $CD90.1^+$ donor T cells by flow cytometry.

We detected a 7.8-fold greater number of primed $CD90.1^+$ donor cells than the previously naïve $CD90.1^+$ donor cells on average, indicating that the primed T cells underwent clonal expansion to a greater extent than the naïve T cells which were transferred in equal number (**Fig. 9C).** When we compared the pre-transfer CXCR5 expression in the $CD90.1^+$ donor cells to the post-transfer CXCR5 expression following the i.p. allergen exposure, we found that both the primed and the naïve T cells upregulated CXCR5 expression (**Fig. 9D).** However, after the secondary allergen exposure, the primed T cells had a 1.8-fold higher MFI for CXCR5

compared to the naïve T cells, with a minor 1.4-fold increase in MFI for PD-1 **(Fig. 9E).** These data suggest that the primed T cells possessed an enhanced capacity to upregulate canonical markers of Tfh cells and particularly CXCR5, which is critical for facilitating Tfh cell migration to the T-B border.[33] Next, we assessed the frequency of $CXCR5^+$ T cells and $CXCR5^+$ $PD\text{-}1^+$ Tfh cells among the $CD44^{hi}$ $CD90.1^+$ donor cells **(Fig. 9F, G)**. We found that 58% of primed T cells were $CXCR5^+$ T cells, which was significantly greater than among the previously naïve T cells, wherein 34% were $CXCR5^+$ T cells **(Fig. 9G)**. Tfh cells made up 32% of primed T cells, a 1.7-fold increase in frequency compared to previously naïve T cells **(Fig. 9G)**. Collectively, these data suggest that primed T cells are predisposed to upregulate CXCR5 and differentiate into Tfh cells upon secondary allergen exposures. In addition, we assessed IL-4 transcription through 4get-linked GFP within the $CXCR5^+$ T cells and Tfh cells, given the critical function of IL-4 in driving IgE CSR.[47] Indeed, we found that IL-4 transcription levels were 2.5-fold higher among $CXCR5^+$ T cells and 2.9-fold higher among Tfh cells descended from primed T cells in comparison to previously naïve T cells, indicating enhanced Th2 polarization **(Fig. 9H).**

Therefore, although primed T cells were undifferentiated to allergy-associated T helper lineages at the time of their generation **(Fig. 6)**, they act as potent precursors of Th2-polarized Tfh-like cells that highly express CXCR5 and IL-4 during a recall response. The enhanced upregulation of CXCR5 and IL-4 in primed T cells upon recall suggested an enhanced ability of these cells to localize to the T-B border and

induce IgE CSR in naïve B cells, as this is thought to be the primary location of CSR.[33] Lastly, we assessed whether the strong Tfh phenotypic signature of the primed T cells upon recall was associated with enhanced serum OVA-IgE production via ELISA. We found that the TCRβ-KO recipients that received primed T cells produced greater levels of OVA-IgE after the i.p. injection than the TCRβ-KO recipients that received naïve T cells **(Fig. 9I)**. Furthermore, we investigated the saturation of IgE bound to FcεRI on circulating basophils as a surrogate measure of clinical reactivity and to further quantify IgE production. We found that among circulating basophils ($CD45^+$ $CD200R3^+$ $Fc\varepsilon RI\alpha^+$) from TCRβ-KO recipients which received primed T cells, 70% had what we deemed as a high saturation of bound IgE and 26% had low saturation of bound IgE following the i.p. allergen exposure **(Fig. 9J).** When compared to circulating basophils from recipients of previously naïve T cells, only 23% of basophils were highly saturated with receptor-bound IgE, which was 3.0-fold lower than in recipients of primed T cells **(Fig. 9J).** These results confirm that the predisposition of primed T cells to upregulate Tfh-like signatures and transcribe IL-4 upon secondary allergen exposures is associated with their enhanced functionality and potency to localize to B cell zones in SLOs and drive *de novo* IgE CSR in naïve B cells. In conclusion, the plasticity of non-lineage differentiated primed T cells further demonstrates their role in holding IgE memory.

