Aus dem Institut für Laboratoriumsmedizin und Pathobiochemie
Molekulare Diagnostik

Geschäftsführender Direktor: Prof. Dr. Harald Renz

des Fachbereichs Medizin der Philipps-Universität Marburg

Elucidating the Role of IgA in Allergic Disease

Inaugural-Dissertation

zur Erlangung des Doktorgrades der Medizinwissenschaften

(Dr. rer. med.)

Dem Fachbereich Medizin der

Philipps-Universität Marburg

in Zusammenarbeit mit

Dem Fachbereich Immunologie der

Boston Children's Hospital

vorgelegt von

Yasmeen El Ansari

aus Dhahran, Saudi Arabia

Marburg, 2024

Angenommen vom Fachbereich Medizin der Philipps-Universität Marburg

am: 25.04.2024

Gedruckt mit Genehmigung des Fachbereichs

Dekanin: Prof. Dr. D.Hilfiker-Kleiner

Referent: Prof. Dr. H. Renz

Korreferentin: Prof. Dr. S. Bauer

Table of Contents

List of figures and tables

List of tables

List of Figures

Figure 20. Flow cytometric evaluation of T follicular helper and regulatory cells, and B cells

Figure 21. Flow cytometric evaluation of T helper subsets

Figure 22. Analysis of the fecal microbiome in IgA+/- and IgA-/- mice

1. Introduction

1.1 Brief History of Allergy

In the past century, there has been a steep increase in the prevalence of allergic disorders. Characterized by an abnormal immune response, termed "hypersensitivity," to normally harmless substances, they include asthma, atopic dermatitis, allergic rhinitis, and food allergy. Despite its recent global rise, hypersensitivity is not a new concept, having been described as early as 2641 BCE, after the death of an Egyptian pharaoh by a bumblebee sting (Krombach et al., 2004). In the 19th century, the term "hay fever" was coined by John Bostock to describe eye and nose sensitivities to hay (Ramachandran and Aronson, 2011). However, the science of modern allergy began with Clemens von Pirquet, also known for his description serum sickness, who coined the word allergy to describe the interaction of antibodies with allergens (Huber, 2006).

Mast cells, the effector cells of allergy, were discovered in the 19th century by Paul Ehrlich, who observed cells with large granules and referred to them as "*Mastzellen"*. These granulocytes are found in the mucosal and connective tissues of the skin, lungs, and gastrointestinal tract. Their granules contain substances such as histamine, proteoglycans, cytokines, proteases, and other mediators (Ghably et al., 2015). The discovery of reagin by Prausnitz and Kustner in the early 20th century as the circulating factor responsible for eliciting allergic reactions provided a link between serum proteins and mast cells in mediating hyperreactivity (Prausnitz and Kustner, 1921). After four decades, the active component of reagin was identified by the Ishizakas and co-workers as IgE, the fifth antibody isotype (Bennich et al., 1968). A series of key advancements in the allergy field subsequently uncovered the processes whereby mast cells become sensitized when IgE binds to its high affinity receptor, FcεRI on their surface, and upon allergen exposure are activated and release their preformed granule contents as well as very rapidly synthesizing eicosanoid mediators to induce hypersensitivity reactions. Discoveries about mast cells and IgE biology were eventually followed by the identification of T-helper type 2 (Th2) cells, which produce cytokines such as IL-4, IL-5, and IL-13, which coordinate the production of IgE, the formation of the key bone marrow-derived effector cells of allergy and the

induction of type 2 tissue inflammation underlying allergic diseases (Mosmann and Coffman, 1987).

Allergy research has advanced remarkably over the years from what was once a collection of anecdotal observations to a sophisticated science that generates hundreds of publications per year. Recent advancements and the cutting edge of the field include the descriptions of numerous functional T cell subtypes such as T regulatory cells and T follicular helper cells, as well as demonstrations of the critical effects of the gut, airway, and skin microbiome on the pathogenesis of allergic diseases.

1.2 Epidemiology and impact on public health

According to the Centers for Disease Control and Prevention (CDC), the 2021 National Health Interview Survey reports that 1 in 3 U.S adults, or 1 in 4 U.S children have seasonal allergies, eczema, or food allergy (Figure 1) (Ng and Boersma, 2023). The European Academy of Allergology and Clinical Immunology (EAACI) reports a similar prevalence to that seen in the United States with 30% of Europeans having allergic symptoms (EAACI, 2015). The rise of allergies worldwide has caused a significant burden on the healthcare sector, with increased doctor visits and hospitalization. Allergies also reduce productivity in settings of work and school, and impair daily functions, thus causing frustrations that decrease overall quality of life. There is now an increasing need for allergy/immunology specialists, as well as advancements in allergy research, to help provide novel medications that are affordable and effective (Baiardini et al., 2006).

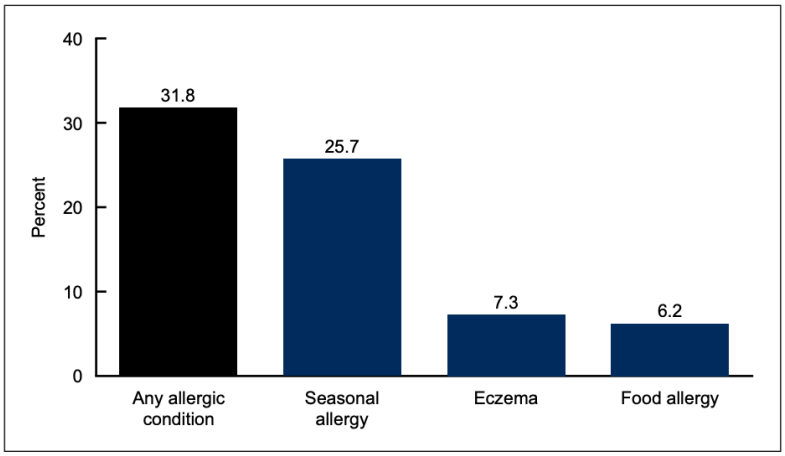


Figure 1. Percent of adults with diagnosed allergic conditions (seasonal allergies, eczema, or food allergy) by the National Health Interview Survey in 2021 (Ng and Boersma, 2023).

1.3 Etiology and risk factors

It is widely accepted that allergies are caused by a complex interplay of genetic and environmental factors. Significant diversity exists within the literature regarding how various genetic and environmental factors impact the likelihood of atopic sensitization and allergic conditions in children. Investigations regarding the influences of factors like owning pets, gender, smoke exposure, air pollutants, use of antibiotics, and atopic sensitization during early life have implicated all of them to varying extents (Ober and Yao, 2011).

1.3.1 Genetics

Food allergy, allergic rhinitis, asthma and atopic dermatitis are all highly heritable. Numerous twin studies have revealed high concordance rates between monozygotic twins in comparison to dizygotic twins, suggesting a strong genetic influence in food allergy, atopic dermatitis, asthma, and allergic rhinitis (Laitinen et al., 1998; Larsen et al., 1986; Rasanen et al., 1998; Sicherer et al., 2000). Despite strong genetic evidence, identifying genes that account for increased risk has been a major challenge.

Polymorphisms in HLA alleles HLA-DPA1 and HLA-DQA1, and IL13 have all been associated with IgE levels in preschool children (Chang et al., 2013). Genome wide

association studies (GWAS) have identified the gene cluster locus (SERPINB) to be associated with increased susceptibility to atopic dermatitis and asthma, and others (HLA-DQB1) with food allergy (Hong et al., 2015). Both orosomucoid 1-like protein 3 (ORMDL), and dysregulated activity of a disintegrin and metalloprotease 10 (ADAM10) has also been implicated in asthma pathogenesis. Variants of Mucosa-Associated Lymphoid Tissue Lymphoma Translocation Protein 1 (MALT1) were found to be associated with peanut allergy in LEAP study participants (Winters et al., 2019). While GWAS has identified individual genes to be associated with asthma, atopic dermatitis, and food allergy, it was surprising that no genes were found to be shared between the different allergic disorders. It was only recently that efforts have been made to identify overlapping genes, with 30 shared genetic loci between the aforementioned allergic disorders (Zhu et al., 2018).

Many of the shared genes were found to be expressed in the skin. Variants of the filaggrin gene (FLG), that encodes a skin integrity protein, have been some of the most extensively studied examples genetically determined risk for allergy. Forty loss of function mutations have been identified and shown to be a major predisposing factor for atopic dermatitis patients (Akiyama, 2010). Susceptibility to allergic rhinitis, and asthma is also higher in patients with FLG mutations (van den Oord and Sheikh, 2009). There is mounting evidence suggesting their association with food allergy (Brown et al., 2011). Kalb and colleagues found FLG mutations to be associated with food allergies independent of eczema including: egg, milk, peanut, soy, cashew, and walnut (Kalb et al., 2022). Animal studies have proven useful to dissect the mechanisms whereby cutaneous antigen exposure can cause food allergies and how FLG variants might influence these pathways. Mice heterozygous for $(Flg)^{ft}$ and $Tmem79^{ma}$ ($FT^{+/-}$) (skin barrier mutations), exhibit an increase in temperature drop, and antigen-specific IgE responses after skin sensitization to allergen and oral challenge compared to controls. Furthermore, $FT^{+/-}$ pups of allergic mothers undergoing the same sensitization protocol also exhibited increased responsiveness to anaphylaxis, with more mast cells, and Th2 cytokines in their skin and intestines. The pathways by which FLG mutations enhance allergic sensitization following cutaneous exposure are mediated by IL-33, as ST2 blockade suppresses anaphylaxis (Walker et al., 2018). It has been

hypothesized that the mutations cause a defective skin barrier, and increased allergen penetration, increasing likelihood of cutaneous sensitization to food antigens, and the development of food allergies (Osawa et al., 2011).

1.3.2 The Hygiene Hypothesis and dysbiosis

While genetic elements, such as barrier defects and immune dysregulation, as well as genetic variations affecting the Th2 response, have been linked to the vulnerability to allergies, it is clear from the rate of increase of these disorders that environmental factors must play a key role. Development of the hygiene hypothesis was the first attempt to explain the alarming increase in allergic disorders, postulating that the changed exposures to pathogenic microbes early in childhood alters the development of the immune system, leading to an increase in allergic diseases (Strachan, 1989). This hypothesis has now been expanded to also consider effects brought about by commensal microbiota, as well as the elements that alter microbial colonization. These factors involve dietary habits, the child's upbringing environment, breast feeding, the administration of antibiotics, and the method of childbirth (Renz and Skevaki, 2021).

Currently, research focuses on how the microbiota foster immune tolerance to allergens. While a direct cause-and-effect relationship is yet to be determined, changes in the composition of both skin and gut microbiota have been linked to allergies, with an important impact on Treg function. More specifically, diverse early life microbial colonization has been proposed to play to protect from allergies, whereas constrained diversity and dysbiosis increases susceptibility. Observations from human microbiome studies have been supported by work done in germ-free mice that develop elevated IgE levels, suggesting that microbial colonization is important in the suppression of IgE mediated allergies (Cahenzli et al., 2013). Recent work done by Abdel Gadir et al., demonstrates that patients with food allergies have gut dysbiosis characterized by a decrease in *Subdoligranulum variabile* and *Clostridiales* species. In a food allergy prone mouse model (IL4ra F709), these microbes can provide protection, mediated by RORγT^{+} Treg cells (Abdel-Gadir et al., 2019).

Effects of early-life microbial exposure have emerged most clearly in studies of children raised in urbanized vs farming environments. The upbringing environment of a child has now been found to be associated with a decrease or increase in prevalence of allergic disease, such as asthma. Living on traditional farms provides asthma-protective effects, attributed by particular environmental microbes, including *Lactococcus lactis* (Debarry et al., 2007) *Staphylococcus sciuri*, (Hagner et al., 2013) and *Bacillus licheniformis* (Vogel et al., 2008) *Acinetobacter lwoffii*, has recently stood out as a protective bacterial species, identified by multiple independent studies, (Brand et al., 2011; Conrad et al., 2009; Debarry et al., 2007). The protective effects of intranasal administration of *Acinetobacter lwoffii*, requires IL-6, and IL-10 (Cinicola et al., 2022).

These studies, among others have established a robust foundation for recognizing significant microbial characteristics that are common among patients that are afflicted by one type of allergic disease. Only several studies have investigated the links between infant microbial compositions and various separate outcomes related to allergic diseases. Work done by Hoskin et al., by investigating infant stool microbiome at age 3 months and 1 year, found that children diagnosed at age 5 years had delayed infant microbiota maturation compared to children who had no history of allergic sensitization. By taking an aggregate approach, researchers were able to find infant microbiota shifts even before diagnoses of multiple allergic diseases (asthma, allergic rhinitis, food allergy, or atopic dermatitis). Their work reveals that changes in infant microbiota maturation may be the commonality that all allergic diseases share (Hoskinson et al., 2023).

1.4 Allergy Diagnosis and Management

Classical immediate allergic reactions are driven by IgE-mediated activation of mast cells and/or basophils, the key effector cells of immediate hypersensitivity. IgE-mediated allergies are diagnosed based on clinical presentation, physical examinations, laboratory tests and family history of atopy. Typically, positive skin testing, and the detection of allergen-specific IgE in the blood, are combined with clinical information to establish a diagnosis. Skin testing involves the application of allergen extracts, along with positive histamine, and negative saline controls by a prick or puncture of the skin. Positive tests are

characterized by the appearance of a “wheal and flare”. Some individuals with allergen specific IgE antibodies, especially in the low/moderate range, will not exhibit allergic symptoms upon allergen exposure. In the case of food allergies, oral food challenges are often utilized to rule in or out food allergy in these subjects with only modest IgE responses (Nowak-Wegrzyn et al., 2017). Food allergies are managed by dietary food avoidance, with a contingency anaphylaxis treatment plan, which includes the prescription of an epinephrine auto-injector. Oral immunotherapy (OIT) has emerged as a disease modifying treatment option for food allergies. It involves the ingestion of incrementally increasing amounts of allergens or allergen products by IgE-sensitized allergic patients in hopes they will be able to tolerate varying amounts of allergenic food without reacting. Palforzia, the first standardized OIT regimen was approved by the FDA in 2020 for the treatment of peanut allergies.

1.5 Pathophysiology

Contact with allergens, whether by inhalation, ingestion or injection, can trigger an array of hypersensitivity reactions, the most serious being anaphylaxis. Anaphylaxis is a systemic reaction that arises when hypersensitivity reactions simultaneously affect multiple organ systems, including the skin, gastrointestinal tract, cardiovascular system, respiratory system, and central nervous system. Gastrointestinal manifestations of anaphylaxis include diarrhea, nausea, cramps, and vomiting. Cardiovascular responses include diminished cardiac output, hypotension and tachycardia, which, along with plasma loss via vascular leak, can lead to hypovolemic and cardiogenic shock. Angioedema and urticaria are common cutaneous manifestations of immediate hypersensitivity (Yu et al., 2016).

IgE is unique in its ability to induce immediate pathological responses by sensing minute quantities of antigens. Crosslinking of FcεR1 activates constitutively associated Lyn tyrosine kinase, which, in turn, phosphorylates tyrosine residues within the Immunoreceptor Tyrosine-based Activation Motif’s (ITAM’s) in the cytosolic domains of the β- and γ-chains of FcεRI. This facilitates the binding of spleen tyrosine kinase (Syk) to the phosphorylated ITAM’s, which results in a conformational change in Syk. Syk when

activated, phosphorylates the linker for the activation of T cells (LAT), and other scaffold proteins such as SLP76 which serve as docking sites for multiple signaling intermediates, including VAV, GAB2, GADS, and PLC-γ. Phosphorylation of LAT also activates the PI3K and protein tyrosine kinase BTK pathway. The actions of PLC-γ and the activation of PI3K and BTK pathways lead to the immediate release of preformed mediators (histamine, proteoglycans, proteases) contained in mast cell granules, very rapid synthesis of vasoactive lipid mediators (LTC4, LTD4, and LTE4), and transcriptional activation of genes encoding several pro-allergic cytokines and chemokines (Siraganian et al., 2010). A range of physiological responses is elicited within minutes by the preformed and lipid mediators and is termed an immediate hypersensitivity reaction. Histamine, prostaglandins, leukotrienes, and serine proteases induce, increased vascular permeability and vasodilation, resulting in erythema and tissue edema. In the airways they induce smooth muscle constriction and mucus production (Nowak-Wegrzyn et al., 2017). Such responses may be play key protective effector functions in the expulsion of helminths and inactivation of insect and reptile venoms but are pathogenic when triggered in response to harmless antigens(Tsai et al., 2015). A late phase reaction, mediated by chemokines and cytokines produced by mast cells and basophils occurs hours after activation and is associated with an the influx of basophils and eosinophils into affected tissues (Iype and Fux, 2021).

1.6 Mechanisms of immune sensitization

A unifying theme in current understanding of allergic sensitization is that disruption of epithelial barriers in the airways, skin, or gut (due to toxins, physical trauma or infection) can lead to Th2 mediated responses. This concept has been most thoroughly developed in studies of atopic dermatitis, where a disrupted skin barrier due to filaggrin mutations, increases the risk of atopic dermatitis and food allergy (Marenholz et al., 2006). In the setting of food allergy, damage to intestinal epithelial cells, leads to the production of proinflammatory cytokines IL-25, thymic stromal lymphopoietin (TSLP), and IL-33 that activate dendritic cells (DCs) and innate lymphocyte cells 2 (ILC2s). Gastrointestinal mucosal DC’s that have picked up and processed allergens absorbed from the gut migrate to draining mesenteric lymph nodes (mLN) and upregulate Jag-1 which interacts with

Notch receptors on T cells, reprogramming Tregs to Th2 and Th17 phenotypes. In the skin, CD301b$^+$ DC's also induce Th2 skewing effects in a model of cutaneous allergen exposure. Dewan et al., demonstrate that substance P produced by TRPV1+ sensory neurons l stimulate CD301b dendritic cells through the Mas-related G-protein coupled receptor member A1 (MRGPRA1). CD301b$^+$ DC's then migrate to draining lymph nodes to initiate Th2 responses (Perner et al., 2020). Naïve CD4$^+$ T cells in the lymph nodes are primed into Th2 and Tfh cells. Tfh cells interact with B cells in lymphoid follicles to induce class switching to IgE, a primary event in sensitization. IgE binds to its high affinity receptor FcεRI on mast cells, and basophils. IgE-mediated activation of these cells results in the induction of Th2-promoting cytokines, including IL-4 in a feed forward loop that further amplifies emerging Th2 responses, and the production of more IgE (Burton et al., 2014b; Yu et al., 2016). A current model of the induction of a IgE mediated allergic immune response is given in Figure 2.

1.6.1 ILC2

Type 2 innate lymphoid cells (ILC2) are predominantly found at mucosal barriers and function like Th2 cells but lack TCRs. They release large amounts of IL-5, IL-13, IL-4, IL-25 and IL-9, when activated (Schwartz et al., 2017). ILC2s further promote allergic inflammation and suppress the production and function of allergen-specific regulatory T cells. IgE-activated mast cells can drive the intestinal expansion of ILC2 that produce IL4, Il-5, IL-9, and IL-13, and contribute to allergy phenotypes (Moro et al., 2010; Neill et al., 2010; Noval Rivas et al., 2016). In the absence of IgE and mast cells, ILC2 expansion is impaired (Burton et al., 2018a).

1.6.2 Tfh

Found in germinal centers, T follicular helper (Tfh) cells engage in cognate interactions with B cells that promote class switching and generation of high-affinity antibodies. This Th type was first described in the 2000s and is now recognized as vital for IgE responses. Tfh express the transcription factor Bcl-6, along with the surface markers PD-1 and CXCR5 (which guides their localization to lymphoid follicles, and produce cytokines such

as IL-21, IL-4, and IL-13. The use of genetically modified mice in which Tfh cell development is impaired by conditional deletion of Bcl6 or the IL-6 receptor results in diminished IgE levels in multiple allergy models (Dolence et al., 2018; Gowthaman et al., 2019; Kobayashi et al., 2017). Paradoxically, classical Tfh's express IL-21, a cytokine known to suppress IgE switching in B cells. Recent studies by Eisenbarth and colleagues revealed the existence of a novel Tfh subset, Tfh13s, that express Bcl-6 and produce IL-13 and can act synergistically with IL-4 to induce IgE class switching (Gowthaman et al., 2019).

1.6.3 IgE^+ B cells

IgE production by B cells is a central mechanism in allergic responses. IL-4 signaling is critical in driving IgE production. Mast cells, basophils, natural killer T cells (NKT), Th2 cells, and Tfh's have all been reported to produce IL-4. IL-4 interacts with IL-4/IL-13 receptor on B cells to induce germline transcription and then class switch recombination (CSR) to IgE. CSR involves the transcriptional activation of Cε, nucleotide modification of switch region (Sε), followed by the introduction of double stranded breaks in Sμ and Sε by activation-induced cytidine deaminase (AID). Lastly, these double stranded breaks are annealed by nonhomologous end-joining by bringing together the VDJ and Cε regions to finalize the switching process to create a functional IgE. Sometimes, B cells can switch first from IgM to IgG, and then undergo a second switch to IgE (Oettgen, 2016).

The Iε promoter controls the transcription of the ε-locus and contains binding sites for signal transducer and activator of transcription (STAT) 6, and nuclear factor κB (NF-kB). IL-4 and/or IL-13 bind to their receptors on B cells to activate STAT6. Engagement of CD40L on T cells with CD40 on B cells acts as a second signal to activate NF-kB, which, along with STAT6, drives IgE class switching. B cells can also switch to IgE without T cell help after the engagement of B cell–activating factor of the TNF family (BAFF) on DCs and monocytes with transmembrane activator and CAML interactor (TACI) on B cells that have already been stimulated by cytokines. McCoy et al demonstrated that IgE production can occur in mice and humans that have no T cells or MHC II, and this ‘‘natural IgE" with unknown function increases with age. These mice cannot mount effective antigen-specific

IgE responses, and their natural IgE shows no evidence of somatic hypermutation (McCoy et al., 2006). While IL-4 is indispensable, it is not sufficient for optimal IgE production, which also requires IL-13 as shown by Gowthaman *et al*. Their work identified a novel subset of IL-4- and IL-13-producing Tfh that are needed to produce IgE with a high affinity to the antigen (Gowthaman et al., 2019).

IgE^+ B cells are short-lived in germinal centers, a site important for antibody maturation, and the generation of long-lived plasma cells (Erazo et al., 2007). Mice deficient in Blimp-1, a transcriptional regulator of plasma cell differentiation, have increased numbers of IgE^+ germinal center B cells, indicating that IgE^+ B cells are predisposed to transition into short-lived plasma cells. It was previously believed that IgE^+ B cells are absent from long-lived compartments even though it has always been hard to justify this view in light of the persistence of significant allergen specific IgE levels in food allergic subjects avoiding allergen and in pollen sensitive patients even outside of pollen season. Evidence from a chronic allergic mouse model by Asrat *et al.* supports the presence of IgE^+ long-lived plasma cells in the bone marrow that are derived from sequential class switching of IgG1. The same study also identifies IgE^+ plasma cells in the bone marrow of human allergic donors, which are capable of inducing mast cell degranulation in mice (Asrat et al., 2020). Indirect evidence from human blood samples, such as the persistence of IgE over several years, even after targeting IL-5/IL5R and IL-4/IL-13, suggests the presence of long-lived cells that secrete IgE (Pitlick and Pongdee, 2022). The sites of IgE production are likely to be in mucosal tissues. The presence of epsilon germline transcripts and IgE switch excision circles in the respiratory mucosa of allergen-exposed patients with seasonal rhinitis has suggested local formation of cells in allergen-exposed tissue (Cameron et al., 2003). More recent findings from single cell RNA sequencing in the intestine of food allergic subjects have revealed IgE^+ B cells and plasmablasts in the stomach and duodenum in peanut allergic patients (Hoh et al., 2020). The thymus may serve as the site of production of natural IgE in mice. Kwon et al show that thymic plasma cells that produce IgE depend on IL-4 production by NKT cells, using mixed bone marrow chimeras. They also show that thymus derived IgE causes mast cell expansion in the gut and skin in a model of food

anaphylaxis (Kwon et al., 2022). This work opens a new avenue of research in terms of source and origin of homeostatic vs antigen specific IgE^+ B cells.

Just as the sites of active IgE production have been elusive over the past decades, so have the cells harboring IgE memory. Circulating IgE^+ memory B cells are exceedingly rare. A combination of experimental approaches including clonal lineage analysis of IgG and IgE B cell families in allergic patients and studies of animals lacking IgM have indicated that both the formation of high-affinity IgE responses and the maintenance of memory reside in IgG^+ B cells and that elicitation of an anamnestic IgE response requires IgE isotype switching of this IgG^+ memory pool (Aranda et al., 2023; Xiong et al., 2012; Zhang et al., 2010).

1.6.4 Allergic effector cells

Mast cells play a vital role in allergic pathogenesis. They are stationed at the interface between our bodies and the environment and serve as effectors of anaphylactic reactions. They are long lived cells, arising locally from blood borne bone marrow-derived progenitors and increase by several-fold in patients with IgE-dependent hypersensitivity disorders (Stone et al., 2010). FcεRI is the high-affinity IgE receptor on mast cells that triggers a signaling cascade that results in mediator release and cytokine production. The vasoactive mediators released by mast cells induce immediate reactions, such as vascular leak with tissue edema, vasodilation with tissue erythema, smooth muscle contraction, and mucus production (Oettgen, 2016). These host responses confer a vital adaptive advantage in expelling helminths and removing venoms but are pathogenic when triggered in response to harmless antigens such as food, dust, or pollen. IgE- FcεRI aggregation on mast cells also leads to the production of a plethora of cytokines that exert both pro-inflammatory and immunomodulatory effects. As prolific producers of pro-Th2 cytokines, mast cells amplify adaptive allergic responses in asthma, contact hypersensitivity, and food allergy (El Ansari et al., 2020). Studies involving mice deficient in mast cells or IgE antibodies have revealed impaired migration of dermal dendritic cells and T cell activation during the sensitization phase of the immune response, indicating that mast cells play a crucial adjuvant role in immune sensitization (Bryce et al., 2005; Galli and Tsai, 2010). In recent years, it has become clear that in the setting of food allergy, mast cells not only function as effector cells

of hypersensitivity reactions but also as inducers of emerging immune responses, such as Th2 expansion and suppression of Treg development and function (Burton et al., 2014b).

Similar to mast cells, basophils release inflammatory mediators upon IgE- FcεRI receptor crosslinking. However, they differ from mast cells in several ways. They originate and mature in the bone marrow (mast cells mature in tissue) and are released into the bloodstream, where they circulate, representing only 0.5-1% of blood leukocytes. Unlike mast cells, they do not express c-Kit and have a much shorter lifespan of a few days (Poto et al., 2022). They appear to have a non-redundant role in the delayed hypersensitivity response that occurs days after antigen-IgE-Fc cross-linking. For instance, their role in priming chronic allergic inflammation has been demonstrated in a model of passive cutaneous anaphylaxis by passively sensitizing mice with IgE and injecting their ears with antigen. Obata *et al.,* showed that the inflammatory response that occurs 3-4 days after ear swelling can be elicited in the absence of mast cells and T cells. However, this response was absent in mice deficient in basophils (Mcpt8DTR) which exhibit a reduction in inflammatory cell infiltration and ear swelling (Obata et al., 2007). Basophil recruitment to the skin was recently shown to be mediated by IL-3 producing T cells (Hachem et al., 2023). Basophils have also been reported to enhance humoral Th2 responses when activated by IgD. In humans, in vitro data demonstrates IgD to interact with an unknown receptor on basophils to induce the release of IL-4 (Chen et al., 2009). Work done by Shan et al., expands on this by identifying Galectin-9 and CD44 to interact with IgD in mice to induce basophil release of IL-4, IL-5, and IL-13, which induces IgG1 and IgE production by B cells (Shan et al., 2018).

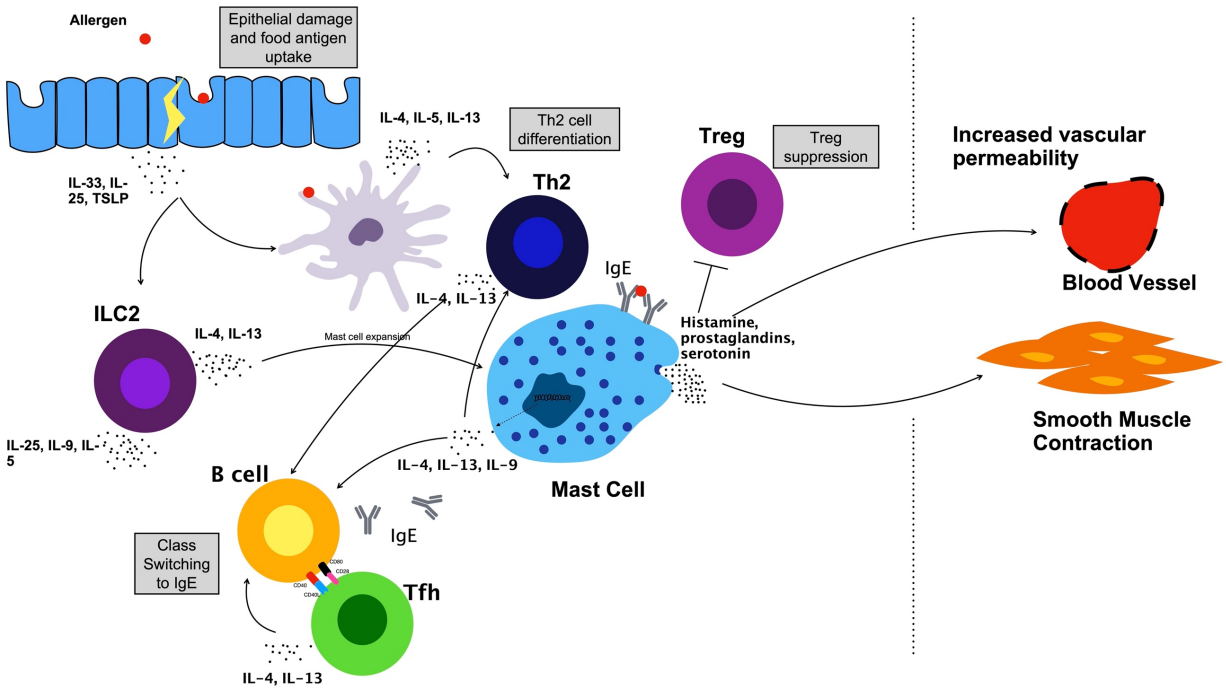


Figure 2. IgE dependent activation of mast cells in in the gut leads to Th2 cell-mediated immune responses. During sensitization, damage to the epithelial barrier in the gastrointestinal tract (due to trauma or toxins) leads to increased antigen entry and secretion of epithelium derived IL-33, IL25, and thymic stromal lymphopoietin (TSLP). Upon antigen recognition and processing by antigen presenting cells, IL-33, IL-25, and TSLP promote dendritic cell differentiation into a Th2 promoting functional phenotype. Mature APCs migrate to lymph nodes (not shown) to prime naive T cells and promote their differentiation into Th2 cells. This response drives ILC2 expansion and B cell class switching to IgE. T follicular helper cells also produce IL-4, and IL-13 to help generate high affinity IgE. IgE binds to its high affinity receptor FcεR1 on mast cells, and upon subsequent antigen-receptor cross linking cause them to degranulate. Products of degranulation such as histamine cause increased vascular permeability and smooth muscle contraction. Mast cell produced cytokines such as IL-4 and IL-13 further amplify the Th2 response by acting on B cells to produce more IgE, and further promote expansion of ILC2s. ILC2s can also drive mast cell expansion via IL-4, and IL-13. Mast cells also suppresses Treg function further promoting an allergic response and inhibiting tolerance.

1.7 Mechanisms of immune tolerance

Immune tolerance is the default immune response to ingested antigens, and microbes and is mediated by the induction of antigen-specific regulatory T cells (Treg). Breakdown of tolerance is a key feature of allergic sensitization. Tolerance is defined as a state of sustained systemic antigenic unresponsiveness. Effective induction of immune tolerance in physiologic immune responses to food antigen ingestion accounts for the fact that the

majority of humans are exposed to an array of allergens and never develop allergies. Several mechanisms underlie the induction of tolerance. CX3CR1+ macrophages, found in the gut mucosa, can extend their dendrites via gap junctions in the gut epithelium to capture antigens and can induce tolerance by secreting IL-10. They then transfer antigens to a subset of DC's that express CD103 which are thought to be vital in the induction of tolerance. In parallel, CD103+ DCs that produce retinoic acid (RA) and transforming growth factor-β (TGF-β) induce naïve CD4+ T cells to differentiate into Foxp3+ regulatory (Treg) cells. CD103+ DCs also program Tregs to home back to the lamina propria, in an RA mediated fashion dependent on upregulation of CCR9 and integrin α4β7. Zbtb46-DTR mice with ablated conventional DC's (cDCs) present with a reduction of Treg cells, making them critical cells in oral tolerance (Esterhazy et al., 2016; Yin et al., 2021).

In the lamina propria, Tregs expand with the help of resident CX3CR1+ macrophages. Tregs regulate multiple mucosal immune responses. They express inducible T cell co-stimulator (ICOS), and CTLA-4, IL-10, IL-35, and TGF-β, which act to maintain a state of T cell tolerance to dietary and microbial antigens. They also suppress the production of type 2 cytokines, and IgE, as well as hinder the function of mast cells and basophils (Gri et al., 2008). The migration of Tregs back to the gut is essential for the induction of oral tolerance, as mice deficient in gut-homing receptors β7 integrin, MADCAM and CCR9, demonstrate impaired oral tolerance (Cassani et al., 2011; Hadis et al., 2011).

Evidence supporting the physiologic role of Treg in the induction and maintenance of oral tolerance comes from several human and mouse studies. Children with cow milk allergy who develop natural oral tolerance have increased $CD4^{+}CD25^{+}$ Treg's in their peripheral blood and reduced proliferative milk-specific T cells (Karlsson et al., 2004; Shreffler et al., 2009). Similarly, in patients with egg and peanut tolerance, stimulation of peripheral mononuclear cells has been shown to expand $Foxp3^{+}$ T cells in tolerant subjects more efficiently than in patients with persistent egg and peanut allergies (Qamar et al., 2015).

The role of Treg cells in oral tolerance has further been supported by allergic mouse studies. Il4raF709 mice, which are more susceptible to allergy, mice have Th2 like Tregs

(GATA3^{+} Foxp3^{+}) that produce IL-4 (Noval Rivas et al., 2015). In response to OIT, allergic mice also have impaired Treg responses (Burton et al., 2014b).

The adoptive transfer of peripherally induced antigen-specific regulatory cells has been shown to transfer tolerance, establishing them pivotal cells in this process (Hadis et al., 2011). Mutations in Foxp3 are associated with a breakdown in tolerance and the development of allergies, as children with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), who harbor mutations in Foxp3, can present with atopic dermatitis, eczema, or food allergies (Nieves et al., 2004). Mice with Foxp3 loss of function mutations also develop atopic dermatitis-like lesions (Brunkow et al., 2001). Together, these studies indicate a suppressive role for Tregs in allergies.

1.8 Antibodies in tolerance

Prophylactic inoculation of allergen has been used since 1911 to desensitize grass pollen in patients with seasonal allergic rhinitis (Noon, 1953). This process, subcutaneous immunotherapy (SCIT) involves repeated administration of allergen for a period of months to years. It currently stands as the only disease-modifying treatment for respiratory allergies mediated by IgE. However, it is not devoid of limitations, including accompanying side effects including severe allergic reactions (which preclude its use in food allergy), low patient adherence, and ineffectiveness in some patients. In subjects undergoing SCIT there is a strong correlation between effective outcomes and the generation of allergen specific IgG antibody responses. It has long been known that IgG antibodies can inhibit IgE induced mast cell degranulation (Bruhns et al., 2005; Malbec and Daeron, 2007). It has been proposed that these IgG's intercept allergens, binding to and hiding their epitopes and render them invisible to IgE on sensitized effector cells such as mast cells (Shamji et al., 2022).

In food allergy, oral immunotherapy (OIT) involves the ingestion of incrementally increasing amounts of allergens or allergen products by IgE-sensitized allergic patients. Upon completion of immunotherapy course, patients can tolerate significant amounts without a reaction. Despite adopting a state of unresponsiveness after completion of OIT,

patients still have high levels of antigen-specific IgE, levels that would normally be associated with significant reactions (Santos et al., 2020). This has long suggested that OIT induces factors that suppress IgE-mediated reactions. It is now greatly appreciated that sublingual and oral immunotherapy induce strong allergen-specific IgG responses in both mouse models and in human subjects (Burton et al., 2014a; Caubet et al., 2012; Tsai et al., 2020). Furthermore, both subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT) have been successful in conferring clinical benefit in allergic rhinitis patients, with numerous studies showing an induction of IgG antibodies (Jutel et al., 2005; Oefner et al., 2012; Reisinger et al., 2005). The natural resolution of allergies have also been associated with increasing IgG levels (Ruiter et al., 2007; Savilahti et al., 2010).

The mechanisms by which IgG inhibit IgE-mediated mast cell activation have been studied extensively in mouse models. Work done by Burton and colleagues using bone marrow derived mast cells (BMMCs) sensitized with peanut-IgE, shows that IgG from sera of mice that underwent peanut OIT can inhibit mast cell degranulation (Burton et al., 2014a). When FcγRIIb$^{-/-}$ BMMCs are used, suppression by IgG is not observed, indicating the importance of suppressive signals from this inhibitory receptor. Steric blocking has commonly been presumed to be the dominant mechanism by which IgG inhibits IgE-mediated reactions, but this study nicely uses genetic tools and dose response analyses to show that while at high concentrations IgG this is true, that at orders of magnitude lower levels, IgG acts via FcγRIIb$^{-/-}$. IgG: FcγRIIb-mediated inhibition of IgE-sensitized mast cells has also been observed *in vivo*. Enhanced anaphylaxis was exhibited in FcγRIIb-deficient mice, and passive transfer of IgG inhibits anaphylaxis in wildtype but not FcγRIIb-deficient mice, indicating a requirement for the inhibitory receptor in controlling IgE-mediated anaphylaxis (Burton et al., 2018b).

By utilizing sera from peanut allergic patients who have undergone OIT, Burton et al tested the suppressive activity of the sera using an indirect basophil activation test (iBAT). IgE-containing serum is added to non-allergic donor blood, and then whole blood is stimulated with peanut extract. Granule extrusion by the measurement of CD63 on basophils is analyzed by flow cytometry. Adding pre-OIT sera alone lead to increased basophil activation after peanut stimulation. The addition of post-OIT serum resulted in lower

degranulation than that induced by pre-OIT sera alone. Suppression by IgG was also determined to be FcγRIIb-mediated (Burton et al., 2018b).

IgG can also inhibit the production of Th2 cytokines (IL-4, IL-13) in BMMC cultures. This is also true *in vivo*, where IgG administered prophylactically before allergen ingestion prevents the development of IgE and Th2 responses. Repeated administration of OVA in inherently atopic mice Il4raF709 leads to the production of OVA-specific Th2 cells and expansion of mast cells. The administration of IgG suppresses these Th2 and mast cells and permits the expansion of Tregs. OVA-IgG treated animals also have lower IgG and are resistant to anaphylaxis in comparison to control treated mice. These findings establish that IgG not only blocks IgE-mediated hypersensitivity reactions, but also blocks the development of food allergies by suppressing mast cell Th2 adjuvant function (Burton et al., 2018b). Together, these findings reveal an underappreciated role for IgG in protecting against food allergy.

1.9 IgA

As IgG regulates both immediate hypersensitivity and emerging Th2/Treg responses IgA, as the major gut immunoglobulin has garnered significant interest in recent years as also possibly being involved in oral tolerance. IgA shares the basic structure of all other antibodies but can exist as a monomer and a dimer joined by the joining chain (J-chain) (Figure 3). In humans, dimeric IgA is more prevalent in the gut, whereas monomeric IgA is predominantly found in serum. IgA in mice is mainly polymeric in both the serum and mucosal secretions. Human IgA is divided into two subclasses, IgA1 and IgA2, whereas mouse IgA has only one. IgA1 has a long hinge region, which is advantageous for antigen recognition but is susceptible to bacterial proteases (Figure 3) (de Sousa-Pereira and Woof, 2019; Mkaddem et al., 2014).

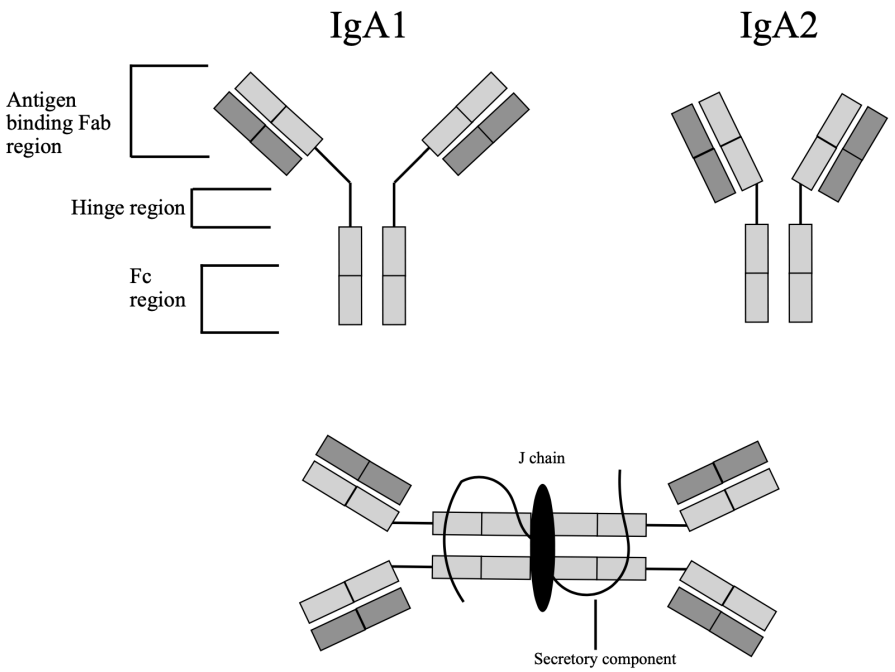


Figure 3. Schematic diagram of the structure of IgA1, IgA2, and secretory IgA (joined by a J-chain).

1.9.1 Induction and regulation of IgA

IgA is produced mainly in secondary lymphoid structures such as Peyer's patches (PP), mesenteric lymph nodes, and isolated lymphoid follicles (ILF), but can also be made in extrafollicular sites such as the lamina propria. Peyer's patches are thought to be the main inductive sites for IgA. Craig and Cebra were the first to show that these specialized structures contain IgA^+ plasma cell precursors. They demonstrated that Peyer's patch cells could reconstitute IgA^+ cells in lethally irradiated rabbits (Craig and Cebra, 1971). Peyer's patches are characterized by germinal centers that promote T-B cell interactions, class switch recombination, and somatic hypermutation. They contain a high number of B cells, four to six times more than T cells, and are rich in IgA-inducing cytokines, such as transforming growth factor beta (TGF-β), IL-4, IL-6, and IL-10. The subepithelial dome of the Peyer's patches is where IgA class switching is initiated, and contains mesenchymal cells that are important for IgA induction (Nagashima et al., 2017).

TGF-β, along with engagement of CD40 ligand (CD40L) on T cells with CD40 on B cells, induces IgA class switching and the generation of antigen-specific IgA B cells. Mice lacking the TGF-β receptor have impaired systemic and intestinal IgA responses (Cazac and Roes, 2000). Although Peyer's patches are important, they are not essential for IgA induction. This was shown in mice that did not develop Peyer's patches after injection of the lymphotoxin-B receptor fused with IgG Fc (LTβR-Ig). Despite the lack of Peyer's patches, these mice have a large number of IgA^+ plasma cells in their lamina propria after oral immunization (Yamamoto et al., 2004).

IgA^+ B cells can also migrate to mesenteric lymph nodes, where they proliferate, and differentiate into plasmablasts. IgA plasmablasts upregulate gut-homing receptors α4β7, and CCR9 and then return to the gut in response to CCL25 production by the intestinal epithelial cells. Mice lacking mesenteric lymph nodes, Peyer's patches, and isolated lymphoid follicles, (LT-a-deficient mice and double LT-a-TNF-deficient mice) have reduced IgA secreting plasma cells in their intestinal lamina propria, making mLNs and ILFs major induction sites for IgA (Kang et al., 2002; Ryffel et al., 1998). They are however, non-essential, as Id2-deficient mice, and RORγT-deficient mice, containing no organized lymphoid follicles, still retain the ability to produce antigen specific IgA B cells in the lamina propria (Eberl and Littman, 2004).

While the lamina propria contains reduced IgA producing B cells in mice lacking Peyer's patches, mLNs, and ILF, their presence points to the lamina propria as an inductive site for IgA. The intestinal lamina propria contains precursor IgM^+ B cells of IgA plasma cells (Fagarasan et al., 2001). It has been observed that these can migrate from either Peyer's patches or bone marrow to the lamina propria, where DC's encounter antigen to stimulate $CD4^+$ T cells to initiate IgA responses with the help of CD40L, IL-10 and TGF-β (Cerutti et al., 1998).

T-independent IgA responses can also occur in the lamina propria. BAFF and APRIL produced by lamina propria pDCs act on B cells via TACI to induce IgA class switching without T cell help (Tezuka et al., 2011). Even though decreased, CD40 knockout mice and

T cell knockout mice still retain the ability to mount intestinal IgA responses to microbes and commensals (Macpherson and Uhr, 2004).