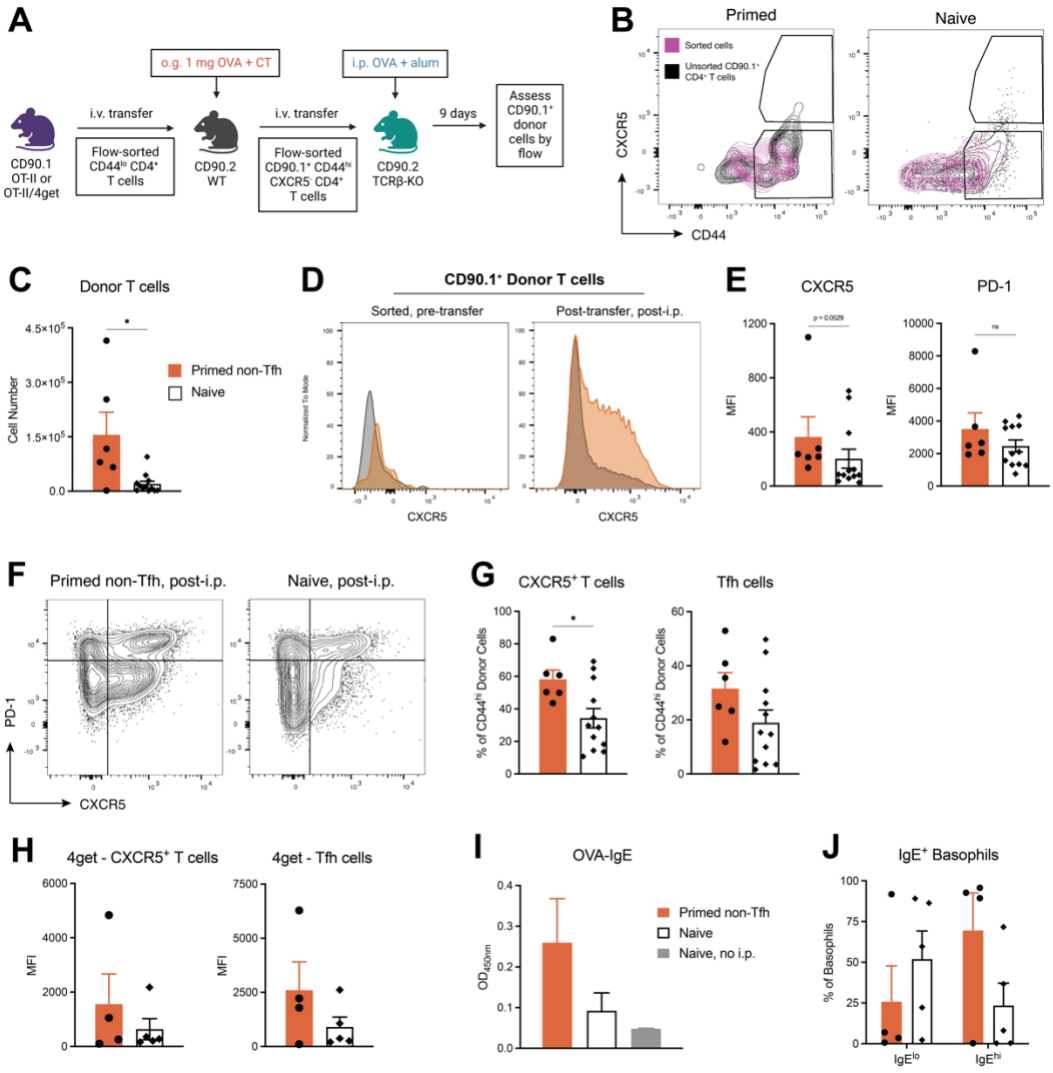

Fig. 9: Primed CD4$^+$ T cells possess enhanced capacity to upregulate Tfh-like signatures upon recall and drive IgE CSR.
(A) Schematic of experimentation. **(B)** Flow cytometry plots of CD44 and CXCR5 expression on donor CD90.1$^+$ CD4$^+$ T cells from primed WT mice and naive OT-II mice. Unsorted cells pictured in black and sorted cells pictured in purple. **(C)** Number of donor CD90.1$^+$ CD4$^+$ T cells detected in TCRβ-KO recipients. **(D)** Histograms of CXCR5 expression on donor CD90.1$^+$ CD4$^+$ T cells prior to transfer and following transfer into TCRβ-KO recipients. **(E-H, J)** Pooled cells from the mLNs and spleen (E-H) or blood (J) were analyzed by flow 9 days post OVA + alum injection. **(E)** MFI of CXCR5 and PD-1 on donor CD90.1$^+$ CD44^{hi} CD4$^+$ T cells. **(F)** Concatenated flow cytometry plots of CXCR5 and PD-1 expression on donor CD90.1$^+$ CD44^{hi} CD4$^+$ T cells. **(G)** Frequency of CXCR5$^+$ T cells and CXCR5$^+$ PD-1$^+$ Tfh cells among donor CD90.1$^+$ CD44^{hi} CD4$^+$ T cells. **(H)** MFI of 4get-linked GFP on donor CD90.1$^+$ CD44^{hi} CXCR5$^+$ PD-1$^+$ Tfh cells. **(I)** Serum OVA-specific IgE. **(J)** Frequency of IgE^{lo} vs. IgE^{hi} basophils among CD45$^+$ CD200R3$^+$ FcεRIα$^+$ basophils. * $p < 0.05$ via Mann-Whitney test. n=2-5 per group, three independent experiments, plotted as means ± SEMs.

Mechanistic requirements for seeding primed memory CD4$^+$ T cells and for their productive recall upon allergen re-exposures.

S1PR-mediated immune cell migration is required for seeding memory CD4$^+$ T cells during priming.

We next determined the involvement of immune cell trafficking between tissue sites during both the priming exposure and the allergen re-exposures. This aimed to assess the geography of immunological events associated with priming and where these primed CD4$^+$ T cells reside once in the memory phase. To assess lymphocyte trafficking, we administered FTY720 (Fingolimod) during priming or during and following the allergen re-exposures. FTY720 is an immunomodulatory drug that acts as a potent agonist of the sphingosine-1-phosphate receptors (S1PRs) following its phosphorylation, which inhibits lymphocyte egress from primary and peripheral lymphoid organs, but does not directly affect T cell activation nor does it reposition tissue-resident memory T cells to secondary lymphoid organs.[53,68,69] Mice were treated with FTY720 in their drinking water for 5 days prior to allergen exposure, which depleted circulating T cells by 98% and circulating B cells by 90% (data not shown). When administered during priming, FTY720 treatment was continued for 1 month after the gavage to ensure that lymphocyte trafficking was impeded during the entire primary response **(Fig. 10A).** After cessation of FTY720 treatment, circulating lymphocytes returned to naïve levels 14 days later (data not shown), at which point the mice were administered three 1 mg OVA-alone re-

exposures every other day **(Fig. 10A)**. We then assessed the antibody response and clinical reactivity of the mice 14 days after the final gavage re-exposure by conducting ELISAs on serum and performing an i.p. allergen challenge.

The mice which received FTY720 treatment during priming with 1 mg OVA + CT were not capable of generating OVA-specific IgE or IgG1 upon subsequent allergen gavage re-exposures **(Fig. 10B-C).** Furthermore, these mice did not respond clinically following challenge, with no significant differences in temperature, hemoconcentration, or clinical score compared to naïve mice **(Fig. 10D-F).** These results demonstrate that the seeding of functional memory $CD4^+$ T cells following priming requires S1PR-mediated immune cell migration, which we hypothesize involves the migration of primed $CD4^+$ T cells from SLOs to multiple tissue sites where they reside in the memory phase.