1.9.2 Function

IgA, which is secreted into the lumen of the gut and respiratory tract, plays a crucial role at mucosal surfaces by serving as the first line of humoral defense. It provides protection by immune exclusion mechanisms, binding and entrapping dietary components and microorganisms. It reduces the motility of bacteria by downmodulating the expression of bacterial adhesion factors, blocking their attachment to the epithelium. It also neutralizes bacterial toxins and enzymes and can facilitate antigen sampling by interacting with microfold (M) cells, an epithelial cell specialized in antigen capture. Furthermore, IgA can retrograde transport microorganisms that have breached the epithelial barrier and entered the mucosa back into the lumen in a process facilitated by the polymeric Ig receptor (pIgR) (Cerutti and Rescigno, 2008).

IgA also plays an important role in maintaining intestinal homeostasis by interacting with the local microbiota. The best evidence for this comes from studies with germ-free mice that completely lack intestinal microbiota. These animals have reduced intestinal IgA. Specific pathogen free (SPF) mice that have limited exposure to pathogenic microbes but retain a diverse commensal microbiome have abundant IgA in their serum and stool, whereas germ free and axenic mice have reduced IgA levels (Benveniste et al., 1971). More evidence for the role of IgA in controlling the composition of the microbiota comes from AID-deficient mice have B cells that cannot class-switch to IgA and have an increase in anaerobic bacteria in the small intestine. $pIgR^{-/-}$ mice, which have no secretory IgA and hence no IgA in the gut lumen, also exhibit altered microbiomes, with a decrease abundance of *Bifidobacterium spp*., and increased abundance of *Helicobacter spp* in their cecum (Reikvam et al., 2012). $IgA^{-/-}$ mice also show altered microbiota shift from newborn to adult, with increased colonization of *Enterobacteriaceae* family (Mirpuri et al., 2014). Furthermore, IgA deficient patients exhibit an altered gut microbiota composition in comparison to healthy controls. While the lack of IgA is compensated by IgM, IgM displayed less binding specificity than IgA, also highlighting a non-redundant role for IgA

in controlling the microbiota (Catanzaro et al., 2019). Both $IgA^{-/-}$ mice and strains with low IgA levels, can still develop IgG responses against commensals. This only occurs in wild-type mice when bacteria or antigen is injected systemically, indicating an integral role for IgA in limiting the penetration of commensal bacteria (Cong et al., 2009; Macpherson and Uhr, 2004).

To date, efforts to identify the specific bacteria that enhance IgA production have not been fruitful. One group however found that the genus *Anaeroplasma* in the microbiota is associated with increased TGF-B expression, and class switch recombination to IgA in the PP (Beller et al., 2020).

1.9.3 IgA receptors

IgA can interact with a variety of receptors on many different cell types to induce protective and inflammatory reactions. It can provide mucosal immune protection by binding to the polymeric Ig receptor (pIgR) through the J-chain on the basolateral side of epithelial cells and is transported to the luminal side of the gut. Bound IgA is proteolytically cleaved from pIgR, generating a secretory component known as secretory IgA (SIgA). SIgA complexes then entrap dietary antigens and microbes in the mucus layer (Cerutti and Rescigno, 2008). pIgR KO mice are more vulnerable to DSS-induced colitis and more susceptible to *Salmonella typhimurium* infection (Murthy et al., 2006; Wijburg et al., 2006).

The principal myeloid IgA receptor in humans is FcαRI (CD89), which plays an important antimicrobial role when bound to IgA. It facilitates pathogen clearance via DCs, neutrophils, and phagocytes. This receptor can induce responses including phagocytosis, respiratory burst, degranulation, and cytokine production. The interaction of monomeric IgA with FcαRI results in the recruitment of SHP-1, leading to an inhibitory signal, whereas interaction with multimeric ligands leads to Syk recruitment and cell activation (Mkaddem et al., 2014).

Mice lack the FcαR receptor gene but express a receptor, Fcα/μR, similar to pIgR, which binds to both IgM and IgA. It is expressed in marginal zone B-cells and follicular dendritic cells. Studies using Fcα/μR knockout mice have indicated a regulatory function for this receptor in humoral immune responses against T-independent antigens. Fcα/μR KO mice immunized with T-independent antigens showed increased induction of $IgG3^+$ B cells and enhanced germinal center formation. Both FcαR and Fcα/μR are absent in mast cells and basophils (Wines and Hogarth, 2006).

1.9.4 IgA deficiency and allergy

Research exploring the impact of IgA on the development of atopic disorders has centered on the study of patients with selective IgA deficiency, the most common form of primary immunodeficiency. Affected patients experience an array of infectious, autoimmune, and allergic symptoms (Morawska et al., 2021). More than 50% of patients with IgA deficiency are asymptomatic. Those affected by the deficiency experience increased incidence of sinopulmonary and gastrointestinal infections, autoimmune diseases, and increased susceptibility to allergies. While evidence supporting the role of IgA in infection and autoimmunity is clear, the mechanisms whereby IgA might affect allergic responses requires further investigation (Cinicola et al., 2022).

The prevalence of atopy among patients with IgA deficiency varies among studies, perhaps due to the populations surveyed. A study conducted in Turkey showed that 45.7% of IgA-deficient patients have eczema, asthma, rhinitis, or atopic dermatitis (Erkocoglu et al., 2017). In Iran, reports have described 84% of patients as having allergies (Aghamohammadi et al., 2009). The IgA-allergy correlation appears to be weaker in subjects in China, with only 17% having allergic symptoms, mostly due to drugs (Wang et al., 2020). These findings indicate heterogeneity among study populations, which likely have both unique genetic backgrounds and environmental exposure histories.

Gualdi et al's study involving 102 patients with selective IgA deficiency (SIgAD), revealed that 57.84% of these cases had atopic dermatitis, although only 10.17% of these patients

had high IgE (Gualdi et al., 2015). Another study by Magen et al. indicated a slightly lower occurrence of AD in SIgAD, standing at 4.6%. Nevertheless, this prevalence remained significantly higher than what was observed in the control group (Magen et al., 2017).

Conflicting outcomes have emerged from studies examining the correlation between asthma and selective IgA Deficiency (SIgAD). Most analyses have indicated an increased prevalence of asthma among individuals with SIgAD (Aghamohammadi et al., 2009; Erkocoglu et al., 2017; Zivkovic et al., 2019). However, a case-control study conducted by Jorgensen et al. did not reveal any differences in prevalence when comparing SIgAD patients with the control group (Jorgensen et al. 2013). Asthmatic patients also exhibit a higher likelihood of being diagnosed with SIgAD/CVID than those without asthma (Urm et al., 2013). Elevated levels of salivary IgA have also been associated with a reduced likelihood of wheezing in sensitized infants (Sandin et al., 2011). As for food allergy, the available SIgAD data remain limited, with some studies reporting a low or normal range for IgA in food allergic patients (Latcham et al., 2003; Shahin et al., 2020)

Several investigations have shown a relationship between low IgA levels and the development of allergic diseases. Reports by Ludviksson et al, found an increased prevalence of allergic [i]symptoms in patients with low IgA levels (Ludviksson et al., 1992), and Asperen et al., demonstrated that children born to atopic parents have a higher incidence of salivary IgA deficiency (van Asperen et al., 1985). Low serum or salivary IgA levels have also been correlated with positive skin prick tests and development of allergic rhinitis or atopic eczema in children (Ludviksson et al., 2005). Inversely, Kukkonen et al. found that infants with high fecal IgA levels had a reduced risk of IgE-associated diseases (Kukkonen et al., 2010). Secretory IgA levels have also been associated with a decreased risk of allergy (Fageras et al., 2011; Sandin et al., 2011). Collectively these findings suggest that IgA deficient patients, or patients with impaired IgA production face a higher susceptibility to atopic disorders.

1.10 IgA, Microbiome, Allergy, and Tolerance

The abundance of IgA at mucosal surfaces bestows it with a key role in defense and immune tolerance. Exposure to inhaled or ingested antigens, and the colonization of these surfaces with large numbers of commensal microbes, creates a complex environment for IgA to function. IgA is believed to contribute to oral tolerance and immune non responsiveness through methods of immune exclusion to both dietary antigens and microbes. In allergic mouse models induction of food-specific IgA in the gut appears to require the presence of an adjuvant. Zhang et al observed minimal peanut-specific IgA in the stool of peanut fed mice compared to mice sensitized with peanut alongside cholera toxin (Zhang et al., 2020).

Some studies have examined relationships between serum or stool IgA levels and the induction of oral tolerance or allergy. For instance, Frossard and colleagues showed that serum IgA goes up in mice enterally sensitized with β-lactoglobulin (BLG) and cholera toxin with low levels of fecal IgA, whereas tolerant mice previously fed BLG in drinking water, had low serum BLG-specific IgA and high fecal IgA (Frossard et al., 2004). This result points to important functional differences between serum and fecal IgA in tolerance and additional studies are needed to define these distinctions.

The best evidence for the role of IgA in oral tolerance comes from work done by Elesela and colleagues. They demonstrated that IgA immune complexed with TNP/OVA can protect against Th2 responses in both the gut and lung. Administration of IgA TNP/OVA immune complexes intratracheally, reduced Th2 cytokines, diarrhea, and temperature loss in models of allergic airway disease, and food allergy. *In vitro* data demonstrated that IgA induces tolerance by driving TGFβ and IL-10 production by DC's (Elesela et al., 2023).

There is growing evidence that certain gut bacteria have the ability to promote or suppress the development of immune tolerance to foods. The microbiome is thought to regulate macrophages, innate lymphoid cells, and T cells which suppress or promote allergic responses. Several studies have shown correlations between the composition of the skin and

gut microbiota and an increased risk of allergies. One study demonstrated low phylogenetic diversity of the gut microbiota in adults with self-reported nut and seasonal allergies. Patients presented with an increased relative abundance of *Bacteroidales* and a reduced relative abundance of *Clostridiales* in their gut microbiota (Hua et al., 2016). Specifically, *Clostridia* species have repeatedly been associated with the ability to suppress food allergies in mouse studies (Atarashi et al., 2013; Atarashi et al., 2011; Stefka et al., 2014). Selective colonization of gnotobiotic mice with Clostridia-containing microbiota has been shown to protect against food allergies. They promote the production of IL-22 by RORγt^{+} innate lymphoid cells and T cells, leading to enhanced intestinal permeability through the production of mucus and antimicrobial peptides. *Clostridia* colonization also induces isotype class-switching to IgA, with colonized mice having elevated fecal IgA levels compared to uncolonized gnotobiotic mice (Stefka et al., 2014). The link between IgA, the microbiota, and tolerance to food antigens is still not well understood.

2. Hypothesis and Aims

The presence of food specific IgE antibodies is required for the elicitation of mast cell-driven hypersensitivity reactions. However, there are significant gaps in our understanding of the regulation and function of IgE. The mechanisms that both drive and restrain allergic sensitization (i.e., the induction of food antigen-specific IgE production in some individuals) are not fully understood. Furthermore, the critical cofactors, acting in concert with IgE to amplify or attenuate effector cell activation and immediate hypersensitivity responses, are not known. A major conundrum in the food allergy field has been that not all subjects with IgE responses exhibit symptoms upon ingesting allergenic foods (Nowak-Wegrzyn et al., 2017). We hypothesize that the inconsistent relationship between IgE responses and clinical symptoms is due to the presence of factors that inhibit IgE-mediated effector cell activation in some subjects. We have shown that one such inhibitor is food-specific IgG. IgG antibodies signal via the inhibitory IgG receptor, FcgR2b, to block IgE-induced effector cells activation.

Given the known protective roles of immunoglobulin A (IgA) at mucosal surfaces, we hypothesize that IgA might exert a similar suppressive role in the allergic disease. Such a function is suggested by several observations. Among primary immunodeficiencies, the lack of IgA is most common (Morawska et al., 2021). While the incidence of reoccurring sinopulmonary infections is high in IgA-deficient individuals as one might predict, there are multiple reports showing that the probability of atopy is also increased (Aghamohammadi et al., 2009; Erkocoglu et al., 2017; Gualdi et al., 2015; Shahin et al., 2020). IgA has also been shown to provide protective effects in mouse models of asthma and food allergy (Elesela et al., 2023). Furthermore, our group and many others have shown that antigen-specific IgA plasma levels increase after allergen immunotherapy in humans (Burton et al., 2014a; Francis et al., 2008; Shamji et al., 2021). Collectively these observations suggest that impaired IgA responses are correlated with the development of allergic diseases suggesting a protective role for this antibody isotype.

We hypothesize that IgA plays a central protective role both in the prevention of allergic immunological sensitization and in the elicitation of effector responses. This project will test that hypothesis in 2 specific aims:

Aim 1. *Evaluate the interaction of IgA with mast cells and basophils and its effects on their function.* Our preliminary findings suggest that IgA antibodies suppress IgE-induced degranulation via a receptor mediated process. We aim to characterize the physical process by which IgA binds to and inhibits mast cell degranulation and how glycosylation patterns on IgA effects these mechanisms. We hypothesize that the suppressive effects of IgA will also affect mast cell signaling and cytokine production. This hypothesis will be tested using cultured bone-marrow derived mast cells (BMMC) activated with IgE: antigen in the presence of antigen-specific IgA. Furthermore, we plan to assess the effects of human IgA enriched from post OIT sera, on basophil degranulation, in a basophil activation test.

Aim 2. *Evaluate the effects of IgA on emerging immune allergic responses*. We generated a new line of IgA-deficient mice ($IgA^{-/-}$), on the BALB/c background (using CRISPR/CAS9). These new mice have an intact IgH locus and overcome an artifactual limitation of IgE responses in $IgA^{-/-}$ previously generated by others rendering them suitable for studies of allergy pathogenesis. We hypothesize that IgA is an important attenuator of Th2 responses and that IgA deficient mice will present with a Th2-dominant phenotype at baseline, characterized by elevated IgE levels, increased mast cells, and a decrease in T regulatory cells in comparison to littermate IgA sufficient controls. Furthermore, we anticipate an altered gut microbiome in $IgA^{-/-}$ mice and predict that this dysbiosis contributes to an increased susceptibility to the observed allergic phenotype.

3.Materials and Methods

3.1 Materials

Table 1. Animals

BALB/c WT mice	Jackson Laboratory, USA
CRISPR $IgA^{-/-}$ mice	Made by the Genome Manipulation Core at Boston Children's Hospital

Table 2. Chemicals

Bovine Serum Albumin (BSA)	Gemini, USA
Fetal Bovine Serum (FBS)	Gemini, USA
Non essential amino acids	Thermofisher Scientific, USA
Recombinant mouse IL-3	Peprotech, USA
Recombinant mouse SCF	Peprotech, USA
Sodium Pyruvate	Thermofisher Scientific, USA
2-mercaptoethanol (Bme)	Sigma, USA
Streptomycin	Thermofisher Scientific, USA
HEPES	Thermofisher Scientific, USA
Gentamicin	Thermofisher Scientific, USA
Penicillin/Streptomycin	Thermofisher Scientific, USA
RPMI 1640 + L-glutamine	Thermofisher Scientific, USA
Anti-TNP IgA	ATCC, USA
Anti-TNP IgE	ATCC, USA
LS columns	Miltenyi, USA
Sulphuric Acid 2N	Sigma, USA
Super Signal TMB Substrate	Thermofisher Scientific, USA
PNPP	Thermofisher Scientific, USA
Streptavidin	Sigma, USA
10 x PBS	Corning, USA
10X HBSS	Thermofisher Scientific, USA
NucBlue Stain	Thermofisher Scientific, USA
10X RBC Lysis	Biolegend
TNP-BSA	LGC Biosearch Technologies, Germany
EGTA	Sigma, USA
Sialic Acid (NANA)	Vector Labs, USA

Table 3. Kits

CD3 T cell Isolation Kit, mouse	Miltenyi, USA
CBA and cytokine flex sets	BD Biosciences, USA
Anti-human IgA CaptureSelect affinity column	Thermofisher Scientific, USA

Flow CAST Basophil Activation Test	Bühlmann Laboratories, Switzerland
Pierce FITC antibody labeling kit	Thermofisher Scientific, USA
Protein L column	Thermofisher Scientific, USA
RNeasy Kit	Qiagen, USA
MCPT1 ELISA Kit	Invitrogen, USA
Foxp3 Staining Bufffer Kit	Thermofisher Scientific, USA

Table 4. Instruments

LSR Fortessa	BD Biosciences, USA
EVOS M7000	Thermofisher Scientific, USA
Cytospin3	Shandon, USA
QuantStudio	

Table 7. Software

BD FACS Diva	BD Biosciences, USA
GraphPad Prism	GraphPad Software, La Jolla (CA),USA

3.2 Buffers

FACS buffer

25% FCS, 1x PBS

MACS buffer

Cell culture medium

RPMI-1640: 10% fetal bovine serum, 10mM HEPES buffer, 100 mg/ml streptomycin, 100U/ml penicillin 10mg/ml gentamicin, 1% Minimum Essential Medium non-essential amino acids, 1 mM sodium pyruvate, and 55mm 2-mercaptoethanol,

ELISA coating buffer

1x PBS

ELISA blocking buffer

2% BSA in 1x PBS

ELISA wash buffer

0.1% Tween in 1x PBS

3.3 Materials and Methods

3.3.1 Mouse Anti-TNP IgA Production and Purification

Frozen TIB-194 hybridoma cells (ATCC, Manassas, VA) were thawed according to manufacturer's instructions, transferred, and diluted with warmed RPMI-1640 (10% FBS, 100U/ml penicillin). Cells were centrifuged at 300 x g for 5 minutes, and the diluted freezing additive was removed and replaced with new media. Cells were propagated by the addition of fresh media approximately every 2-3 days. As cells multiplied, they were transferred into bigger flasks. Culture supernatants were tested by a sandwich ELISA for presence of anti-TNP IgA. Supernatants were collected and run over a protein L column (ThermoFisher Scientific, Waltham, MA). IgA was then concentrated and dialyzed with Amicon Ultra Centrifugal Filter Devices carrying a 100 kDa cut-off (Millipore Sigma, Darmstadt, Germany) and filter-sterilized with 0.2mM syringe filters (Millex, EMD Millipore, Billerica, MA)

3.3.2 Mast Cell Culture

BALB/cJ mouse bone marrow was cultured in RPMI-1640 media supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 10mM HEPES buffer, 100 mg/ml streptomycin, 100U/ml penicillin 10mg/ml gentamicin, 1% Minimum Essential Medium non-essential amino acids, 1 mM sodium pyruvate, and 55mm 2-mercaptoethanol, (all from ThermoFisher Scientific, Waltham, MA) in the presence of both 10-20 ng/ml of IL-3 and SCF (Shenandoah Biotechnology, Warwick, PA) for the differentiation into BMMCs. BMMCs were cultured for 4-6 weeks until cultures reached 90% purity. The purity of BMMCs were assessed by flow cytometry (c-Kit$^+$ FcεRIa$^+$).

3.3.3 LAMP-1

For the lysosomal-associated membrane protein 1 (LAMP-1) assay, mouse BMMC's (0.5 x 10^6/ml) were incubated overnight with anti-TNP (50 ng/ml, BD Biosciences, Franklin Lakes, NJ), and anti-TNP IgA (100 μg/ml, TIB-194 hybridoma, ATCC, Manassas, VA) in

100 µl. Cells are then washed with RPMI to remove unbound antibody and centrifuged at 1400 RPM for 5 minutes, and then stimulated for 10 mins at 37°C with TNP BSA (50 ng/ml, Biosearch Technologies, Teddington, UK) while simultaneously being stained with, BV605 anti-c-Kit (Biolegend, San Diego, CA), fixable viability dye eFluor 780 (eBioscience, San Diego, CA), and PE anti-LAMP-1. For antigen specificity experiments, anti-OVA IgA (gifted by Dr. Duane Wesemann), was used alongside anti-TNP IgA.

3.3.4 Measuring phospho-Syk

For measurements of phospho-Syk by flow cytometry, mouse BMMCs were incubated with anti-TNP IgE, and anti-TNP IgA as above, and stimulated at 37°C for zero, one and two minutes. Cells were fixed and stained using Thermofisher Scientific Protocol C: Two-step Protocol for Fixation/Methanol. Briefly, cells were resuspended at a concentration of 1 x 10^6/ml and added to FACS tubes and stimulated as conditions above at 37C. At the end of stimulation cells were fixed by adding 100 µl of IC Fixation buffer, and vortexed to mix. Samples are then incubated for 10 minutes at room temperature in the dark. Samples are then centrifuged at 600 x g for 5 minutes at room temperature, and supernatant is discarded. Cell pellet is resuspended by adding 1ml of 100% methanol, and vortexed, and incubated for 30 minutes in the fridge. Cells are washed with FACS buffer and centrifuged at 600 x g for 5 minutes. Supernatant is discarded and cells are resuspended at 1x107 cells/ml in FACS buffer. Cells were stained with mouse PE anti-phospho-Syk (Cell Signaling Technologies, Danvers, MA), in the presence of mouse Fc block and assessed on an LSR Fortessa.

3.3.5 Cytometric Bead Array (CBA)

For the cytokine release assay, BMMCs were stimulated for 6 hours per conditions above. Splenocytes were stimulated with PMA (500 ng/ml) and ionomycin (500 ng/ml) for 3 days. Supernatants were collected and cytokines were measured using a bead-based immunoassay called Cytometric Bead Array (CBA). This allows for the simultaneous detection of multiple cytokines in one sample. Capture beads are initially mixed with samples/standards and then incubated with PE-conjugated detection antibodies to form

complexes. After incubation and washing steps, the samples are analyzed by flow cytometry. The Mouse/Rat Soluble Protein Master Buffer kit, along with the cytokine flex sets for IL-13, IL-6, TNF-α, IL-4, and IL-10 were used according to manufacturer's instructions (BD Biosciences, Franklin Lakes, NJ).

3.3.6 IgA Binding Experiments (Calcium chelation, Deglycosylation and Desialylation, and imaging)

For IgA binding experiments, mouse BMMC's (0.5 x 10^6/ml) were incubated overnight with anti-TNP IgA (100 µg/ml, TIB-194 hybridoma, ATCC, VA, USA) in 100 µl of 1X HBSS in a 96 well v-bottom plate. Cells are then washed with HBSS to remove unbound antibody and centrifuged at 1400 RPM for 5 minutes. Cells were stained and run on a flow cytometer (refer to section 3.3.)

For imaging experiments, BMMCs were IgE/IgA sensitized as per the conditions above and stained with NucBlue Live Cell Stain (Thermofisher Scientific, Carlsbad, CA), and anti-mouse Fc block and FITC anti-IgA (BD Biosciences, Franklin Lakes, NJ), or isotype control. Cells were resuspended in 200 µl of FACS buffer and then cytospun onto a slide at a speed of 200 RPM for five minutes. Slides were visualized and imaged using an EVOS M7000 microscope (Thermofisher Scientific, WA, USA).

For IgA binding experiments with EGTA. 80 mM of EGTA was added to 1X HBSS media for 30 minutes to chelate calcium prior to resuspending BMMC's in that same media. IgA surface expression was measured as detailed above in section. For sialic acid blocking experiments, BMMC's were preincubated with 100 µg/ml of sialic acid (Vector Labs, CA, USA) for 30 minutes before continuation with the IgA binding protocol as described above.

For removal of N-linked glycans, anti-TNP IgA was incubated with PNGase F (New England Bioscience, Ipswich, MA) at 37°C for 72 hours according to manufacturer's instructions. For desialylation (removal of terminal sialic acid residues), anti-TNP IgA was digested with neuraminidase from Vibrio cholerae (Sigma, MO, USA) at 37°C for 24 hours

according to manufacturer's instructions. The reactions (control IgA in glycobuffer, and digested IgA in glycobuffer and neuraminidase) were then incubated at 65°C for 10 min. to deactivate the enzymes. Digestion efficacy was assessed by lectin blot analysis probing with biotinylated SNA (Vector Labs, CA, USA).

For sialic acid blocking experiments, BMMC's were preincubated with 100 μg/ml of sialic acid (N-acetylneuraminic Acid NANA) from (Vector Labs, CA, USA) before IgA binding experiments were performed as above.

3.3.7 Flow Cytometry

In vitro

IgA Binding Experiments

T cells were purified from a BALB/cJ spleen by MACS sorting using the CD3ε Microbead kit according to manufacturers instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). BMMCs were incubated with 100 μg/ml anti-TNP IgA or anti-TNP OVA in 1X HBSS for 30 minutes at 4°C. Purified T cells were also incubated with 100 μg/ml anti-TNP IgA in 1X HBSS for 30 minutes at 4°C. Mast cells were stained for BV605 anti-c-Kit (2B8) , PE anti-FcεRI (MAR-1), fixable viability dye eFluor 780 (eBioscience, San Diego, CA), and FITC anti-IgA (mA-6E1) (BD Biosciences, Franklin Lakes, NJ) in the presence of anti-mouse Fc block (Biolegend, San Diego, CA), in FACS buffer (1X PBS, 10% FBS) at 4°C for 30 minutes. T cells were stained for PE anti-CD3 (17A2) (eBioscience, San Diego, CA), BV605 anti-CD4 (Biolegend, San Diego, CA), FITC anti-IgA (BD Biosciences, Franklin Lakes, NJ), and fixable viability dye eFluor 780 (eBioscience, San Diego, CA), in FACS buffer.

In vivo

$IgA^{-/-}$ Experiments

Flow cytometric analysis of T effector cells in IgA deficient mice and controls was done by staining with the following antibodies : AF700 anti-CD45 (30-F11), BV605 anti-CD4 (RM4-5), BV510 anti-CD3 (17A2), eFluor 450 anti-Foxp3 (FJK-16s), ef660 anti-GATA-3 (TWAJ), PE anti-ROR-γt (B2D), PerCP/Cy5.5 anti-T-bet (4B10), ef780 anti-Helios (22F6). For Tfh's and Tfr's: AF700 anti-CD45 (30-F11), FITC anti-CD4 (RM4-5), BV605 anti-

PD-1 (J43), APC anti-CXCR5, PE-Cy7 anti-BCL6 (K112.91), PE anti-B220 (RA3-6B2) eFluor 450 anti-Foxp3 (FJK-16s). Mast cells were identified by staining with : AF700 anti-CD45 (30-F11), BV605 anti-c-Kit (104D2), PE-Cy7 anti Fcer1a (MAR-1), BV421 anti-Siglec F (E50-2440), PE anti-IgE (R35-72), and FITC lineage negative (CD3 (145-2C11), CD4 (RM4-5), CD8 (53–6.7), B220 (RA3-6B2), Gr-1(RB6-8C5), CD11b (M1/70), CD11c (N418), TCRβ (H57-597), NKp46 (29A1.4) and TCRγδ (GL3). B cells were stained with: AF700 anti-CD45 (30-F11), BV711 anti-CD19 (, BV421 anti-B220 (RA3-6B2), PE anti-FAS (SA367H8), PerCP/Cy5.5 anti-GL7 (GL7), FITC anti-IgA (C10-3), APC anti-IgG1 (RMG1-1), BV605 anti-IgM (RMM-1). Viability dyes (eFluor506 and e780) were used from (eBioscience, San Diego, CA). The Foxp3 Transcription Factor buffer set from (eBioscience, San Diego, CA) was used for intracellular staining of transcription factors. Single cells were gated on by FSC-H and FSC-W signals, and dead cells were excluded based on the staining of eFluor 780 and 506 fixable viability dye (eBioscience, San Diego, CA), Analysis was done by LSR Fortessa (BD Biosciences, Franklin Lakes, NJ) and data was processed using Flowjo (Tree Star Inc, Ashland, OR).

3.3.8 Isolation of Peritoneal Lavage, Peyer's patches, mLN, and spleen

Peritoneal lavage was isolated from six-week-old BALB/cJ females by injection of 5 ml of 1X HBSS (using a 5ml syringe (23-G) into the peritoneal cavity to dislodge attached cells. Mice abdomens were lightly massaged to help dislodge peritoneal cells. Cells were pelleted and resuspended in 1X HBSS containing 100 µg/ml of anti-TNP IgA – incubated for 30 minutes at 4°C. Cells were then washed in FACS buffer and stained with mouse AF700 anti-CD45 (Biolegend, San Diego, CA), APC anti-CD11b (eBioscience, San Diego, CA), BV605 anti-c-Kit (Biolegend, San Diego, CA), PE anti-FcεRIα (Biolegend, San Diego, CA), and FITC anti-IgA (BD Biosciences, Franklin Lakes, NJ.

At 6-8 months of age, IgA deficient mice and their controls were sacrificed with CO_2 asphyxiation, and then bled via cardiac puncture. Peyer's patches, mLN and spleen were harvested from mice, and homogenized in 1X PBS containing 2% FBS. Cells were then washed once in PBS containing 2% FBS and used for experiments. Peritoneal lavage was

obtained by injecting 1X PBS into the peritoneal cavity using a 27g needle. Peritoneum is gently massaged to dislodge any cells, and fluid is collected back in to the needle.

3.3.9 Passive Systemic Anaphylaxis

8 week old wildtype mice were passively sensitized to TNP-OVA by intraperitoneal injection of αTNP IgE (2 μg/mouse (BD, Franklin Lakes, NJ, USA). Some mice were treated with 80 μg αTNP IgA (TIB-194 hybridoma, ATCC, Manassas, VA). Anaphylaxis was induced by *i.p* injection of 2 μg TNP OVA the next day. Anaphylaxis was measured by monitoring core body temperature using microchip IPTT-300 transponders (Bio Medic Data Systems, Waterford, WI, USA) implanted subcutaneously under the nape while mice are under anesthesia (isofluorane). Transponders are typically injected 12 hrs prior to challenging the mice. Readings were taken using the DAS-6001 console (Bio Medic Data Systems, Waterford, WI, USA) every 5 minutes for one hour.

3.3.10 Human IgA Purification and Anti-Peanut IgA Quantification from Post OIT sera

As OIT induces a strong food allergen-specific IgA response, we used sera obtained from patients who had undergone peanut OIT as a source of peanut-specific IgA (25). Post OIT sera were pooled from several patients and IgA was purified using an anti-human IgA CaptureSelect affinity column as per manufacturer's instructions (ThermoFisher Scientific, Carlsbad, CA). The IgA-enriched eluate from this column was then run through a Protein G column as per manufacturer's instructions to further deplete it of IgG (ThermoFisher Scientific, Carlsbad, CA). The IgA was concentrated to a volume equivalent to that of the original input serum and dialyzed with Amicon Ultra Centrifugal Filter Devices carrying a 100 kDa cut-off (Millipore Sigma, Darmstadt, Germany) and filter-sterilized with 0.2 μm syringe filters (Millex, EMD Millipore, Billerica, MA). Total IgA and IgG was quantified using the Human IgA and IgG ELISA kits (ThermoFisher Scientific, Carlsbad, CA).

3.3.11 Human Basophil Activation Test

Basophil activation tests (BAT) were performed using the Flow CAST Basophil Activation Test kit (Bühlmann Laboratories, Schönenbuch, Switzerland). 50 μl aliquots of whole

blood from peanut allergic patients were pre incubated with 125-1000 µg/ml of purified IgA for 2-4 hours at 37°C in 100 µl of basophil stimulation buffer. Samples were incubated for l5 min at 37°C with 3.6x10-4 µg/ml of crude peanut extract (CPE) and staining antibodies for human PE anti-CCR3 (Biolegend, San Diego, CA) and FITC anti-CD63 (BD Biosciences, Franklin Lakes, NJ). Anti-FcεRI stimulation was used as a positive control for basophil activation. After red blood cell lysis, cells were washed and assessed for activation by flow cytometry. Basophils were identified as SSClowCCR3^{+} and activated cells identified based on CD63 expression. Around 200 basophils were evaluated for each sample. Peripheral blood from peanut allergic donors was obtained with informed consent under a protocol approved by the Institutional Review Board of Boston Children's Hospital.

3.3.12 Fluorescent Labeling of Human IgA and IgA Binding Studies on Human Cell Types

Human IgA purified from post OIT sera was fluorescently labeled using the Pierce FITC antibody labeling kit (Thermofisher Scientific, Rockford, IL) according to the manufacturer's instructions. For IgA binding experiments, donor whole blood was stained with labeled IgA, along with antibodies to human leukocyte markers PE anti-CCR3 (5E8), APC anti-FcεR1(AER-37 (CRA-1)), AF700 anti-CD3 (HIT3a), PE-Cy7 anti-CD16 (B73.1), BV510 anti-CD14 (63D3) all from (Biolegend, San Diego, CA) for 30 minutes at 37°C.

3.3.13 IgE Class Switching B cell cultures

To induce class switching to IgE, splenocytes were cultured in 96 U-bottom 96-well plates at (10^6/ml) in complete RPMI 1640. B cells were stimulated with IL-4 (50 ng/ml) R&D (Minneapolis, MN), and anti-CD40 Millipore Sigma (1 µg/ml) (Burlington, MA). Some cultures lasted for 4 days, for analysis of IgE+ B cells by flow cytometry. Other cultures lasted for 7 days to assess splenocyte supernatant for IgE by ELISA.

3.3.14 Generation of IgA Knockout Mice (IgA$^{-/-}$) by CRISPR/Cas9

CRISPR/CAS9 (Clustered Regularly Interspaced Short Palindromic Repeats) has revolutionized the field of genetics/immunology and is widely used to generate gene knockouts in mice, allowing researchers to study the function of specific genes and their role in various biological processes. The guide RNA is specific to the target DNA sequence and the Cas9 nuclease induces a double-strand break (DSB) at the target site. Within cells, these DSBs are mended through two distinct repair pathways: 1) non-homologous end joining (NHEJ), and 2) homology-directed repair (HDR). NHEJ is a highly efficient but error-prone mechanism that actively mends DSBs. During this repair process, it often introduces insertions or deletions (InDels) of nucleotides, frequently resulting in the creation of a premature stop codon. This, in turn, leads to the loss of gene function, effectively generating a gene knockout (KO). Homology-directed repair (HDR) is a repair mechanism that, while less efficient than NHEJ, offers a higher level of precision. It is employed to make precise alterations by providing a donor template, which can be either single or double-stranded DNA, containing specific modifications and bordered by homologous arms.

Our selected gene of interest is the IgA locus, therefore four guide RNA's (gRNA) were designed to target the mouse BALB/c Igha gene (Gene ID: 238447) (refer to table below for gRNA sequences). 2 nmol of gRNA was ordered through IDT (Coralville, IA), along with 5 nmol of tracrRNA and 100 ug Cas9 protein for injections. All four gRNA's were injected together into BALB/c females (The Jackson Laboratory) to increase chances of successful deletion of the Igha gene. The generation of IgA knockout mice ($IgA^{-/-}$)was all done by the Gene Manipulation and Genome Editing Core at Boston Children's Hospital. Only four pups were born from the injected pseudopregnant recipients, and two of the four founders were used for these experiments.

Table 6 : Guide RNA sequences

Guide RNA	Sequence	Exon
gRNA-1	TCACATTCATCGTGCCGGAA GGG	Exon 1
gRNA-2	CGGCACGATGAATGTGACCTGGG	Exon 1

gRNA-3	GGCAGGTGGGAAGTTTACGG TGG	Exon 1
gRNA-4	GGACACACTGTAGCAGCCGC AGG	Exon 2

Genotyping was first done by polymerase chain reaction (PCR), using primers (Fwd: AGCTGACCCCCTAACGTTCTT; Rev: AAAGCCCTGCTGGAGTCATTTG) for exon 1, and (Fwd: TGAGCAGGGAACGTCATACAAT; Rev: ATCCAGAAAAGGGGACAGCAAAG) for exon 2. To confirm genotypes, PCR products were sent to GENEWIZ for sanger sequencing. Blood was later taken from the mice at three weeks of age, and IgA deficiency was confirmed using an IgA ELISA. We later set up a qPCR-based genotyping protocol with Transnetyx (Cordova, TN). All founders were backcrossed onto BALB/c mice 8-10 generations to remove possible off-target mutations. All $IgA^{-/-}$ lines were housed under specific pathogen-free conditions and used according to the guidelines of the institutional Animal Research Committees at the Boston Children's Hospital. To generate littermate controls, $IgA^{-/-}$ females were crossed with $IgA^{+/-}$ males.

3.3.15 Enzyme linked immunosorbent assay (ELISA)

Measurement of immunoglobulins

Immunoglobulin levels were measured by their respective ELISAs. 3 μg/ml (50 μl/well) of anti-mouse IgA (BD, Franklin Lakes, NJ, USA), IgG1, IgG2a, IgG2b, IgG3, IgE and IgM (Southern Biotechnologies, Birmingham, AL, USA) were coated in 1X PBS on flat bottom 96 well plates overnight at 4 °C. Plates were washed 4X with 0.05% Tween 20 in 1X PBS and blocked with 200 μl of 2% BSA in 1X PBS for 2 hours at room temperature. Plates were then washed again 4X with wash buffer. Standards and samples were diluted accordingly (standards for IgG1, IgG2a, IgG2b, IgG3, IgE and IgM (BD, Franklin Lakes, NJ), plated (50 μl/well), and incubated overnight at 4 °C. Plates were washed 4X after overnight incubation, and (50 μl/well) of the following detection antibodies were used at a dilution of 1:4000 (IgA-HRP, IgM-HRP, IgG1-HRP, IgE-HRP from Southern Biotechnologies), for one hour and 1:2000 (IgG2a AP, IgG3 AP, IgG2b AP from Southern

Biotechnologies, for two hours. HRP conjugated plates were detected using (50 µl/well) of TMB substrate (ThermoFisher Scientific, Carlsbad, CA, USA), and AP plates were detected using (100 µl/well) of PNPP (ThermoFisher Scientific, Carlsbad, CA, USA). HRP (horseradish peroxidase) plates were stopped by the addition of (50 µl/well) of 2M sulfuric acid, and measured at 450 nm using an ELISA reader. AP (alkaline phosphatase) plates were measured at 405 nm using an ELISA reader.

The murine mast cell protease 1 (MMCP-1) ELISA kit from (eBioscience, San Jose, CA, USA) was used to measure MMCP-1 in mouse serum according to manufacturer's instructions.

3.3.16 Blood basophil staining

At 6-8 months of age, mice lightly anesthetized using isoflurane for retro-orbital bleeding using heparinized capillary tubes (Fisher Scientific, USA). Blood was collected in Eppendorf tubes and 100 µl of whole blood was stained with the following antibodies: AF700 anti-CD45 (30-F11), BV510 anti-CD3 (17A2), FITC anti-CD19 (145-2C11), PerCP-Cy5.5 anti-CD49b (HMα2), PE anti-IgE (RME-1), and viability dye (e780) for 30 minutes at 4°C. Cells were lysed and fixed using 1-step Fix/Lyse solution (ThermoFisher Scientific, Carlsbad, CA) for 30 minutes. Cells were centrifuged at 1400 RPM for 5 minutes, and then washed twice with FACS buffer.

3.3.17 Microbiome Analysis

Stool samples from 6-month female $IgA^{+/-}$ and 4 female $IgA^{-/-}$ were sent to Transnetyx (Cordova, TN) for microbiome analysis using whole genome DNA sequencing (shallow shotgun). Relative abundances pertaining to different taxa was generated by the OneCodex system in partnership with Transnetyx.

3.3.18 RNA extraction, reverse transcription, and real time quantitative polymerase chain reaction (qPCR) of small intestinal tissue

Small intestinal tissue was weighed so they do not exceed 30 mg. 10 µl of Bme was added to the RLT buffer provided by the Qiagen RNeasy mini kit for animal tissues (Qiagen, MA, USA). 700 µl of RLT+Bme was added per sample to 2ml bead tubes, in addition to one bead. Samples were homogenized by a bead beater (Fisherbrand™ Bead Mill 24 Homogenizer) at speed four for two minutes. RNA was extracted using Qiagen RNeasy mini kit according to manufacturers protocol. RNA concentration was determined using a spectrophotometer (Nanodrop One, Thermofisher, USA) at an absorbance of 260nm. After RNA extraction, the RNA was transcribed to complimentary DNA (cDNA). The reaction involved the addition of 1 µg of RNA, 4 µl of 5X iScript reaction mix, 1 µl of reverse transcriptase, and water to make up a total volume of 20 µl (Biorad, CA, USA). qPCR allows for the real-time detection of fluorescent signals from DNA that has been amplified. PCR amplification was done by using Fast Advanced Master Mix (Thermofisher, USA), which contains the AmpliTaq Fast DNA polymerase, dNTPS, ROX dye, and buffer components, and run on QuantStudios 3 (AppliedBiosystems, Thermofisher, USA). cDNA was first diluted a 1:2, and 1.6 µl of sample was added along with 10 µl of 10X Fast Taqman, 1 µl of probe, and 7.4 µl of water in a 96 well qPCR plate. B-actin was used as a housekeeping gene. The following reaction conditions and probes were used.

Table 7: Reaction conditions for qPCR

	Time (seconds)	Temperature (°C)	Number of Cycles
Polymerase activation	20 seconds	95°C	1 cycle
Denaturing	1 second	95°C	40 cycles
Anneal/Extend	20 seconds	60°C	

Table 8: Primers for qPCR (all from Thermofisher, USA)

Gene	Assay ID
B-actin	Mm02619580_g1
IL-4	Mm00445259_m1

Il-6	Mm00446190_m1
IL-10	Mm01288386_m1
TGF-β	Mm01178820_m1
IL-13	Mm00434204_m1

3.3.19 Statistical Analysis

Graphs were generated using GraphPadPrism (Graph Pad Software). For groups of 3 or more, ANOVA was used. Unpaired T-test's were used to compare cell types in $IgA^{-/-}$ mice vs littermate controls. Data shown is the mean ± SEM (n= 3-9/group). *P< 0.05, **P<.01, ***P < .001****P < .0001.

4. Results Project 1

These Results are adapted from :

Yasmeen S. El Ansari, Cynthia Kanagaratham, Oliver T. Burton, Jenna V. Santos, Brianna-Marie A. Hollister, Owen L. Lewis, Harald Renz, and Hans C. Oettgen**. Allergen-Specific IgA Antibodies Block IgE-Mediated Activation of Mast Cells and Basophils.** Front Immunol. 2022 July 5.

With the permission of the Journal of Frontiers in Immunology

4.1 IgA Antibodies Inhibit IgE-Induced Mouse Mast Cell Degranulation in an Antigen-Specific Manner

In order to evaluate the effects of IgA antibodies on IgE-mediated mast cell activation, we took advantage of the availability of monoclonal hapten-specific IgA antibodies as well as the well-characterized experimental model system of cultured BMMCs in which murine bone marrow cells are incubated with IL-3 and SCF to differentiate to c-Kit^{+} FcεRI^{+} mast cells. Our own investigations as well as others have shown that IgG antibodies can inhibit IgE-mediated BMMC activation in a manner that is antigen specific and FcγR2b -dependent (e.g. the effect is not observed in FcγR2b$^{-/-}$ BMMCs) (14, 15). To test the effects of IgA on IgE-mediated mast cell activation, BMMCs were incubated with IgE ± IgA monoclonal antibodies specific for the hapten, trinitrophenyl (TNP) prior to antigen exposure. Cells were then washed to remove unbound antibody, and then stimulated for 10-minutes with (TNP-BSA). LAMP-1, which is extruded to the plasma membrane surface during granule fusion, was measured by flow cytometry as an indicator of mast cell degranulation.

In the absence of anti-TNP IgE, TNP-BSA induced no detectable BMMC degranulation, with LAMP-1 expression only 0.1% ± 0.02% (Figure 4). Anti-TNP IgE-sensitized BMMCs exposed to TNP-BSA exhibited a robust induction of surface LAMP-1 on the cell surface (16.9% ± 0.50%). Addition of anti-TNP IgA to these cultures resulted in a marked suppression of IgE-induced LAMP-1 expression (3.5% ± 0.20%) (Figure 4). In contrast,

incubating anti-TNP IgE sensitized mast cells with IgA directed against an irrelevant antigen, ovalbumin (OVA), did not impair IgE mediated activation (20.5% ± 0.70%), indicating that IgA mediated suppression is antigen specific.

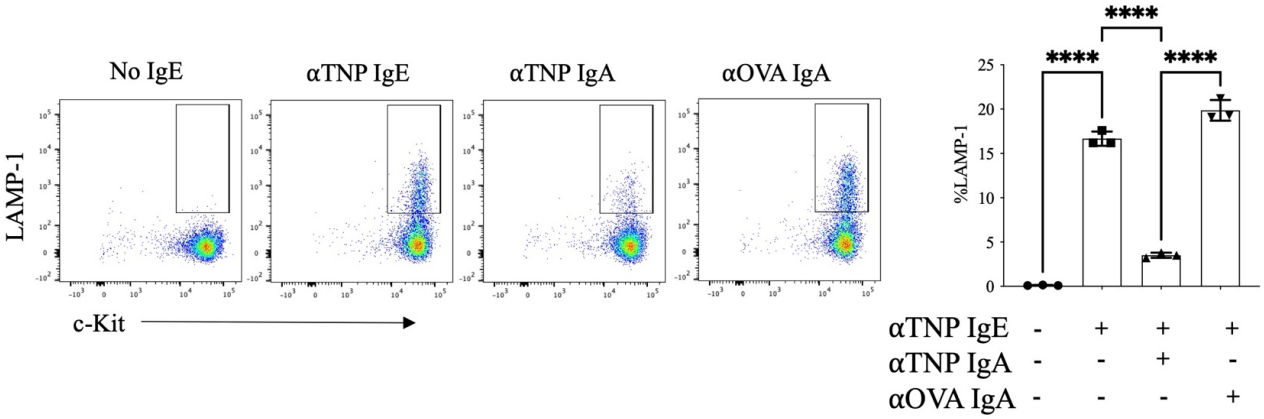


Figure 4. Effects of IgA antibodies on IgE mediated degranulation of bone marrow derived mast cells.