Since FTY720 is a non-selective agonist of S1PRs that can modulate S1PR1, S1PR3, S1PR4, and S1PR5, we cannot determine the exact S1PR that is required for the seeding of primed T cells through these experiments.[53,68] Further experimentation would be required to establish which S1PR receptor on which cells are critically required for the generation of primed memory T cells. Additionally, although FTY720 has been shown to severely impact the number of circulating T cells and B cells in mice,[69] this drug can also impact other immune cells that utilize S1PRs for trafficking, such as certain DC subsets.[70,71] FTY720 treatment during priming may also impede migration of allergen-presenting DCs, which could

obstruct memory $CD4^+$ T cell generation. As such, we cannot definitively conclude that the critical role of S1PR-mediated migration during priming with 1 mg OVA + CT is solely through migration of primed T cells to distal tissue sites.

Primed $CD4^+$ T cells productively drive de novo IgE CSR upon recall at multiple distinct tissue sites independently of S1PR-mediate cell migration.

We also assessed whether primed memory T cells can drive *de novo* IgE CSR within specific tissue sites without recirculating or whether trafficking between SLOs is required for IgE generation. In a separate treatment group, mice were primed with 1 mg OVA + CT and, 1 month later, FTY720 treatment began **(Fig. 10A).** Five days later, mice were administered 1 mg OVA-alone re-exposures every other day while undergoing FTY720 treatment **(Fig. 10A)**. We found that mice which received FTY720 treatment during re-exposures produced robust levels of OVA-specific IgE and IgG1, which was significantly higher than in naïve mice **(Fig. 10B-C).** These mice also experienced anaphylaxis upon allergen challenge, as demonstrated by a drop in core body temperature, elevated hemoconcentration, and high clinical scores which were comparable to a control group which was primed and re-exposed in the absence of FTY720 treatment (**Fig. 10D-F).** These data suggest that after primed $CD4^+$ T cells are seeded following priming, they can be recalled upon secondary allergen exposures in an S1PR-independent manner through tissue-specific immune responses.

We further investigated the site where primed memory $CD4^+$ T cells induce *de novo* CSR and GC reactions in naïve B cells. Therefore, we assessed allergen-specific B cell responses in the spleens, mLNs, and small intestines via flow cytometry analysis 7 days following the final gavage in mice that either did or did not receive FTY720 treatment during the allergen re-exposures **(Fig. 10G-J).** For the following experiments, we did not include the group which received FTY720 treatment during priming with 1 mg OVA + CT, as the results from the allergen challenge indicated that there was no active humoral immunity in these mice. All tissues were kept separate for analysis, with magnetic bead-based enrichments for OVA-specific B cells performed within the spleen samples.

There were no statistically significant increases in the number of OVA-specific B cells detected in the group which received FTY720 treatment during re-exposures compared to naïve mice in the spleen, mLNs, or small intestines **(Fig. 10G).** We detected an average of 14-fold more OVA-specific B cells in the mLNs and 13-fold more OVA-specific B cells in the small intestines of FTY720-treated mice compared to naïve mice **(Fig. 10G).** In the spleens, the number of OVA-specific B cells had a 1.3-fold non-significant decrease in the FTY720 treatment group compared to naïve mice **(Fig. 10G)**, which may be related to the overall decrease in circulating B cells due to FTY720 treatment. However, the frequency of class-switched OVA-specific B cells was significantly higher in FTY720-treated mice compared to naïve mice in all three tissues, with no significant difference between the FTY720 treatment group and the allergic control group **(Fig. 10H).** Within the

spleens, mLNs, and small intestines, the class-switched B cells composed 12%, 73%, and 56% of the OVA-specific B cell repertoire **(Fig. 10H).** The majority of these class-switched B cells were IgG1$^+$ B cells (data not shown). These data demonstrate that following priming, memory CD4$^+$ T cells are seeded in the spleens, mLNs, and small intestines and that, upon secondary allergen exposures, these CD4$^+$ T cells are recalled and induce *de novo* CSR in naïve B cells within each tissue site. Furthermore, this recall of memory CD4$^+$ T cells is independent of S1PR-mediated immune cell migration.

The GC activity followed a similar trend in the mLNs and small intestines, where 66% and 45% of OVA-specific B cells were GC B cells in the FTY720-treated mice, respectively - both significant increases compared to naïve mice **(Fig. 10I).** Meanwhile in the spleens, there was not significant GC activity, wherein 3.5% and 3.4% of OVA-specific B cells were GC B cells in FTY720-treated mice and allergic mice, respectively **(Fig. 10I).** These data indicate that beyond their ability to induce CSR, primed memory CD4$^+$ T cells form cognate pairs with B cells and seed new GCs in multiple tissues upon secondary allergen exposures. These findings are consistent with the predisposition of primed CD4$^+$ T cells to upregulate CXCR5 and localize to B cell zones **(Fig. 9).**

Furthermore, we assessed the number of IgE-secreting OVA-specific short-lived PCs to determine the ability of tissue-resident primed T cells to drive the *de novo* IgE production we observed in FTY720-treated mice **(Fig. 10B)**. We detected

higher numbers of IgE^+ $H+L^{hi}$ $B220^-$ PCs in the spleens, mLNs, and small intestines of the FTY720 treatment group compared to the allergic and naïve groups **(Fig. 10J)**. We concluded that this was related to the inability of short-lived PCs to egress from SLOs and travel to the bone marrow due to ongoing FTY720 treatment. In the spleens and mLNs of the FTY720-treated mice, we detected approximately 64,000 and 900 IgE-secreting PCs, respectively, which was significantly different than the naïve mice **(Fig. 10J).** In the small intestines of FTY720-treated mice, we detected 1200 IgE^+ PCs, though this was not statistically significant **(Fig. 10J).** Thus, we concluded that the largest contribution to IgE production came from the spleens, despite data suggesting that limited GC activity was ongoing in the spleens **(Fig. 10I).** These results were consistent with studies suggesting that IgE^+ PC differentiation primarily occurs through the extrafollicular pathway, particularly during the inaugural generation of low-affinity IgE.[2,72,73]

Further, the recall of these memory $CD4^+$ T cells occurs independent of S1PR-mediated migration in the spleen, mLNs, and small intestines upon secondary allergen exposures. Within these distinct tissue sites, resident memory $CD4^+$ T cells induce CSR and IgE^+ PC differentiation in naïve B cells and initiate GC reactions.