Flow cytometry plots from a representative experiment (left) and aggregate data (n=3) bar plots (right) of percent LAMP-1 expression of IgE sensitized BMMCs following antigen exposure. BMMCs were sensitized with anti-TNP IgE (αTNP IgE) (50 ng/ml). Subsequently, some cells were co-incubated with anti-TNP IgA (αTNP IgA) (100 µg/ml) (purified from TIB-194 hybridoma) or anti-OVA IgA [αOVA IgA (100 µg/ml)]. Cells were primed with antibodies overnight, then washed and stimulated with, 50 ng/ml of TNP-BSA for 10 minutes before assessing activation by staining with anti-mouse c-Kit, and LAMP-1. Statistical analysis done by ANOVA. Data shown mean ± SEM of one experiment representative of three independent experiments. ****P < .0001.

4.2 IgA Binds to Bone Marrow Derived Mouse Mast Cells and Peritoneal Mouse Mast Cells

The observation that IgA antibodies blocked IgE-induced activation of allergen-exposed BMMCs, led us to consider the possibility that IgA might be physically interacting with these mast cells. Surface expression of IgA on c-Kit$^+$ FcεRI$^+$ cells was measured by flow cytometry following incubation of BMMCs with anti-TNP IgA for 30 minutes in HBSS. In

the presence of IgA, BMMCs uniformly stained with anti-IgA FITC in a dose dependent manner (Figure 5, upper panel), however no IgA binding was observed with purified splenic T cells (lower panel). Immunofluorescence imaging of BMMCs, incubated with or without anti-TNP IgA similarly revealed the presence of staining (green) on mast cells incubated with IgA (Figure 6). In order to evaluate IgA binding to primary mast cells, we examined peritoneal lavage cells. Staining for surface IgA after incubation with anti-TNP IgA, revealed IgA binding to CD11b^{-} c-Kit^{+} FcεRI^{+} mast cells isolated from the peritoneum (Figure 7).

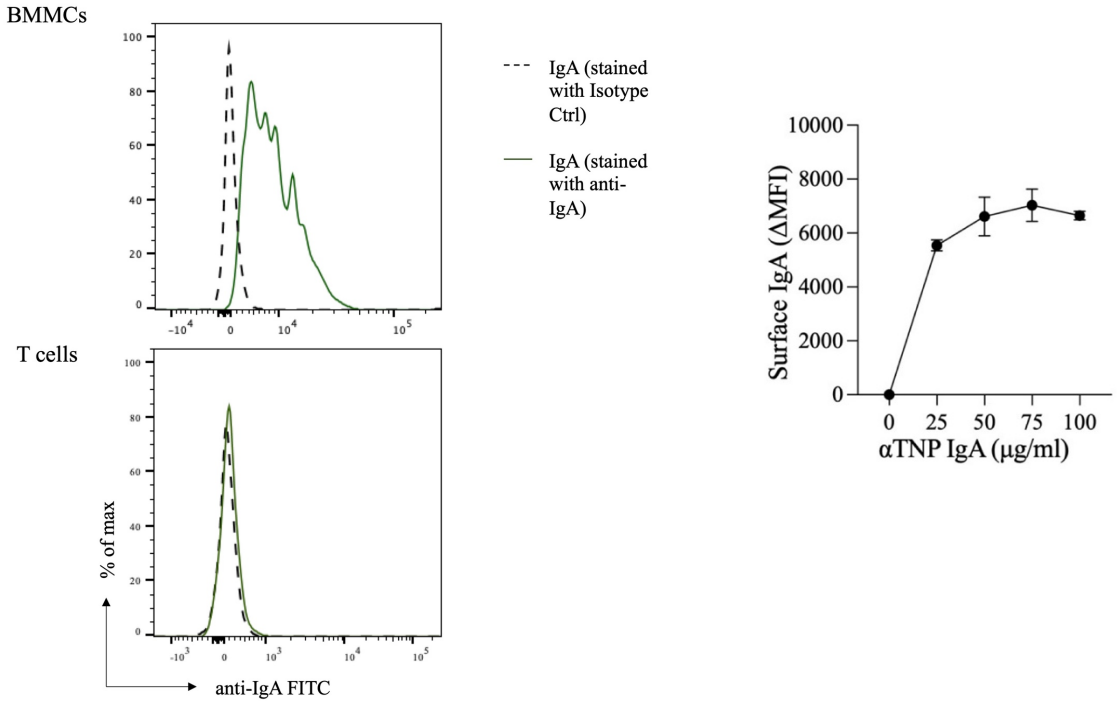


Figure 5. Surface IgA staining on bone marrow derived mast cells

Representative histogram of αTNP IgA binding to BMMCs (top) and purified splenic T cells (bottom), and a dose response curve of αTNP IgA binding to BMMCs (right). Mast cells incubated with or without anti-TNP IgA were stained with antibodies for c-Kit, FcεRIα, and IgA and analyzed by flow cytometry. Purified splenic T cells incubated with or without anti-TNP IgA were stained with antibodies for CD3, CD4, and IgA and analyzed by flow cytometry. Data shown is one experiment representative of six independent experiments.

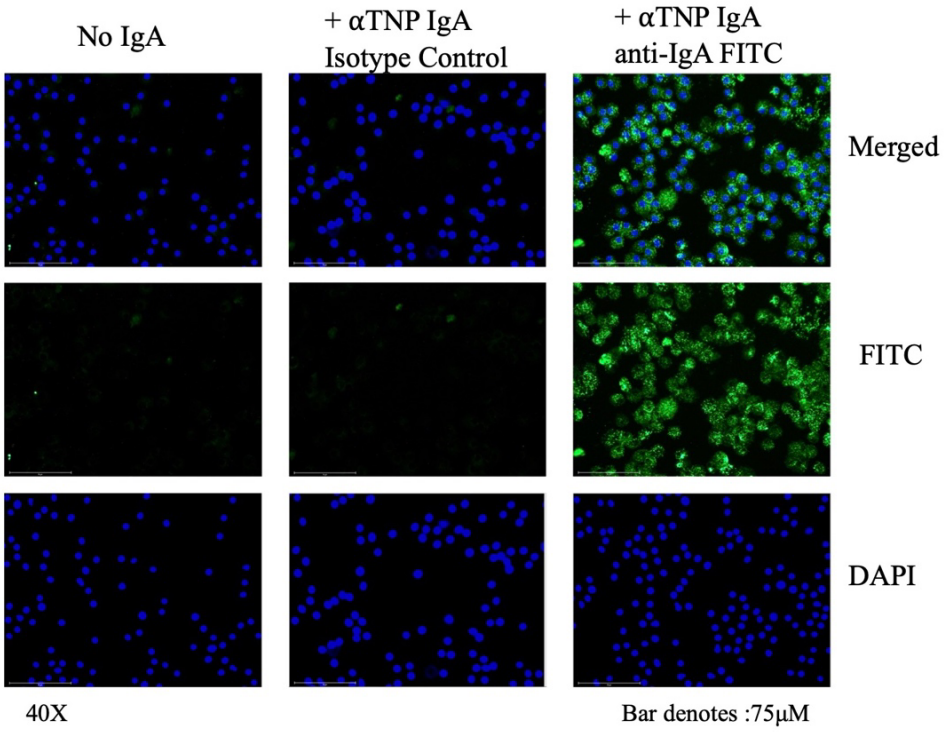


Figure 6. Immunofluorescence analysis of IgA binding to BMMCs.

Representative photomicrographs (40x) of BMMCs incubated with or without 100µg/ml of αTNP IgA in HBSS for 30 minutes at 4°C. BMMCs were stained for nucleus (blue), IgA (green), or isotype control (middle), and merged (right). Scale bars: 75 µm. Images were taken using an EVOS M7000 microscope. Data shown is one experiment representative of two independent experiments.

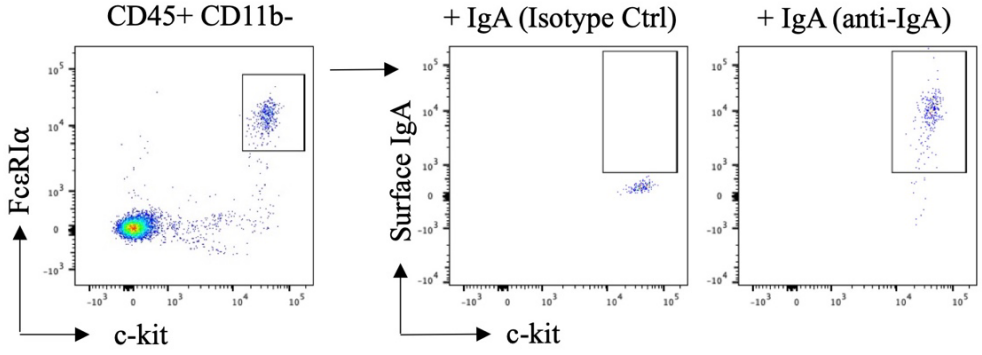


Figure 7. Surface IgA staining on peritoneal mast cells

Representative flow cytometry plots of IgA staining on peritoneal cavity mast cells, identified by the presence of CD45, c-Kit, FcεRIα. Data shown is one experiment representative of two independent experiments.

4.3 IgA Binding to Mast Cells Is Calcium- and Sialic Acid-Dependent

As mast cells do not express any known IgA receptors, we sought to characterize the physical properties of IgA binding to BMMCs. To test calcium dependence, exogenous calcium was chelated by addition of EDTA to BMMC suspensions prior to incubation with IgA. Calcium and magnesium removal dramatically impaired IgA binding with a decrease in MFI from 1485 ± 81.33 to 59.33 ± 59.33 (Figure 8A) but had no effect on IgE binding (Figure 8C). We similarly observed that BMMCs suspended in calcium-free PBS failed to bind IgA (Figure 8A). IgA binding was also impaired in cultures that had EGTA, which specifically targets calcium (Figure 8B).

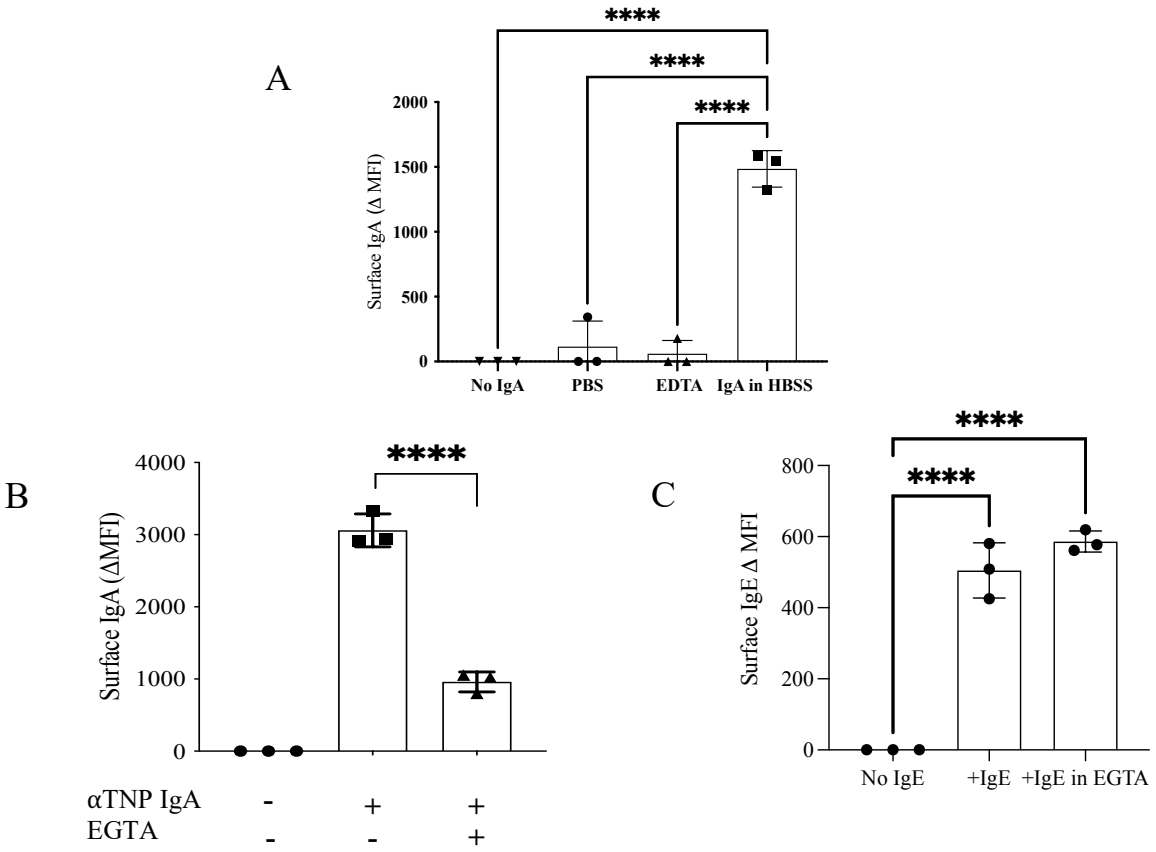


Figure 8. Effects of calcium chelation on IgA binding

(A) Mean fluorescence intensity (MFI) of BMMCs stained with anti-IgA after incubation with anti-TNP IgA in PBS, EDTA, or HBSS. (B) Mean fluorescence intensity (MFI) of BMMCs stained with anti-IgA after incubation with anti-TNP IgA in the absence or presence of EGTA. (C) Mean fluorescence intensity (MFI) of BMMCs stained with anti-IgE after incubation with anti-TNP IgE, with or without EGTA. Statistical analysis done by ANOVA. Data shown mean ± SEM of one experiment representative of three independent experiments. ****P < .0001.

The abrogated IgA binding after calcium chelation suggested interaction with a calcium dependent receptor. Some such receptors are lectins, leading us to consider that the carbohydrate moieties on IgA might be mediating its interaction with BMMCs. To test this, we treated anti-TNP IgA with either PNGase F to remove all N-linked sugars, or neuraminidase to remove terminal sialic acid residues. Either N-deglycosylation of anti-TNP IgA or the removal of sialic acid rendered the antibody completely unable to bind to BMMCs (Figure 9A) without affecting its ability to bind to TNP-OVA (Figure 9B). To corroborate this sialic acid dependence of IgA binding to BMMCs, we incubated cells with sialic acid for 30 minutes prior to addition of IgA and observed suppression of IgA binding to undetectable levels (Figure 9C).

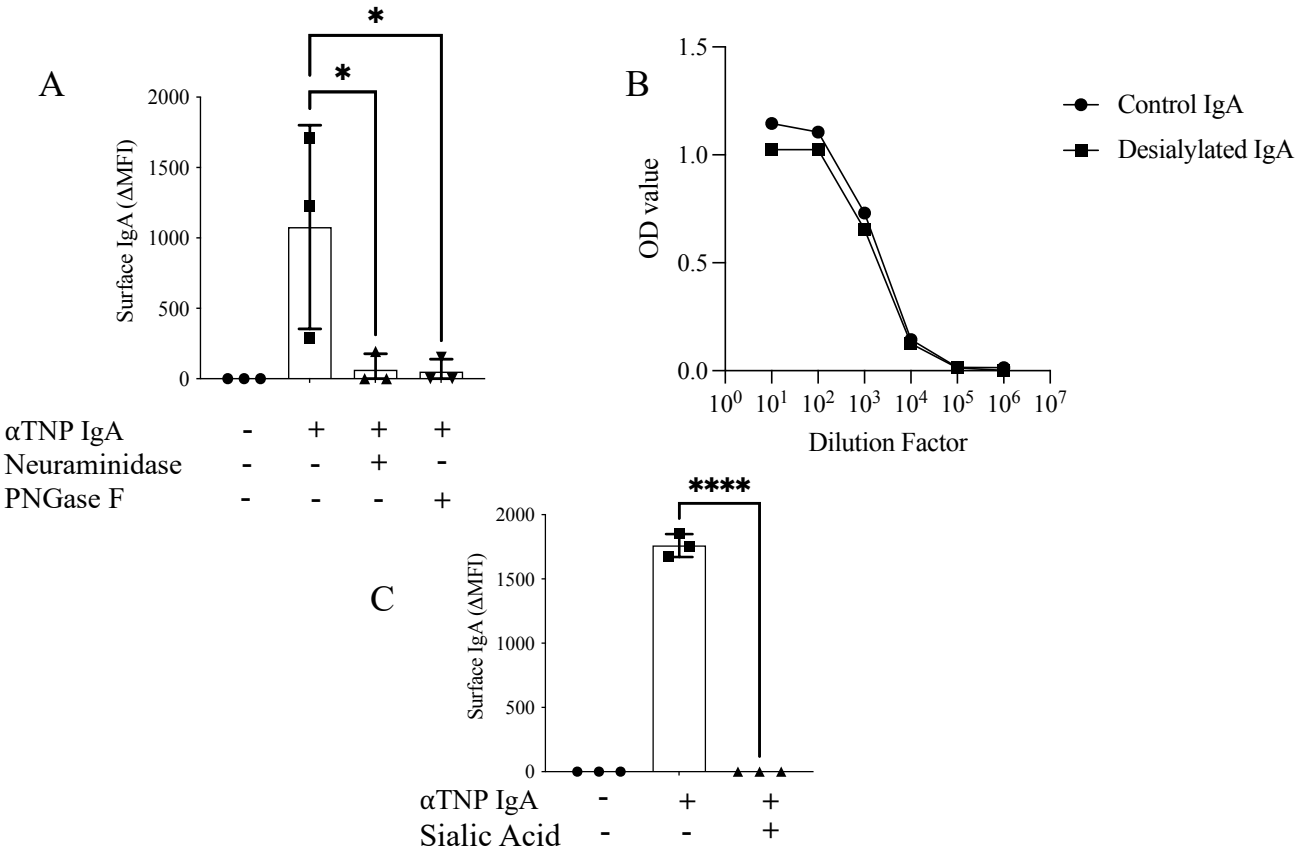


Figure 9. Analysis of sialic acid requirement for IgA binding.

(A) MFI of BMMCs incubated with untreated anti-TNP IgA, desialylated anti-TNP IgA or N-deglycosylated anti-TNP IgA in HBSS for 30 minutes at 4°C. Mast cells incubated with or without anti-TNP IgA were stained with antibodies for c-Kit, FcεRIα, and IgA and analyzed by flow cytometry. (B) Effects of sialic acid competition on IgA binding to BMMCs. MFI of BMMCs stained with anti-IgA with or without preincubation with sialic acid. Statistical analysis done by ANOVA. Data shown mean ± SEM of one experiment representative of three independent experiments. ****P < .0001.

Using desialylated IgA, we also confirmed a functional requirement for sialic acid in the ability of IgA to suppress IgE-mediated mast cell activation. We observed that while untreated IgA inhibited LAMP-1 induction (8.8% ± 0.06% in treated compared to 16.2% ± 1.2% in controls), desialylated anti-TNP IgA was incapable of suppressing IgE-mediated BMMC degranulation (18.3% ± 2.3%) (Figure 10). We could not similarly establish the effects of calcium depletion on the inhibitory function of IgA antibodies since it is not possible to perform mast cell activation assays in the absence of calcium. These experiments reveal that the binding of IgA to BMMCs is dependent both on ambient calcium concentration and on sialylation of the IgA antibodies and that sialyation is also critical for functional inhibition of mast cell activation by IgA.

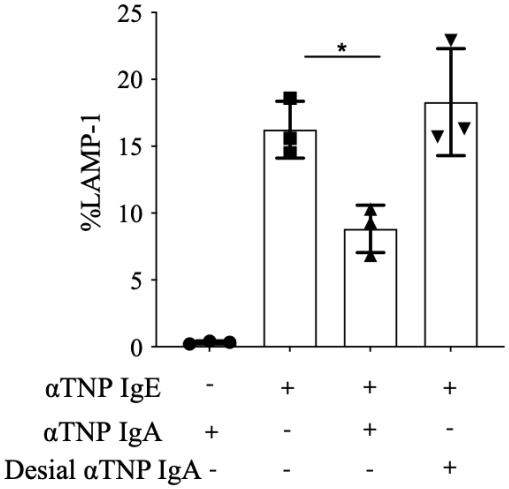


Figure 10. Consequences of sialic acid removal on the inhibitory effects of IgA.

Bar plot of percent degranulation across three replicates of LAMP-1 induction in IgE sensitized BMMCs incubated with or without anti-TNP IgA, or desialylated anti-TNP (desial αTNP) IgA. Statistical analysis done by one-way analysis of variance (ANOVA). Data shown mean ± SEM of one experiment representative of three independent experiments. *P < .05.

4.4 IgA Inhibits Syk Phosphorylation and Cytokine Production in IgE-Activated Bone Marrow Derived Mast Cells

IgE-antigen receptor crosslinking on BMMCs results in the phosphorylation of the protein tyrosine kinase (PTK) Syk. Activation of a PTK signaling cascade downstream of Syk drives many of the phenotypes of activated mast cells, including degranulation, synthesis of arachidonate-derived lipid mediators and induction of cytokine transcription. To establish if IgA antibodies block this critical signaling pathway, we evaluated their effects on phosphorylated-Syk (p-Syk), the active signaling form of this PTK. BMMCs loaded with anti-TNP IgE and stimulated with TNP-BSA exhibited rapid increases in p-Syk levels, peaking one minute after stimulation (MFI 298 ± 7.6) and then quickly decreasing (Figure 11). In contrast, allergen-exposed BMMCs sensitized with anti-TNP IgE and incubated with anti-TNP IgA exhibited attenuated p-Syk induction (176.6 ± 4.7) 1 minute after stimulation.

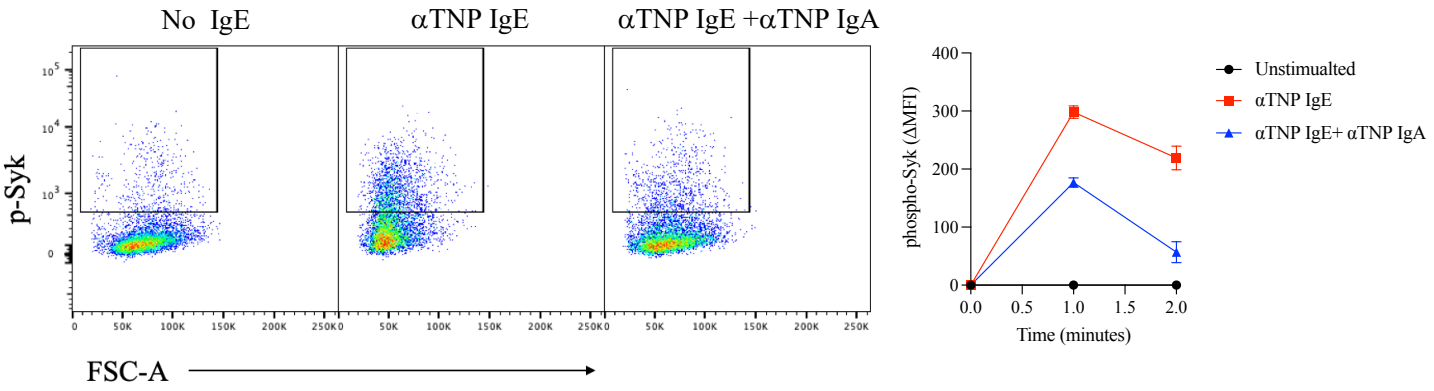


Figure 11. Effect of IgA antibodies on IgE-induced phosphorylation of Syk by activated bone marrow derived mast cells

IgE sensitized BMMCs were incubated with or without αTNP IgA and stimulated with antigen for up to two minutes followed by measurement of phosphorylated-Syk (phospho-Syk) using flow cytometry (left panel). Mean fluorescence intensity (MFI) of BMMCs stained with anti-phospho-Syk after incubation with anti-TNP IgE/and anti-TNP IgA (right panel). This experiment is representative of three replicates.

Cytokine production is an important property of activated mast cells and is critical for the generation and tissue recruitment of the effector cells of inflammation as well as for the expansion of type 2 adaptive immune responses. We evaluated the effects of antigen-specific IgA on IgE-induced cytokine production by BMMCs. As expected, IgE-sensitized BMMCs exposed to antigen produce IL-13, TNF-α, and IL-6. Addition of IgA to these IgE-sensitized BMMCs prior to antigen stimulation resulted in a complete suppression of the production of these cytokines (Figure 12). These results indicate that IgA acts early in the FcεRI signaling cascade, inhibiting formation of the most proximal signaling intermediate, p-Syk and that IgA-mediated blockade of mast cell activation extends beyond degranulation to also affect cytokine production.

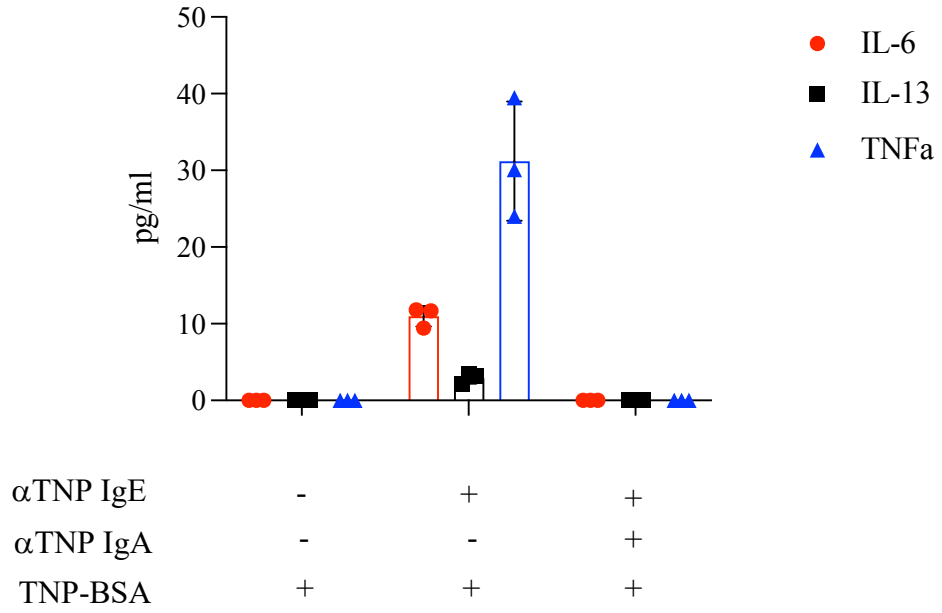


Figure 12. IgA effects on cytokine production.

Cytokine (IL-6, IL-13 and TNF-α) levels in the supernatants of anti-TNP IgE sensitized BMMC incubated with or without anti-TNP IgA and stimulated with TNP-BSA for 6 hours. Statistical analysis done by ANOVA. Data shown mean ± SEM of one experiment representative of three independent experiments. ***P < .001.

4.5 IgA inhibits Passive Systemic Anaphylaxis

In the setting of allergen exposure *in vivo*, the mediators rapidly released by mast cells and basophils following IgE crosslinking can drive systemic anaphylaxis, the most severe manifestation of the immediate hypersensitivity response. In order to establish if the inhibitory effects of IgA on IgE-mediated activation we observed in cultured mast cells would translate to a suppression of anaphylaxis, we used the mouse model of passive systemic anaphylaxis. Mice were sensitized by injection of αTNP IgE *i.p.* and treated with αTNP-IgA. Core body temperature was measured as an indicator of anaphylaxis manifesting as hypothermia caused by diversion of blood flow to the skin of mice following vasodilation induced by challenge with TNP-OVA. Challenged mice sensitized with IgE exhibited the expected dropped in core body temperature following TNP-OVA challenge, with a drop of 1.77° ± 0.55 ° below baseline at 30 minutes with gradual recovery over the next 30 minutes (Figure 13). However, IgE-sensitized mice treated with αTNP IgA were protected from anaphylaxis and only dropped 0.27 ° ± 0.69 ° at the same timepoint (Figure 13). Mouse mast cell protease 1 (MMCP-1) serum concentrations were measured after anaphylaxis and were also lower in αTNP IgA treated animals (537.9 ng/ml ± 70.79) vs untreated (1495 ng/ml ± 163.6) (Figure 13 right panel).

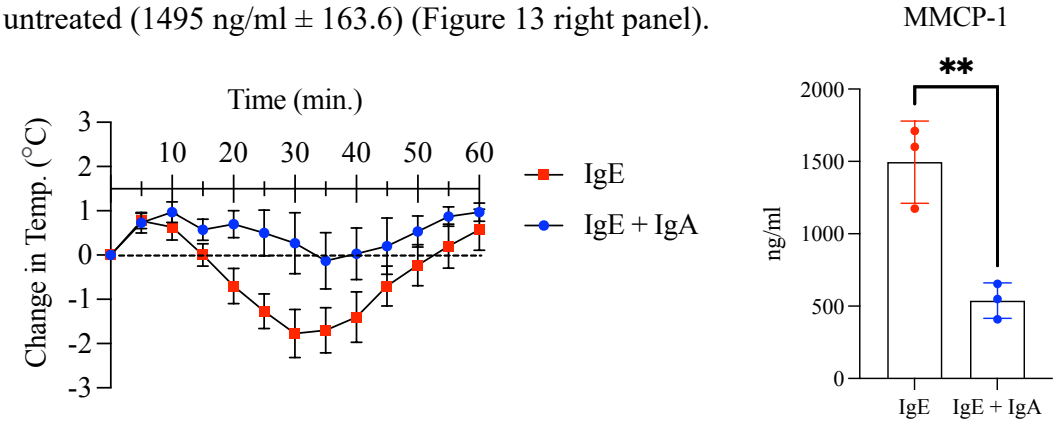


Figure 13. Effect of IgA on passive systemic anaphylaxis

Core temperature change (left) and MMCP-1 levels (right) in wildtype mice treated with or without anti-TNP IgA, sensitized with anti-TNP IgE and challenged with TNP-OVA. Data is representative of two experiments with n = 3/group. Statistical analysis by ANOVA. **P<.01

4.6 IgA Antibodies Inhibit the Peanut-Induced Activation of Basophils From Allergic Subjects

Peanut-specific IgG and IgA antibodies are both present in the plasma of subjects with peanut allergy and both of their levels increase after OIT. We have observed that IgE-mediated activation of basophils is potently suppressed by IgG signaling via FcγR2b (Burton et al., 2014a). We took advantage of post OIT sera as a source of PN specific IgA to test whether IgA exerts similar protective effects as PN-IgG in peanut allergic patients. IgA was enriched from these patient sera by affinity chromatography and further purified by IgG depletion using Protein G Sepharose, yielding a preparation containing 2.5 mg/ml IgA and no detectable IgG. Whole blood samples from a peanut allergic donor (with confirmed IgE mediated allergy to peanut) were pre-incubated for two to four hours with varying concentrations of purified IgA (125-1000 µg/ml). The samples were then stimulated with CPE and expression of CD63 was used to as a marker of basophil activation. Basophil activation was markedly suppressed in the presence of IgA (Figure 14 left panel). This inhibitory effect of IgA was dose dependent over a range of IgA amounts from (250-1000 µg/ml) (Figure 14 right panel).

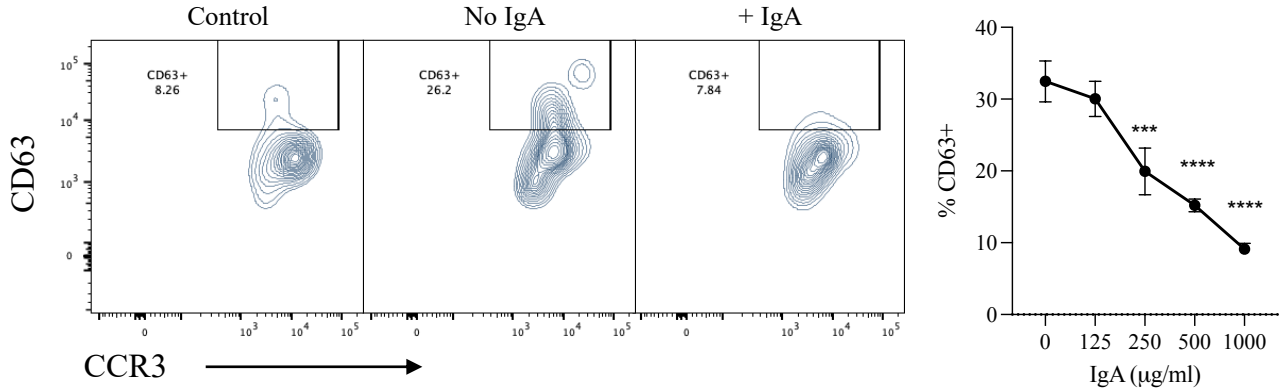


Figure 14. Effects of peanut-specific IgA on basophils from peanut allergic subjects.

(A) Whole blood from peanut allergic patient incubated with or without IgA from peanut allergic subjects and stimulated with CPE followed by measurement of CD63 on basophils using flow cytometry. (B) Dose response analysis of effects of IgA from peanut allergic subjects on peanut induced activation of basophils from a peanut allergic donor. Data shown from one experiment run in triplicate. Statistical analysis by ANOVA. ***P < .001, ****P < .0001

While IgA has been found to interact with several human cell types, including neutrophils and eosinophils (Gimpel et al., 2021; Lamkhioued et al., 1995), its interaction with mast cells and basophils is incompletely characterized. At least one report has suggested an activating effect of IgA antibodies on basophils (Iikura et al., 1998). We sought to determine if IgA might bind to basophils. We stained whole blood with fluorescently labeled human IgA along with antibodies defining the key leukocyte subsets. As expected, IgA was bound by neutrophils but not by T cells (Figure 15). Basophils also exhibited IgA binding with a unimodal fluorescence shift relative to isotype control. These observations indicate that IgA antibodies, like IgG antibodies, bind to and are capable of inhibiting the activation of antigen stimulated basophils from peanut-allergic subjects.

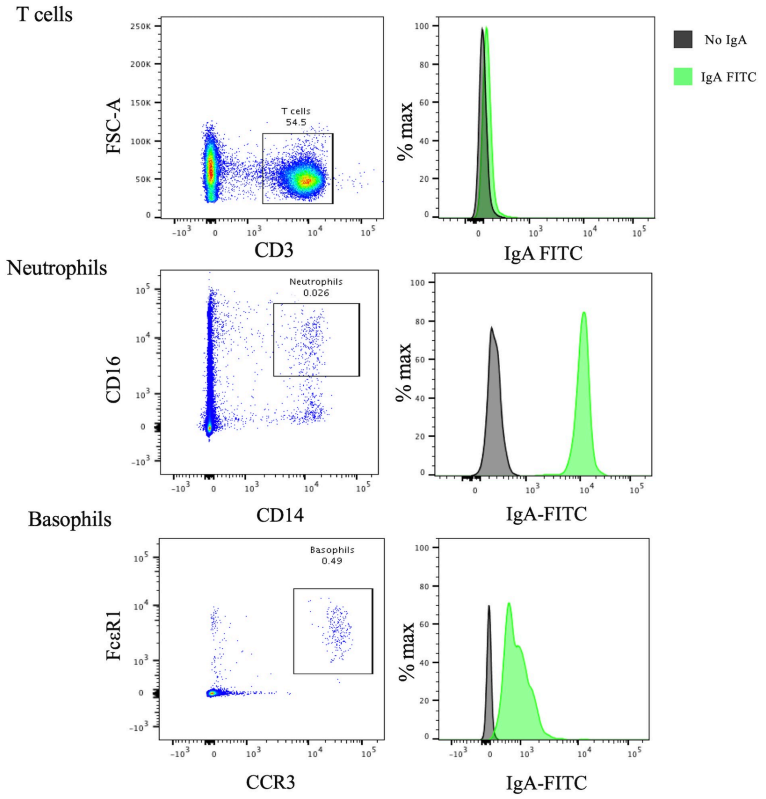


Figure 15. IgA binding to basophils.

Representative flow cytometry plots showing human IgA purified from post-OIT sera labeled with FITC binding to T cells, neutrophils and basophils from whole blood. T cells were identified by the presence of CD3, neutrophils by CD45, CD14, CD16, and basophils by CCR3 and FcεRI.

5. Results Project 2

5.1 Generation of CRISPR IgA$^{-/-}$ mice

Our preliminary work highlighting an inhibitory role for IgA in *in vitro* mast cell cultures and in human basophils from whole blood (Sections 3.1-3.6), led us to hypothesize that IgA, similarly, would be protective in *in vivo* mouse models of acute allergic reactions. As mast cells serve as endogenous adjuvants of developing Type 2 immune responses, we further hypothesized that IgA antibodies, by restraining mast cell activation, might also blunt emerging type 2 responses to allergens encountered at mucosal surfaces. We reasoned that the best approach to test these hypotheses might be to compare allergic responses in normal mice with those of mice with a targeted deletion of the IgA gene, IgA$^{-/-}$ mice. Here I describe our findings supporting these hypotheses, obtained using IgA$^{-/-}$ mice. As detailed below, our preliminary studies using IgA-deficient mice previously generated by others yielded unexpected results, most notably blunted IgE responses. This turned out to be due to an artifactual suppression of IgE switching in these animals by sequences introduced in the process of targeting the IgA gene. To circumvent this limitation, we produced new knockouts using CRISPR and obtained results in line with our hypothesis.

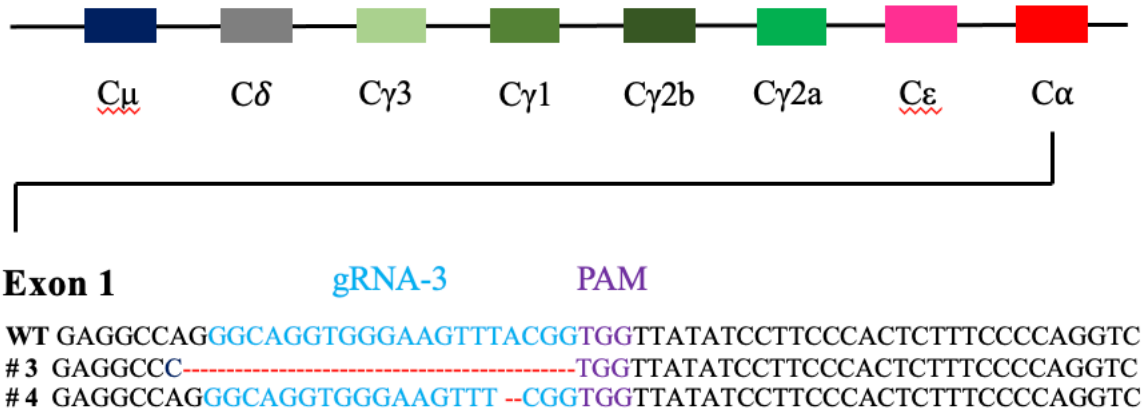
Studies previously done by Arnaboldi and colleagues revealed that IgA$^{-/-}$ mice generated by the classic approach of homologous recombination in embryonic stem cells, exhibited reduced pulmonary inflammation compared to WT animals in a model of lung allergy (Arnaboldi et al., 2005). Using those same mice, which had been generated in the 1990's (Mbawuike et al., 1999), we observed similar trends in a model of experimental oral allergen-induced diarrhea. IgA$^{-/-}$ and WT mice, *i.p* sensitized with OVA and alum, were challenged orally with OVA. We were surprised to find that total IgE was markedly reduced in sensitized IgA$^{-/-}$ mice in comparison to WT mice. They also had less IgE on their mast cells and made less OVA specific IgE but exhibited comparable levels of OVA IgG1 (Supplementary Figure 1A).

These unexpected findings prompted us to undertake a careful examination of the strategy that had been used to generate those IgA$^{-/-}$ mice. We noted that the gene targeting

constructs injected into embryonic stem cells to disrupt the Cα locus inserted a PGK-neo^r cassette into the locus. We reasoned that defective IgE production by these mice might be the result of interference of this strong transcriptional unit, with germline transcription and class switching to the neighboring Cε locus, encoding the IgE heavy chain constant region domains. Such an effect had been described by Seidl and colleagues who showed that insertion of PGK-neo at two sites downstream of Cα inhibits class switching to multiple IgH isotypes (including Cγ3, Cγ2a, Cγ2b, and Cε). To validate our hypothesis that these mice had an artifactual B cell intrinsic genetic inability to switch to IgE, we stimulated their splenocytes with anti-CD40 and IL-4 and observed a reduced percentage of IgE$^+$ B cells and IgE in the culture supernatant (Supplementary Figure 1B.). These findings led us to conclude that these mice, by virtue of their inability to switch to IgE, would be inappropriate for the analysis of the role of IgA in allergic IgE-mediated processes.

To overcome this limitation, we set out to generate a new strain of IgA deficient mice, on the BALB/c background using CRISPR/Cas9 to investigate the effect of IgA deficiency on allergic effector responses. To generate IgA deficient mice using CRISPR/CAS9, guide RNAs were designed to target the mouse Igha locus and injected along with Cas9 into BALB/c zygotes by the Gene Manipulation and Genome Editing Core at Boston Children's Hospital. This approach did not leave behind any transcriptionally active sequences that could affect heavy chain class switch recombination. A few pups had successful deletions, as confirmed by PCR, Sanger sequencing and IgA ELISAs (Figure 16). As expected with the chosen guide RNA's, all deletions were in exon 1 of the Igha locus. Founders were backcrossed onto BALB/c mice for the establishment of our IgA$^{-/-}$ colonies. Founders #3 and #4 were used in experiments.

A



B

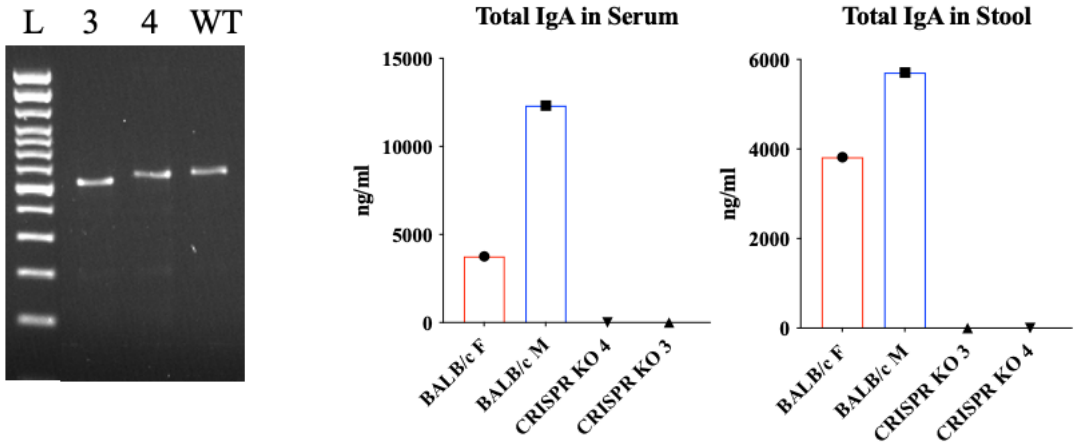


Figure 16. Generation of IgA Knockout mice using CRISPR/Cas9.

(A) Injection of 4 sgRNAs, Cas9, and tracrRNA, resulted in deletions in exon 1 of the Igha gene in two mice, at the gRNA-3 targeting site. (B) PCR analysis of mouse pups #3-4, and WT using primers encompassing target region (left). Serum and stool IgA levels in BALB/c males and females compared to CRISPR IgA$^{-/-}$ founders 3 and 4 (right).

5.2 IgA deficiency results in serum hyperimmunoglobulinemia

Comparison of baseline immunoglobulin levels in the sera of 6-8 month old $IgA^{-/-}$ mice and their littermate controls ($IgA^{+/-}$) revealed several major differences. We found that $IgA^{-/-}$ mice had significantly higher levels of IgM, IgG1, IgG2a, and IgG2b (Figure 17). As expected, IgA was undetectable in the $IgA^{-/-}$ mice, IgG3 levels however were comparable to those of littermate controls (Figure 17). These data nicely corroborates results obtained using previously generated IgA knockout mice which also had elevated IgM, IgG1, and IgG2b in their serum (Mbawuike et al., 1999).

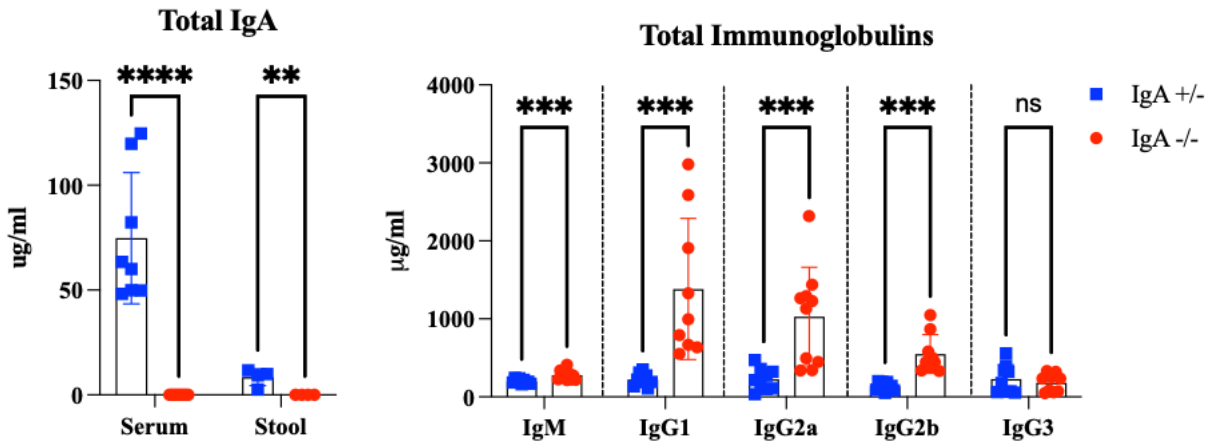


Figure 17. Immunoglobulin levels in the sera of CRISPR $IgA^{-/-}$ mice.