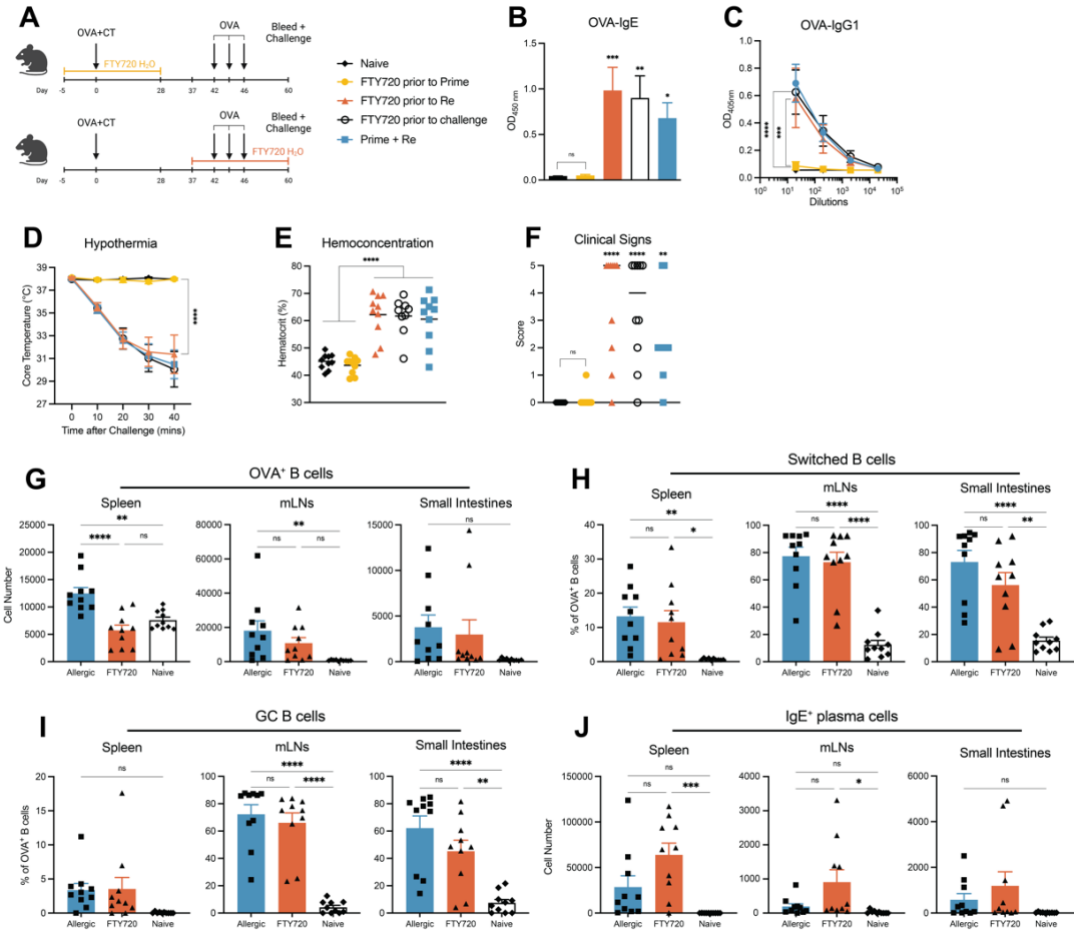

Fig. 10: Lymphocyte migration is not required for the inaugural generation of IgE mediated by memory CD4$^+$ T cells.
(A) Schematic of experimentation. **(B, C)** Serum OVA-specific IgE and IgG1 at 2 weeks following the final gavage re-exposure. **(D)** Core body temperature at 10-minute intervals during systemic challenge with OVA. **(E)** Hemoconcentration at 40 minutes after challenge. **(F)** Scoring of clinical signs during systemic allergen challenge. **(G-J)** WT mice were primed with OVA and CT and were administered OVA-alone gavages either alongside FTY720 treatment or without. Cells from the spleens, mesenteric lymph nodes (mLNs), and small intestines were analyzed by flow cytometry 7 days after the final gavage, with OVA-specific cells identified using OVA B cell tetramers. **(G)** Number of OVA-specific B cells. **(H)** Frequency of switched IgM$^-$ IgD$^-$ OVA-specific B cells. **(I)** Frequency of GL7$^+$ CD38$^-$ OVA-specific GC B cells. **(J)** Number of IgE$^+$ OVA-specific H+L^{hi} B220$^-$ PCs. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$ via one-way or two-way ANOVA. n=5 per group, two independent experiments, plotted as means ± SEMs or medians (F).

Loss of CD40/CD40L costimulation or IL-4Rα signaling completely inhibits primed CD4$^+$ T cells from driving IgE CSR in naïve B cells during recall.