Total IgA, IgM, IgG1, IgG2a, IgG2b, and IgG3 levels measured in sera of 6-8 month old $IgA^{+/-}$ and $IgA^{-/-}$ mice. Statistical analysis done by unpaired t test. Aggregate data shown mean ± SEM of two independent experiments. **P<.01, and ***P<0.001

5.3 IgA deficiency results in elevated serum IgE and MMCP-1 levels

Compared to $IgA^{-/-}$ mice made in the 1990's that have reduced levels of total IgE (Mbawuike et al., 1999), our new CRISPR $IgA^{-/-}$ exhibited increased serum total IgE levels (9338 ng/ml ± 1528) compared to littermate controls (3905 ng/ml ± 602.6) (Figure 18). This divergence increased as the mice aged. This observation was accompanied by an

increase in MFI of IgE on blood basophils (59376 ± 11789 vs 28885 ± 7894 in littermate controls), as well as mast cells in the peritoneal cavity (45333 ± 5731 vs 15904 ± 1462) and small intestine (SI) (47212 ± 10122 vs 15745 ± 3472) (Figure 19). There were no differences in basophil or mast cell percentage by flow cytometry, or absolute count (supplementary Figure 2). Consistent with their increased tissue mast cell burden, serum mast cell granule protease 1 (MMCP-1) levels were elevated in $IgA^{-/-}$ mice (63.06 ng/ml ± 5.725), correlating with the increase in total IgE, and surface IgE on mast cells and basophils compared to littermate controls (24.20 ng/ml ± 9.298) (Figure 18).

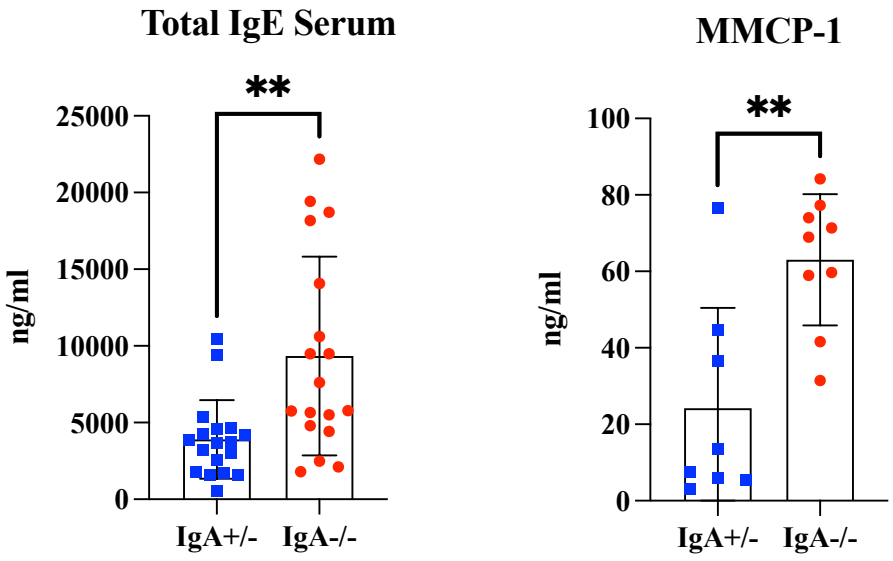


Figure 18. Serum IgE and MMCP-1 levels in $IgA^{+/-}$ and $IgA^{-/-}$ mice.

Total IgE levels (left) and MMCP-1 levels (right) in serum of 6-8 month $IgA^{+/-}$ and $IgA^{-/-}$ mice. Data shown is an aggregate of two independent experiments; n=3-9 mice/group. **P<.01.

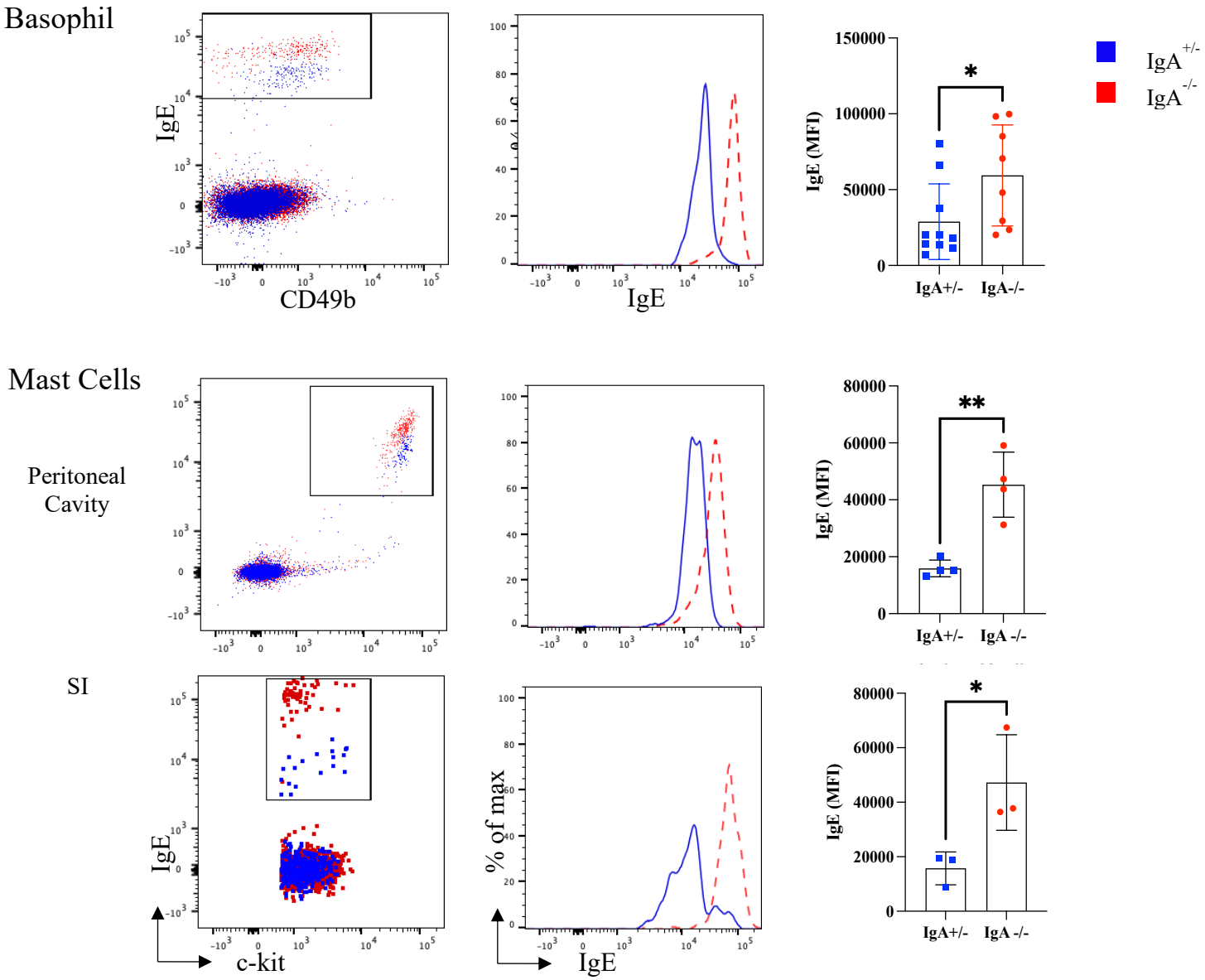


Figure 19. Flow-cytometric evaluation of IgE on basophils and mast cells.

Representative overlapping flow cytometry plots of blood basophils, and mast cells from the peritoneal cavity and small intestine, and histograms of surface IgE on these cells in IgA+/- (blue) and IgA-/- mice (red) (left). Bar plots of mean fluorescence intensity (MFI) of IgE on basophils, and mast cells (right). Blood basophils were identified as CD49b+ IgE+, and mast cells were identified as c-kit^{+}IgE^{+}. Data shown in is representative of three independent experiments; n=3-9 mice/group. *P <.05, and **P<.01.

To investigate whether $IgA^{-/-}$ mice exhibited Th2 skewed immune responses we measured expression of IL-4, IL-13, IL-10, TNFα, TGFb, and IL-6 in the small intestine by real-time quantitative polymerase chain reaction (RT-qPCR). There were no statistically significant differences in the expression of any of these genes. There were also no differences in cytokine production (IL-4, IL-10, IL-13) in $IgA^{+/+}$ vs $IgA^{-/-}$ splenocytes stimulated with PMA and ionomycin (supplementary Figure 3). These data suggest that IgA deficient mice don't have strong T cell cytokine signatures to explain their increased serum IgE or MMCP-1 levels. It is possible that both the elevated MMCP-1 and exaggerated IgE responses arise from Th2 cytokines produced by the expanded tissue pool of mast cells in these animals.

5.4 IgA deficiency results in dysregulated Tfh/Tfr and heightened B cell responses

T follicular helper cells (Tfh), and T follicular regulatory cells (Tfr) are critical for the development of humoral immune responses. Tfh's localize to germinal centers and provide help to B cells to drive expansion and affinity maturation resulting in the generation of somatically mutated B cell clones producing high affinity antibodies. Tfr cells, also found in germinal centers regulate play an opposing role to Tfh cells. Mounting evidence has revealed that the dysregulation of either Tfh or Tfr function contributes to the pathogenesis of autoimmune, and allergic disease (Clement et al., 2019; Fu et al., 2018). Our initial observation of hyperimmunoglobulinemia in $IgA^{-/-}$ mice, suggested an exaggerated Tfh response. Compared to $IgA^{+/-}$, Tfh cells were increased in frequencies in the Peyer's patches of $IgA^{-/-}$ mice (9.4% ± 1.3% vs 3.5% ± 0.3%), with similar expression of BCL-6. In contrast, the Tfh increase observed in Peyer's patches of $IgA^{-/-}$ mice was observed neither in their mesenteric lymph nodes nor their spleens indicating a gut-localized expansion of Tfh (Figure 20A upper panel). Tfr cells were also compromised in $IgA^{-/-}$ mice compared to $IgA^{+/-}$ (4.1% ± 1.7% vs 8.0% ± 0.6%) while unaltered in the spleen (Figure 20 lower panel).

The increases of all immunoglobulin isotypes, and expansion of Tfh cells, indicated a heightened B cell response in $IgA^{-/-}$ mice. To our surprise, percentages of germinal center B cells characterized by CD95 and FAS expression, were similar between IgA deficient and sufficient mice (Figure 20B). However, enumeration of total B cell frequencies, revealed

increased percentages of $CD19^+$ $B220^+$ B cells in the mesenteric lymph nodes of $IgA^{-/-}$ (45.6% ± 1.5% vs 39.4% ± 1.9%), and spleen (56.54%± 1.3% vs 51.14% ± 0.7%) but not Peyer's patches (Figure 20B). $CD19^+$ $B220^+$ B cells were also increased in the blood of $IgA^{-/-}$ mice (supplementary Figure 4A). As expected, $IgA^{-/-}$ mice had no $IgA^+CD19^+B220^+$ B cells in their Peyer's patches, mesenteric lymph nodes or spleens (Figure 20B (right). Consistent with serum immunoglobulin data, $IgA^{-/-}$ mice also exhibited increased $IgG1^+$ $CD19^+B220^+$ cells in the mLN, which was also trending to higher in the gut of IgA deficient mice but not statistically significant (supplementary Figure 5).

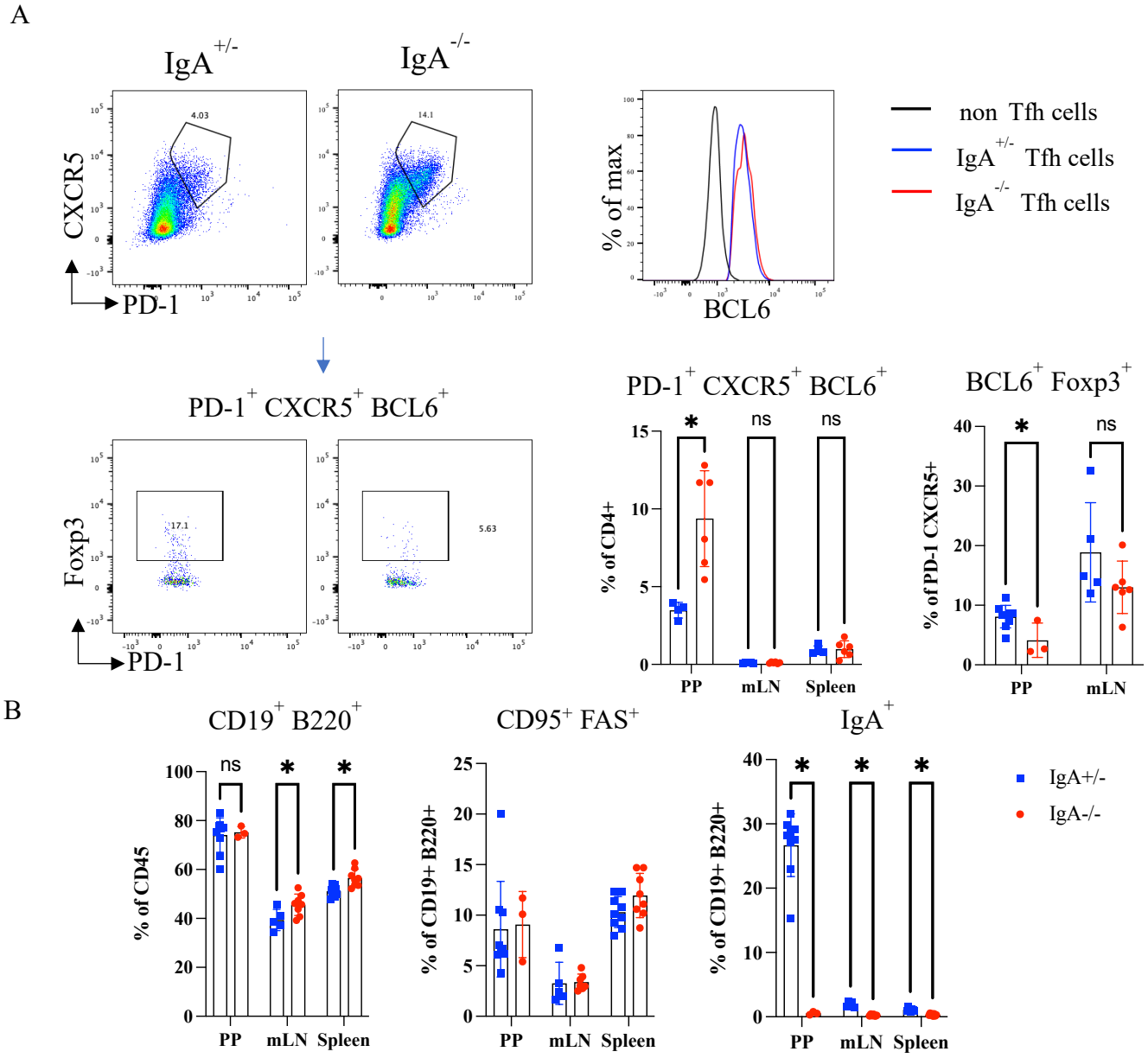


Figure 20. Flow cytometric evaluation of T follicular helper and regulatory cells, and B cells

(A) Representative flow cytometry plots of $CD4^{+}B220^{-}$ $PD\text{-}1^{+}$ $CXCR5^{+}$ Tfh cells and $Foxp3^{+}$ Tfr cells (upper and bottom left), and MFI of BCL6 (upper right) in the Peyer's patches of $IgA^{+/-}$ and $IgA^{-/-}$ mice. Frequencies of Tfh and Tfr cells in PP, mLN, and spleen of $IgA^{+/-}$ and $IgA^{-/-}$ mice (bottom right). (B) Frequencies of $CD19^{+}$ $B220^{+}$ cells, $CD95^{+}$ FAS^{+} cells, and IgA^{+} $CD19^{+}$ $B220^{+}$ in PP, mLN, and spleen of $IgA^{+/-}$ and $IgA^{-/-}$ mice . Data shown in is representative of two independent experiments.; n= 3-9 mice/group. *P <.05.

5.5 IgA deficient mice have decreases in various T helper cell subsets in the Peyer's Patches

IgA$^{-/-}$ mice also exhibited a decreased frequency of T helper cell subsets in the Peyer's patches (GATA3 : 20.14% ± 3.23% vs 29.66% ± 2.16%, RORγT : 2.00% ± 0.49 vs 3.82% ± 0.41%, Foxp3 : 12.82% ±1.04% versus 16.07% ± 0.36%), with decreases in Th2-like Treg cells (Foxp3^{+}Helios^{+}GATA3^{+}) as well compared to littermate controls (1.15% ± 0.1% versus 1.81% ± 0.08%) (Figure 21). There was also an increase in Th17-like Treg cells (Foxp3^{+}Helios^{-}RORγT^{+}) cells only in the mLN's of IgA$^{-/-}$ mice (1.32% ± 0.17% vs 0.78% ± 0.15%) (Figure 21). There were no differences in CD4^{+}CD3^{+}Tbet^{+} cells between IgA$^{+/-}$ and IgA$^{-/-}$ mice in the Peyer's Patches, mLN, or spleen (supplementary Figure 4B).

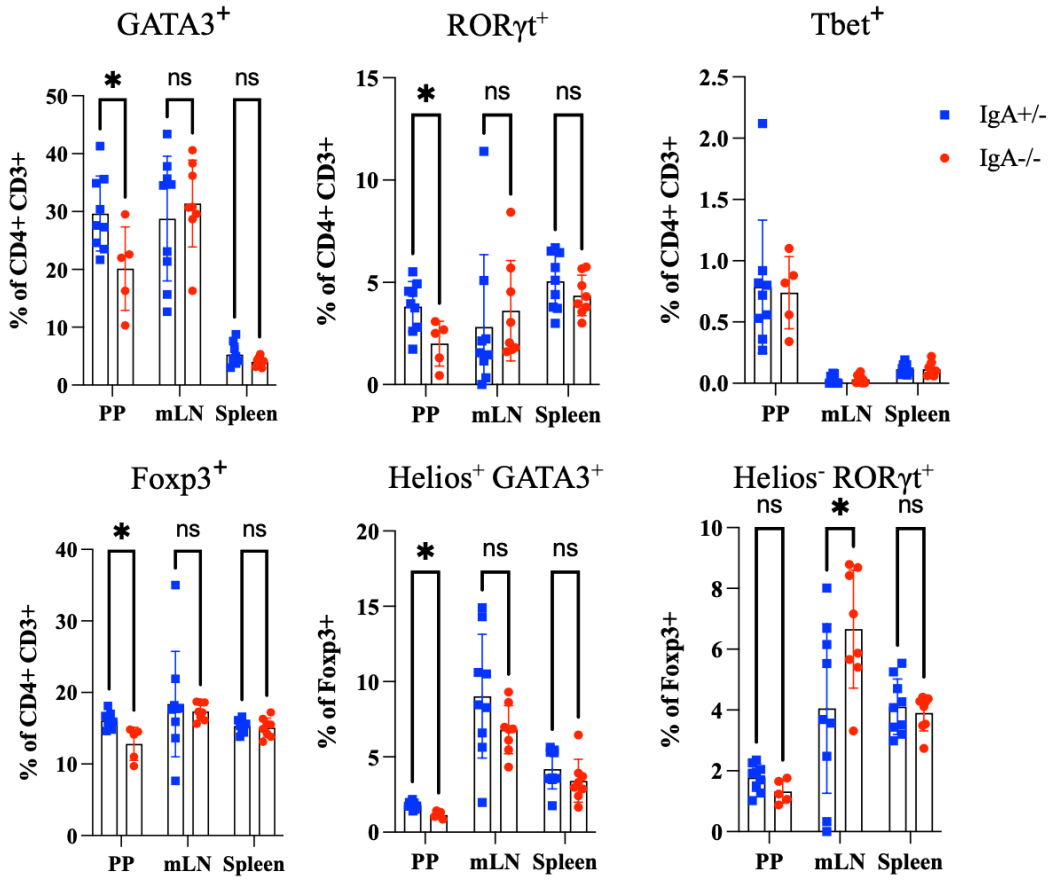


Figure 21. Flow cytometric evaluation of T helper subsets
(A) Frequencies of T helper subsets (Th2, Th17, Th1) (upper panels), along with Treg cells, Th2-like and Th17-like Treg cells (lower panel) in the PP, mLN, and spleen in IgA$^{+/-}$ and IgA$^{-/-}$ mice. Data shown in is representative of three independent experiments.; n= 3-9 mice/group. *P <.05.

5.6 Alterations in the gut microbiota of IgA deficient mice

Given the immense literature on the role of IgA in shaping the gut microbiome, we hypothesized that IgA deficient mice might exhibit alterations in bacterial composition, compared to control mice. To assess the effect of IgA deficiency on microbial composition in the gut, stool samples from $IgA^{+/-}$ and $IgA^{-/-}$ mice were subjected to whole genome sequencing. Using the Shannon Diversity Index for the comparison of alpha diversity in the fecal microbiome of $IgA^{+/-}$ and $IgA^{-/-}$ mice revealed no significant differences (Figure 22A left panel). Beta diversity analysis (Principle coordinate analysis : weighted Unifrac distances) revealed that microbial communities in $IgA^{-/-}$ mice occupied a distinct cluster compared to $IgA^{+/-}$ controls (Figure 22A middle panel). At the phylum level *Firmicutes* were decreased in relative abundance in $IgA^{-/-}$ mice, while *Bacteroidetes* were increased, compared to controls (Figure 22A right panel). There were differences in several other taxa including order, class, and family. The *Lachnospiraceae* family, which belongs to the *Clostridia* order was decreased in $IgA^{-/-}$ mice, while *Porphyromonadaceae* were increased compared to controls. At the species level, both *Porphyromonadaceae*, and *Prevotella* were increased in IgA deficient mice as well (Figure 22C) These results suggest that IgA deficient mice while having similar microbial composition to $IgA^{+/-}$ controls, have significant differences in the relative abundance of several specific taxa.