Next, we sought to determine the molecular interactions required for the generation of IgE from primed memory CD4$^+$ T cells upon allergen re-exposures. First, we blocked CD40/CD40L costimulation through i.p. administration of anti-CD40L monoclonal antibody. CD40/CD40L interactions between Tfh cells and antigen-primed B cells at the T-B border provide signals which enable B cells to make extrafollicular fate decisions, such as CSR, and/or to migrate to the GC.[2] Therefore, we hypothesized that as primed CD4$^+$ T cells differentiated into Tfh cells upon secondary allergen exposures and interacted with naïve B cells to induce IgE CSR **(Fig. 8, 9)**, these cognate interactions would require CD40/CD40L costimulation. As expected, we found that when we primed mice with 1 mg OVA + CT and 1 month later administered anti-CD40L during the allergen re-exposures, mice were incapable of generating OVA-specific IgE or IgG1 responses **(Fig. 11A-B).** Furthermore, they were completely protected from anaphylaxis upon allergen challenge, whereas mice treated with an isotype control (IC) developed severe anaphylaxis **(Fig. 11C-D).**

We next blocked IL-4Rα signaling during recall through i.p. administration of anti-IL-4Rα monoclonal antibody. Given that Tfh-derived IL-4 is critically required for IgE CSR during primary responses,[33] we hypothesized that loss of IL-4 signaling during the recall of primed memory CD4$^+$ T cells would prevent the emergence of

IgE. Indeed, we found that inhibiting IL-4Rα signaling during OVA-alone re-exposures 1 month after priming prevented OVA-specific IgE and IgG1 production **(Fig. 11E-F).** This was associated with a lack of anaphylaxis upon allergen challenge, in stark contrast to IC-treated mice and mice which did not receive antibody treatment, wherein both groups of mice developed hypothermia and increased hemoconcentration **(Fig. 11G-H).** Altogether, these findings indicate that CD40/CD40L interactions and IL-4Rα signaling are critical requirements for memory $CD4^+$ T cells to induce IgE generation from naïve B cells upon recall.

The generation of primed $CD4^+$ T cells is partly OX40L-dependent and strictly CD40L-dependent, but independent of IL-4Rα signaling.

Lastly, we sought to determine the molecular requirements for the initial generation of primed $CD4^+$ T cells following gavage with 1 mg OVA + CT. Here, we provided i.p. injections of different monoclonal antibodies (anti-CD40L, anti-OX40L, and anti-IL-4Rα) to WT mice during priming, waited 1 month to allow for their clearance, and subsequently provided allergen re-exposures. We then assessed serum antibodies and clinical reactivity by allergen challenge 2 weeks after the final gavage re-exposure.

CD40 and CD40L can be expressed on both DCs and $CD4^+$ T cells, though typically CD40 on DCs and CD40L on $CD4^+$ T cells engage in critical interactions for initial T cell activation and memory $CD4^+$ T cell generation.[2,74,75] As expected, when CD40/CD40L interactions were blocked during priming, this completely abrogated

any OVA-specific IgE or IgG1 production upon allergen re-exposures and prevented anaphylaxis upon challenge **(Fig. 11A-D).** These data indicate that CD40/CD40L interactions, likely between DCs and naïve T cells, are critically required for the generation of memory $CD4^+$ T cells following priming with 1 mg OVA + CT.

We also found that when anti-OX40L antibody was administered during priming, this hindered the generation of OVA-specific IgE and IgG1 upon recall, yet did not completely ablate antibody production **(Fig. 11I-J)**. Furthermore, the diminished OVA-specific antibody production in these mice was associated with partial protection from clinical reactivity, as evidenced by less severe reductions in core body temperature and less marked increases in hemoconcentration following allergen challenge compared to IC-treated mice **(Fig. 11K-J).** Therefore, we concluded that the generation of primed $CD4^+$ T cells is only partly-dependent on OX40/OX40L interactions. This is consistent with the literature, which asserts that the administration of CT alongside allergen induces upregulation of OX40L on $CD103^+$ DCs, and these OX40L-expressing DCs drive strong Th2-polarized $CD4^+$ T cell responses.[27,28,38,57–59]

Interestingly, OX40L-expressing DCs are thought to drive IL-4-dependent Th2 cell differentiation, which involves autocrine/paracrine IL-4 signaling within $CD4^+$ T cells.[28] However, we found that most primed $CD4^+$ T cells do not transcribe IL-4 **(Fig. 6I)**, so we next decided to address whether IL-4Rα signaling was a critical

requirement for seeding primed $CD4^+$ T cells. Intriguingly, when we blocked IL-4Rα signaling during priming with 1 mg OVA + CT, we found that this did not obstruct the generation of primed $CD4^+$ T cells. This was evidenced by their intact ability to drive OVA-specific IgE and IgG1 responses upon recall, which was associated with anaphylactic responses upon allergen challenge, similar to the positive control group **(Fig. 11E-H).** These data further distinguish the non-lineage differentiated primed $CD4^+$ T cells we have identified from conventional Th2 cells, given that the requirements for their generation diverge from the IL-4 signaling requirements for Th2 cell differentiation.[28] Altogether, we found that the generation of primed $CD4^+$ T cells is partly dependent on OX40/OX40L and completely dependent on CD40/CD40L costimulation, which are likely involved in cognate interactions between peptide-MHCII-presenting DCs and naïve $CD4^+$ T cells.

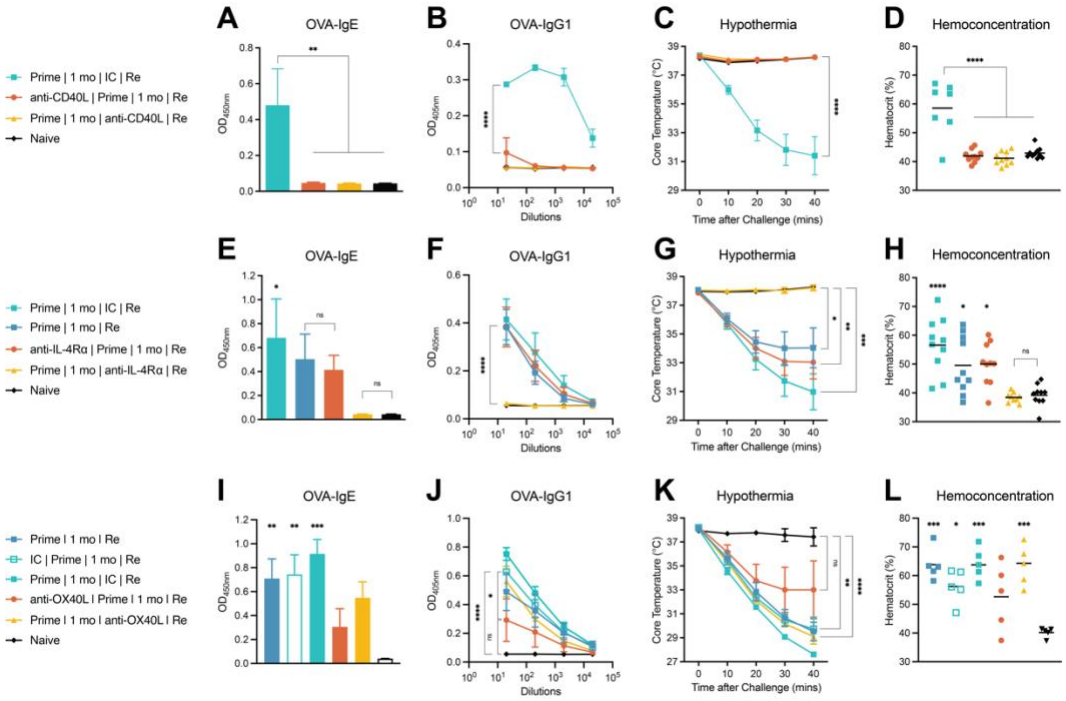

Fig. 11: The generation of primed CD4⁺ T cells is partly OX40L-dependent and strictly CD40L-dependent, but independent of IL-4Rα signaling.
(A-D) WT mice were administered anti-CD40L antibody either during priming or during allergen re-exposures. IC mice were treated with polyclonal Armenian hamster IgG antibody. **(E-H)** WT mice were administered anti-IL-4Rα antibody either during priming or during allergen re-exposures. IC mice were treated with rat IgG2a antibody. **(I-L**) WT mice were administered anti-OX40L antibody either during priming or during allergen re-exposures. IC mice were treated with rat IgG2b antibody. **(A, E, I)** Serum OVA-specific IgE at 2 weeks following the final gavage re-exposure. **(B, F, J)** Serum OVA-specific IgG1 at 2 weeks following the final gavage re-exposure. **(C, G, K)** Core body temperature at 10-minute intervals during systemic challenge with OVA. **(D, H, L)** Hemoconcentration at 40 minutes after challenge. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$ via one-way or two-way ANOVA, with comparison to naive group plotted unless otherwise specified. n=5 per group, two independent experiments (A-H) or one independent experiment (I-L), plotted as means ± SEMs.

DISCUSSION

Food allergy is a phasic disease with a prolonged trajectory, with the production of allergen-specific IgE representing the "final" phase.[17–21] However, the mechanisms underlying the progression to allergen-specific IgE generation are unclear. Though Tfh cells are critical drivers of CSR to IgE during primary responses in mice across multiple type 2 diseases,[33–36,38] the role of *memory* $CD4^+$ T cells in perpetuating IgE memory remains a contentious topic. Using an adapted intragastric model of food allergy, we have demonstrated that the generation of allergen-specific memory $CD4^+$ T cells occurs prior to B cell activation, representing an incipient phase of allergic memory. Upon allergen re-exposures, these memory $CD4^+$ T cells differentiate into IL-4-transcribing Tfh cells and mediate *de novo* IgE CSR in naïve B cells, which drives clinical reactivity. This work demonstrates that $CD4^+$ T cells hold memory of IgE even when MBCs are absent, which were previously the only well-characterized niche of IgE memory.[18,41–44]

MBCs have been the primary focus of studies investigating how IgE memory is maintained. As such, the contribution of MBCs in replenishing IgE^+ PC pools is well characterized, wherein class-switched MBCs undergo sequential CSR to IgE during recall responses, representing the dominant pathway for secondary IgE production.[18,41–43,76] Recent studies by Koenig *et al.*, Aranda *et al.*, and Ota *et al.* have identified a subset of type 2-polarized MBCs termed "MBC2s" which highly express FcεRII (CD23), IL-4Rα, and IGHE which are the primary precursors of IgE PCs during memory responses based on clonal lineage analyses and *in vitro*

cultures.[42–44] However, our work indicates that the presence of allergen-specific MBCs is not strictly required to perpetuate allergen-specific IgE responses. During recall responses, we demonstrated that allergen-specific memory $CD4^+$ T cells induce *de novo* activation of naïve B cells, resulting in GC responses, CSR to IgE and IgG1, and IgE^+ and $IgG1^+$ PC differentiation. This pathway is not specific to allergic immunity. We demonstrated that when primed with allergen in Th1-polarizing conditions, allergen-specific memory $CD4^+$ T cells retained the ability to recruit naïve B cells into GCs and instead drove IgG2b and IgG2c CSR, a Th1-polarized antibody response. Our findings indicate that memory $CD4^+$ T cells can independently maintain memory of an initial antigenic exposure for up to 14 months and drive diverse CSR programs and antibody production upon recall, suggesting that productive recall of cellular memory does not necessarily rely on pre-existing humoral memory. This is consistent with recent work demonstrating that secondary GCs are dominated by naïve-derived B cells.[77–79] Importantly, our work does not preclude the dominant contribution of memory-derived B cell clones to secondary antibody responses demonstrated in the contexts of allergic disease, infections, and vaccination,[42,43,80,81] and instead proposes that upon antigen re-exposures, memory $CD4^+$ T cells may also significantly contribute to driving *de novo* B cell and antibody responses.

Additionally, our work revealed that allergen-specific $CD4^+$ T cells that remain undifferentiated to allergy associated T helper cell lineages (i.e. Tfh and Th2) can be intrinsically programmed to differentiate into Tfh cells upon subsequent allergen

encounters. These memory $CD4^+$ T cells drive divergent CSR programs, which were IgE- and IgG1-dominant when mice were primed with OVA + CT and IgG2b- and IgG2c-dominant when mice were primed with OVA + CpG. Future research should interrogate the transcriptomic and epigenetic differences between the allergen-specific $CD4^+$ T cells generated following priming with OVA + CT versus with OVA + CpG to determine the mechanisms which drive divergent CSR programs upon recall. Performing analyses on primed antigen-specific $CD4^+$ T cells such as SMART-Seq single cell RNA sequencing and ATAC-Seq chromatin accessibility profiling may also elucidate a phenotypic signature that could identify patients who are primed towards allergy before they experience their first known allergic reaction.

The contrasting Th2-polarized versus Th1-polarized CSR programs induced by primed memory $CD4^+$ T cells upon allergen re-exposure are likely dictated by the adjuvant-driven signals in the microenvironment during their initial generation. However, the exact signals critical for generating these divergently-fated memory $CD4^+$ T cells remain unclear and warrant further investigation. CpG ODNs act as an agonist for TLR9, which is thought to induce MyD88 activation in DCs and drive Th1 responses,[59,60] though the critical requirements for priming with OVA + CpG in our model system remain to be elucidated. Intriguingly, we found that seeding of memory $CD4^+$ T cells following OVA + CT priming occurred independently of IL-4Rα signaling with only partial dependence on OX40/OX40L interactions. This was surprising given that CT is thought to upregulate OX40L on

DCs which in turn induces autocrine IL-4 signaling in naïve $CD4^+$ T cells and subsequent Th2 differentiation.[27,28,38,59] Indeed, the expendable nature of IL-4 signaling in the generation of these non-lineage differentiated memory $CD4^+$ T cells further distinguishes them from conventional Th2 cells which require IL-4 during differentiation,[28] and suggests that we have identified a novel $CD4^+$ T cell subset involved in allergic disease.

This novel memory $CD4^+$ T cell subset further differs from typical allergy-associated T helper cell lineages as it lacks classical Tfh-like signatures (i.e., CXCR5, PD-1, and Bcl6). Insufficient CXCR5 expression also suggests that primed $CD4^+$ T cells are inadequate at localizing to B cell zones in SLOs following their generation, which is consistent with the lack of B cell activation we observe following priming with 1 mg OVA + CT. This may be related to the dose of antigen provided, as a gavage with a high dose of 100 mg OVA + CT resulted in Th2 and Tfh cell differentiation and B cell activation, though the exact mechanisms underlying this dose-dependency remain unclear. Lack of CD86 expression following priming with 1 mg OVA + CT suggests that the B cells are not experiencing antigen-specific BCR stimulation, however an analysis of early or early-immediate gene signatures (e.g., Nur77) would be required to determine this definitively. An alternative model is that the B cells are primed during the initial antigenic exposure, but that they return to a naïve-like state due to a lack of sufficient T cell help, as described in the 2017 study by Turner *et al.*[56]

The insufficient T cell help preventing B cell activation may be related to low expression of peptide-MHC II complexes on DCs following priming with 1 mg OVA + CT due to limited antigen availability. This would affect TCR signaling strength and longevity, both of which influence T cell clonal expansion and lineage commitment.[82–84] However, there is contention in the field regarding the role TCR signal strength plays in driving T cell differentiation. Some studies assert that Tfh cell differentiation and memory commitment are promoted by strong peptide-MHC II signals to the TCR,[26,83,84] whereas other studies found that strong TCR signaling promoted Teff cell fates and low TCR signals drove Tfh cell and memory T cell differentiation.[85–87] Yet another study found that tonic TCR signaling influenced fate decisions, wherein individual clones with low tonic TCR signaling promoted Tfh cell differentiation.[88] Importantly, most studies on the initial signals driving T cell differentiation focused on intrinsic differences in TCR specificity and affinity between clones, rather than the impact of a limiting antigen dose. In our system, a low antigen dose during the primary exposure severely limits Tfh cell expansion yet drives the expansion of a non-lineage differentiated Teff cell population. These findings are consistent with literature which demonstrates that sustained antigen availability enhances the magnitude of Tfh cell responses which is directly correlated with enhanced GC activity and high-affinity antibody production,[89,90] but further experimentation is required to delineate the specific mechanisms limiting Tfh cell expansion during priming with 1 mg OVA + CT.

Moreover, non-lineage differentiated $CD4^+$ T cells have also been observed in mice tolerant to food antigens (T_H^{lin-} cells) and are precursors of regulatory T cells,[91] which phenotypically resemble the primed $CD4^+$ T cells we have observed that are precursors of Tfh cells. Work to determine the mechanisms which regulate the diverse recall responses of these phenotypically similar $CD4^+$ T cells may elucidate novel points of intervention applicable in therapeutic design. We found that CD40/CD40L interactions and IL-4Rα signaling were critical requirements during the recall of primed memory $CD4^+$ T cells to induce *de novo* IgE CSR in naïve B cells. Future research is warranted to address whether transiently blocking either of these signaling pathways may divert memory $CD4^+$ T cells away from a pathogenic fate decision towards a regulatory-like fate, such as observed by Hong *et al.* in allergen-specific T_H^{lin-} cells from tolerant mice,[91] or towards a Th1-skewed fate. It is also possible that tertiary allergen re-exposures in the absence of these blockades of signaling pathways would lead to the emergence of IgE and clinical reactivity, which could indicate that fate decisions are immovably programmed in primed $CD4^+$ T cells and that alternative therapeutic approaches would be required. For example, specific deletion of primed $CD4^+$ T cells could eliminate the pathogenic subset, though this would prove technically difficult to achieve in an allergen-specific manner; additionally, in patients who have already progressed to clinical reactivity and have allergen-specific MBCs, this approach may be insufficient to prohibit IgE production.[18,41–43] In light of our findings, both allergen-specific memory $CD4^+$ T cells and MBCs may need to be targeted to prevent relapse of food

allergy upon cessation of therapy, as is frequently observed following OIT.[10–12] Given that the Th2A subset is reduced but not eliminated following immunotherapy in peanut or HDM allergic patients or long-term anti-IL-4Rα blockade in atopic dermatitis patients, this implies that persisting memory $CD4^+$ T cells instigate allergic disease recurrence.[32,65,92]

Conclusion

Altogether, our findings that memory $CD4^+$ T cells drive *de novo* IgE CSR in naïve B cells suggest that memory $CD4^+$ T cells are capable of independently perpetuating allergic disease. We found that memory $CD4^+$ T cells drive IgE CSR at multiple tissue sites following non-adjuvanted allergen exposures, which suggests their potency in maintaining allergic memory. If these findings hold true in patients, this positions primed memory $CD4^+$ T cells as a crucial therapeutic target. Future research ought to investigate the extent of primed memory $CD4^+$ T cell plasticity and whether these cells can be diverted away from an IgE-initiating, pathogenic Tfh cell fate. To conclude, this work has advanced our understanding of the fundamental requirements for the perpetuation of food allergy. This informs the development of efficacious therapeutics that target disease-perpetuating immune cells and lead to long-term remission of food allergy.

APPENDICES

Appendix 1: Flow Cytometry Antibodies for detection of OVA-specific CD4$^+$ T cells

Specificity	Clone	Fluorophore	Step	Dilution	Source
OVA2C/OVA3C:I-A^b-4E tetramers		APC	Pre-stained, 37°C, 45 mins	~10 nM	Jenkins Lab
Fixable Viability Dye eFluor780		APC-Cy7 channel	Extra	1:600	Invitrogen
CD90.1	OX-7	APC	Extra	1:100	BioLegend
TCRβ chain	H57-597	Pacific Blue	Extra	1:100	BioLegend
FoxP3	MF-14	AF488	Intra	1:100	BioLegend
GATA3	L50-823	PE	Intra	1:20	BD Biosciences
Bcl6	K112-91	PE-CF594	Intra	1:50	BD Biosciences
CXCR5	L138D7	PE, PE-Cy7, BV421	Extra, room temp	1:40	BioLegend
ST2	U29-93	BV421	Extra	1:100	BD Biosciences
CD44	IM7	BV510, PerCP/Cy5.5,	Extra	1:100, 1:600	BioLegend
CD62L	MEL-14	BV605, BV650	Extra	1:200	BioLegend
CD69		BV650	Extra	1:100	BioLegend
PD-1	29F.1A12	BV785	Extra	1:100	BioLegend
CD4	GK1.5	PE-Cy7, AF700, BV510	Extra	1:100	BioLegend
CD3	17A2	BV510	Extra	1:100	BioLegend
B220 (Dump)	RA3-6B2	BV711, BV510	Extra	1:600, 1:200	BioLegend
F4/80 (Dump)	BM8	BV711, BV510	Extra	1:400, 1:200	BioLegend
CD11b (Dump)	M1/70	BV711	Extra	1:600	BioLegend
CD11c (Dump)	N418	BV711	Extra	1:600	BioLegend
CD8 (Dump)	53-6.7	BV711, BV510	Extra	1:200	BioLegend

Appendix 2: Flow Cytometry Antibodies for detection of OVA-specific B cells

Specificity	Clone	Fluorophore	Step	Dilution	Source
OVA B cell tetramers		PE, APC	Pre-stained, 4°C, 25 mins		Taylor Lab or made in house
Decoy tetramers		PE-Dylight594, APC-Cy7	Pre-stained, room temp, 10 mins		Taylor Lab or made in house
LIVE/DEAD Fixable Aqua Dead Cell Stain		BV510 channel	Extra	1:600	Invitrogen
Fixable Viability Dye eFluor780		APC-Cy7 channel	Extra	1:600	Invitrogen
B220	RA3-6B2	AF700	Extra	1:100	BioLegend
CD138	281-2	BV605, APC	Extra	1:100	BioLegend
Gamma Immunoglobins Heavy and Light chains (H+L)		Pacific Blue	Intra	1:1000	Invitrogen
IgM	Il/41	BV786	Extra	1:50	BD Biosciences
IgD	11-26c.2a	BV510, BV605	Extra	1:100	BioLegend
IgG1	RMG1-1	BV421, BV650, APC	Intra or Extra	1:200 or 1:100	BioLegend
IgE	RME-1	PE	Intra	1:200	BioLegend
IgG2c	n/a	FITC	Intra	1:100	Southern Biotech
IgG2b	RMG2b-1	APC	Intra	1:100	BioLegend
GL7	GL7	PerCPCy5.5	Extra (2^{nd} stain)	1:400	BioLegend
CD38	90	PE-Cy7	Extra	1:100	BioLegend
CD86	GL-1	FITC	Extra	1:200	BioLegend
F4/80 (Dump)	BM8	BV711	Extra	1:400	BioLegend
CD3 (Dump)	17A2	BV711	Extra	1:200	BioLegend

Appendix 3: Flow Cytometry Antibodies for detection of circulating IgE$^+$ basophils.

Specificity	Clone	Fluorophore	Step	Dilution	Source
Fixable Viability Dye eFluor780		APC-Cy7 channel	Extra	1:600	Invitrogen
IgE	RME-1	FITC	Extra	1:100	BioLegend
CD200R3	Ba13	PE	Extra	1:100	BioLegend
CD45	104	BV421	Extra	1:400	BioLegend
FcεRI	MAR-1	AF700	Extra	1:100	BioLegend

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