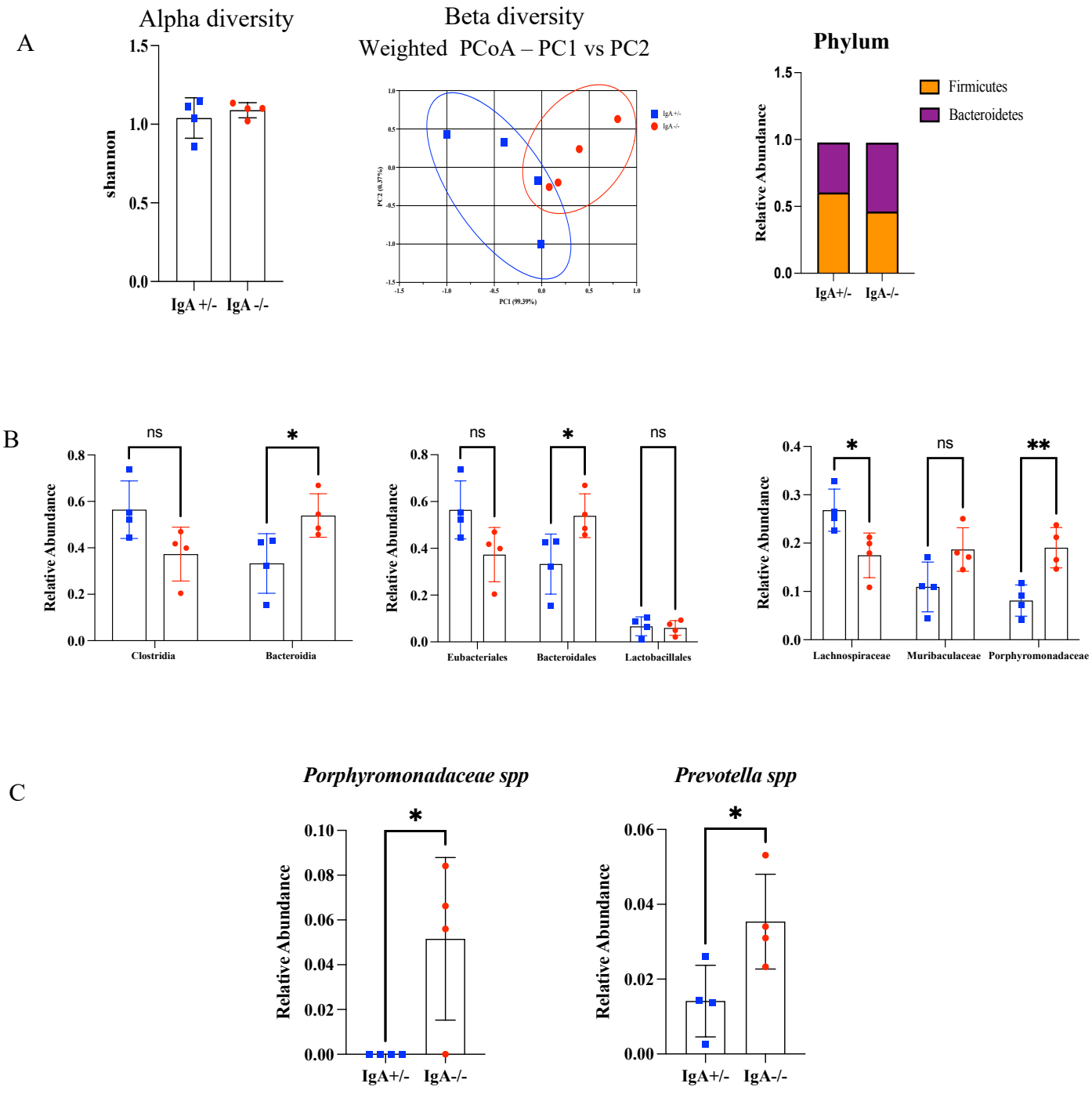


Figure 22. Analysis of the fecal microbiome in IgA$^{+/-}$ and IgA$^{-/-}$ mice

(A) Alpha diversity analyses of fecal microbiota from IgA deficient mice and controls measured by Shannon Diversity indices. (B) Principal Coordinate Analysis plots of weighted UniFrac distances of microbial communities in IgA$^{-/-}$ and IgA$^{+/-}$ mice. Relative abundances of bacterial taxa (phylum, class, order, families, and species). Data shown in is from one experiment.; n= 4 mice/group. *P <.05.

6. Discussion

As the most abundant immunoglobulins at mucosal surfaces, IgA antibodies are well-positioned to act as the initial humoral immune sentinels for ingested and inhaled antigens. They have been extensively characterized with respect to their roles in defense against infections in the airway and gastrointestinal tract, maintenance of gut microbial homeostasis, and regulation of immune responses (Fagarasan and Honjo, 2003). Depending on the involved effector cells and disease processes being studied, both pro- and anti-inflammatory properties have been attributed to IgA (Steffen et al., 2020). Although they are known to play key roles in immune responses at mucosal sites commonly involved in allergic diseases, the physiological functions of IgA in regulating mast cell biology in allergy have not been extensively studied. The projects carried out in this thesis provide valuable new information both about how IgA antibodies directly interact with mast cells and affect their IgE mediated activation (**Project 1**) and how IgA might modulate both immune homeostasis and the emergence of Type 2 immune responses (**Project 2**).

We took advantage of an array of experimental tools including murine cultured mast cells and primary peritoneal mast cells, human basophils, monoclonal hapten-specific IgA, and IgA purified from the sera of subjects that had undergone peanut OIT in **Project 1** to test the hypothesis that antigen-specific IgA antibodies inhibit FcεRI mediated mast cell and basophil activation. We found that IgA binds to mast cells in a manner dependent on calcium and sialic acid and inhibits IgE-induced mast cell degranulation. IgA exerts its function at a receptor-proximal point in the FcεRI-initiated protein tyrosine kinase (PTK) signaling cascade by decreasing phosphorylation of Syk. In addition to blocking degranulation and PTK signaling pathways, we found that IgA suppresses IgE-induced, cytokine production by mast cells. To assess the consequences of inhibitory signaling by IgA *in vivo*, we applied a passive systemic anaphylaxis model and observed inhibition of anaphylaxis, a finding that aligned nicely with a previously reported observation by Strait and colleagues (Strait et al., 2011). The relevance of these findings to humans was demonstrated in our confirmation of IgA's suppressive effects on the allergen-induced activation of basophils from peanut allergic subjects.

Our findings provide new insights into the regulatory roles IgA might play in allergic disease, most notably its ability to suppress IgE-induced mast cell activation and anaphylaxis. The possibility that IgE-mediated mast cell activation might be modulated by other factors has emerged as an important clinical question especially in food allergy where detection of food specific IgE antibodies does not consistently predict clinical reactivity (Keet et al., 2012). Our inference from this clinical observation has been that allergen suppressive factors, most likely antibodies of other isotypes, must exist. Much of the previous work done in our lab has focused on IgG antibodies but given their central function in intestinal immunobiology, we here considered IgA antibodies as likely candidates for regulatory effects.

Consistent with our observations that IgA can inhibit IgE-induced activation of murine bone marrow-derived mast cells (BMMC), Strait and colleagues previously reported that IgA antibodies can suppress IgE-mediated anaphylaxis in mice (Strait et al., 2011). They showed that the suppressive effect of IgA is observed even in knockout mice lacking the murine IgA receptor, Fcα/μR and conclude that IgA likely confers protection by blocking TNP epitopes on absorbed food allergens rather than by a receptor-mediated inhibitory mechanism. Our observations, however, suggest and alternative mechanism, namely that IgA mediated inhibition of mast cell activation is receptor mediated. Specifically, we demonstrate IgA binding to murine mast cells and neutrophils but not lymphocytes, indicating cellular specificity of IgA interaction suggestive of receptor binding. We further observe that IgA association with mast cells is calcium-dependent and that both binding and suppressive function of IgA require the presence of sialic acid. These properties are consistent with a receptor-mediated interaction leading us to consider the possibility that a member of the C-type lectin family of receptors might be exerting the inhibitory effect. However, in a screening survey using BMMCs from mice with targeted deletions of some of the members of this family known to be expressed on mast cells (including SIGN-R1, CD33, and Siglec F), IgA mediated inhibition of IgE activation or IgA binding to BMMCs remained intact (Supplementary Figure 6). Based on reports that IgA might signal via FcγR2b, with Galectin-3 as a binding intermediate (Reljic et al., 2004), we additionally

tested possible suppressive effects of suppressive effects on BMMCs from mice with a targeted deletion of FcγR2b, and observed that suppression remained intact (Supplementary Figure 6B). Thus, while our results strongly implicate a receptor-mediated inhibitory effect of antigen-specific IgA on mast cells, further studies will be required to identify the relevant receptor mediating this effect.

Our finding that IgA in the serum of subjects completing peanut OIT can suppress peanut-induced activation of basophils from allergic subjects, both extends our observation of IgA-mediated suppression of IgE responses to humans and points to a potential regulatory function of IgA in food allergy. Effects of IgA on food tolerance have previously been suggested by several clinical observations. Young children with allergies to cow's milk typically outgrow these allergies later in childhood. The appearance of IgA antibodies specific for cow's milk has been noted to trend in parallel with this resolution of allergy (Savilahti and Savilahti, 2013). Active induction of food unresponsiveness by OIT is also correlated with the induction of IgA responses. We reported marked increases in allergen-specific IgA in a trial of OIT for peanut, an observation subsequently extended by others to both cow's milk and egg OIT (Burton et al., 2014a; Palosuo et al., 2021; Savilahti et al., 2014; Sugimoto et al., 2016; Wright et al., 2016). Murine models have also documented specific IgA responses following food allergen ingestion and OIT (Frossard et al., 2004; Maeta et al., 2018; Wagenaar et al., 2019; Zhang et al., 2020).

In attempts to extend our analysis of IgA effects on allergy from immediate hypersensitivity to characterization of IgA effects on immunological homeostasis and emerging Type 2 immune responses, we focused our studies in Project 2 on comparing the immunological phenotypes of IgA-deficient mice with their IgA-sufficient littermates. In addition to shedding light on these issues, these studies proved fruitful in gaining insights on the role of IgA in controlling commensalism, gastrointestinal T helper cell biology and B cell development. While IgA deficiency is the most common immunodeficiency, mechanisms underlying increased risk of atopy is not well understood. In the second part of our study, we found that our 6-8 month old CRISPR IgA deficient mice present with elevated levels of most immunoglobulin isotypes (IgM, IgG1, IgG2a, and IgG2b). The

success of these studies was made possible by extensive characterization of existing $IgA^{-/-}$ mice previously used by other investigators, work that demonstrated that, unrelated to their IgA deficiency, these animals had a B cell intrinsic genetic inability to produce IgE antibodies. This led to our generation (by CRISPR) of two new IgA-deficient lines, completely capable of making IgE antibodies. These new strains were instrumental to our work and will also be a key resource for investigators studying IgA in the future.

A striking phenotype of $IgA^{-/-}$ mice is hyperimmunoglobulinemia. This led us to believe that IgA deficient mice might have dysregulated Tfh cell responses. Tfh cells are CD4+ cells that provide B cell help in germinal centers. They are identified as $PD1^{hi}CXCR5^{hi}BCL6^{+}$ and play a critical role in driving antibody responses, including the production and regulation of IgE. IL-13 producing Tfh cells have been identified in patients with allergic disease (Gowthaman et al., 2019) We found that Tfh numbers were indeed increased in the Peyer's patches of $IgA^{-/-}$ mice (but not spleen or mLN), implicating a heightened level of B cell help in the gut but not systemically.

Both hypergammaglobulinemia and Tfh expansion are phenotypes shared by previously generated $IgA^{-/-}$ mice. In contrast to our observation of increased Tfh in Peyer's patches but not spleen or mLN, Conrey *et al.* found elevated Tfh levels in the mLNs, and spleen and did not examine Peyer's patches. We speculate that the more extensive, systemic Tfh expansion in these animals could possibly relate to a more immunostimulatory environment in their mouse colony., as mice raised in different institutions have differences in microbial composition due to differences in genotype, diet, cage-type, temperature and bedding (Eberl et al., 2019). Alternatively, it is possible that the dysgammaglobulinemia in these animals related to the retained PGK-neo^{r} cassette in the Igha locus might contribute to an altered microbiome and thereby indirectly modulate Tfh induction.

Our observation of increased Peyer's patch Tfh cells promoted us to also enumerate Tfr cells and other Th subsets. T follicular regulatory cells (Tfr), are a subgroup of Tregs that are $PD1^{hi}CXCR5^{hi}Foxp3^{+}$ that regulate germinal center responses (Tan et al., 2022). Lack of Tfr cells has been associated with dysregulated Tfh cell numbers, and the generation of

both high affinity and low affinity B cells (Baumjohann et al., 2013; Vinuesa et al., 2013). Tfr cells have also been implicated in suppressing IgE-mediated allergies and are reduced in frequency in the tonsils of allergic rhinitis patients (Gonzalez-Figueroa et al., 2021; Schulten et al., 2018). We observed a reduction in Tfr in Peyer's patches. Th2 and Th17 Th subsets as well as Treg were also reduced in the Peyer's patches. T helper subsets were not altered in the mLN or spleen, a finding that was also replicated by Conrey et al. CD4+CD3+Tbet+ (Th1) cells were not altered in any of the compartments.

It is worth noting that a line of CRISPR IgA deficient mice previously generated by Nagashi et al. did not exhibit altered levels of any of the immunoglobulin isotypes in the serum (Nagaishi et al., 2022). We have observed this phenotype to be less evident in younger mice (not shown) and believe this might be because of their analysis of young mice (6 weeks old, versus our evaluation of 6-8 month old mice). Nagashi and colleagues also did not observe increased Tfh numbers in the spleen, Peyer's patches or mLN in their IgA deficient mice, another indicator that Tfh cell responses may track with hyperimmunoglobulinemia and not yet be fully evident at 6 weeks of age. They did however report increases in B cell percentages in the mLN of IgA deficient mice, similar to our own findings. It is important to note that this group did not use littermate controls, a limitation of the study that makes it harder to delineate differences caused by the intestinal microbiome (Nagaishi et al., 2022).

A unique aspect of our study on $IgA^{-/-}$ mice is the demonstration of IgA effects on the development of IgE. We observed, that in addition to increases in IgG, and IgM, IgA deficient mice also exhibit elevated IgE levels, a phenomenon that increases with age. This had not been seen in $IgA^{-/-}$ mice made by Mbawuike et al, which we believe to be due to interference in the germline transcription of the Ighe locus in the presence of a retained transcriptionally active PGK-neo^r cassette in the adjacent Igha locus (Mbawuike et al., 1999; Seidl et al., 1999). Elevated IgE has been reported in many immunodeficiencies including hyper-IgE syndrome, Omenn syndrome, immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX), Wiskott-Aldrich syndrome (WAS), and both Myd88 and DOCK8 deficiency (Ozcan et al., 2008). Studies involving human samples

or mouse models reveal that hyper IgE can result from disturbances in several immunological pathways. Many of these immunodeficiencies display defective functionality or frequency of Treg cells, highlighting the importance of Treg's in controlling aberrant immune responses.

Alterations in the microbiome are also a common characteristic of the aforementioned primary immunodeficiencies and are well known to affect IgE responses. Germ free mice exhibit elevated levels of total IgE and conferring increased gut microbial diversity early in life can completely restore the "hygiene induced" high IgE levels seen in germ free animals (Cahenzli et al., 2013). Using gnotobiotic mouse colonies and gut reconstitution with defined bacterial consortia, Cahenzli *et al.*, later demonstrated the ability of three specific bacterial species (*A. muciniphila* or *E. faecalis, B. coccoides*) to suppress IgE production. In contrast to their suppressive effects on IgE, both *A. muciniphila* and *E. faecalis* enhanced IgA induction and induced increases in Treg frequencies and IL-10 responses (Wyss et al., 2019).

Dysbiosis in IgA deficiency has been examined in both humans and mice, with mixed findings. We found increased abundance of the phyla *Bacteroidetes*, and a decrease in *Firmicutes* in the fecal microbiota of our CRISPR $IgA^{-/-}$ mice. There were several other differences in multiple taxa (class, order, and family), with a clear decrease in bacteria belonging to the *Firmicutes* phylum, particularly from the *Lachnospiraceae* family. There was also an overrepresentation of proinflammatory *Porphyromonadaceae* and *Prevotella* species in $IgA^{-/-}$ mice. Many other studies have highlighted similar findings in IgA deficient mice, with Chang and colleagues reporting decreased relative abundance of *Firmicutes*, and a trend towards increased *Bacteroidetes* in the saliva of 90's $IgA^{-/-}$ mice. A decrease in *Streptococcus*, and increased frequencies of *Aggregatibacer*, *Actinobacillus*, and *Prevotella* was also observed in 90's $IgA^{-/-}$ mice compared to WT mice (Chang et al., 2021). These results were very consistent with our own.

Reports of microbiome signatures in IgA deficient humans are even more varied, with studies noting enrichment in several different species. Moll et al., reported an enrichment of

Erysipelatoclostridium ramosum compared to IgA^{+} household members (Moll et al., 2021). *Escherichia coli,* which is associated with intestinal dysbiosis, and inflammatory disease is also enriched by 26.5-fold in IgA deficient patients (Friman et al., 2002; Moll et al., 2021). Fadlallah et al., found mild dysbiosis in IgA deficient patients, with a depletion of the *Firmicutes* phylum in IgA deficiency, half of which belong to the *Lachnospiraceae* family (consistent with our own findings in $IgA^{-/-}$ mice). Fadlallah and colleagues, also show, that some overrepresented bacteria in IgA deficient patients also comes from the *Firmicutes* phylum, in addition to *Bacteroidetes*. They also demonstrate enrichment of some species of *Prevotella*, also mirroring our own findings in $IgA^{-/-}$ mice, and work done by Chang et al. (Chang et al., 2021; Fadlallah et al., 2018). Taken together, these observations indicate that while alterations in the microbiome in IgA deficiency can vary, many of the same microbes are consistently flagged as enriched or depleted, in both humans and mice, highlighting their importance in eliciting dysregulated antibody responses, or contributing to allergic pathogenesis. Studies involving fecal microbiome transplants in germ free mice will help determine whether the microbiome in IgA deficient mice is the cause of aberrant antibody responses specifically IgE.

Defining the mechanisms contributing to tolerance of ingested antigens is of critical importance in the field of food allergy. Our findings in Projects 1 and 2 provide important new insights in this regard, demonstrating that IgA antibodies, which are known to be physiologically induced after food ingestion and OIT, can block IgE-mediated activation of mast cells and basophils and consequent hypersensitivity reactions, including anaphylaxis. Furthermore, the generation of IgA deficient mice with an intact IgE locus has allowed us to study the effects of IgA deficiency on immunological homeostasis, and systemic antibody responses in the context of allergic disease. We propose that IgA-mediated maintenance of the gut microbiome, is critical in regulating adaptive immune responses, particularly the induction of IgE antibodies. It will be of great interest, going forward, to further delineate the mechanisms mediating this effect, and to consider whether the induction of IgA responses or passive administration of allergen-specific IgA might be utilized as therapies.

7. References

Abdel-Gadir, A., Stephen-Victor, E., Gerber, G.K., Noval Rivas, M., Wang, S., Harb, H., Wang, L., Li, N., Crestani, E., Spielman, S.*, et al.* (2019). Microbiota therapy acts via a regulatory T cell MyD88/RORgammat pathway to suppress food allergy. Nat Med *25*, 1164-1174.

Aghamohammadi, A., Cheraghi, T., Gharagozlou, M., Movahedi, M., Rezaei, N., Yeganeh, M., Parvaneh, N., Abolhassani, H., Pourpak, Z., and Moin, M. (2009). IgA deficiency: correlation between clinical and immunological phenotypes. J Clin Immunol *29*, 130-136.

Akiyama, M. (2010). FLG mutations in ichthyosis vulgaris and atopic eczema: spectrum of mutations and population genetics. Br J Dermatol *162*, 472-477.

Aranda, C.J., Gonzalez-Kozlova, E., Saunders, S.P., Fernandes-Braga, W., Ota, M., Narayanan, S., He, J.S., Del Duca, E., Swaroop, B., Gnjatic, S.*, et al.* (2023). IgG memory B cells expressing IL4R and FCER2 are associated with atopic diseases. Allergy *78*, 752-766.

Arnaboldi, P.M., Behr, M.J., and Metzger, D.W. (2005). Mucosal B cell deficiency in IgA-/- mice abrogates the development of allergic lung inflammation. J Immunol *175*, 1276-1285.

Asrat, S., Kaur, N., Liu, X., Ben, L.H., Kajimura, D., Murphy, A.J., Sleeman, M.A., Limnander, A., and Orengo, J.M. (2020). Chronic allergen exposure drives accumulation of long-lived IgE plasma cells in the bone marrow, giving rise to serological memory. Sci Immunol *5*.

Atarashi, K., Tanoue, T., Oshima, K., Suda, W., Nagano, Y., Nishikawa, H., Fukuda, S., Saito, T., Narushima, S., Hase, K.*, et al.* (2013). Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature *500*, 232-236.

Atarashi, K., Tanoue, T., Shima, T., Imaoka, A., Kuwahara, T., Momose, Y., Cheng, G., Yamasaki, S., Saito, T., Ohba, Y.*, et al.* (2011). Induction of colonic regulatory T cells by indigenous Clostridium species. Science *331*, 337-341.

Baiardini, I., Braido, F., Brandi, S., and Canonica, G.W. (2006). Allergic diseases and their impact on quality of life. Ann Allergy Asthma Immunol *97*, 419-428; quiz 429-430, 476.

Baumjohann, D., Preite, S., Reboldi, A., Ronchi, F., Ansel, K.M., Lanzavecchia, A., and Sallusto, F. (2013). Persistent antigen and germinal center B cells sustain T follicular helper cell responses and phenotype. Immunity *38*, 596-605.

Beller, A., Kruglov, A., Durek, P., von Goetze, V., Werner, K., Heinz, G.A., Ninnemann, J., Lehmann, K., Maier, R., Hoffmann, U.*, et al.* (2020). Specific microbiota enhances intestinal IgA levels by inducing TGF-beta in T follicular helper cells of Peyer's patches in mice. Eur J Immunol *50*, 783-794.

Bennich, H.H., Ishizaka, K., Johansson, S.G., Rowe, D.S., Stanworth, D.R., and Terry, W.D. (1968). Immunoglobulin E: a new class of human immunoglobulin. Immunology *15*, 323-324.

Benveniste, J., Lespinats, G., and Salomon, J. (1971). Serum and secretory IgA in axenic and holoxenic mice. J Immunol *107*, 1656-1662.

Brand, S., Teich, R., Dicke, T., Harb, H., Yildirim, A.O., Tost, J., Schneider-Stock, R., Waterland, R.A., Bauer, U.M., von Mutius, E.*, et al.* (2011). Epigenetic regulation in

murine offspring as a novel mechanism for transmaternal asthma protection induced by microbes. J Allergy Clin Immunol *128*, 618-625 e611-617.
Brown, S.J., Asai, Y., Cordell, H.J., Campbell, L.E., Zhao, Y., Liao, H., Northstone, K., Henderson, J., Alizadehfar, R., Ben-Shoshan, M.*, et al.* (2011). Loss-of-function variants in the filaggrin gene are a significant risk factor for peanut allergy. J Allergy Clin Immunol *127*, 661-667.
Bruhns, P., Fremont, S., and Daeron, M. (2005). Regulation of allergy by Fc receptors. Curr Opin Immunol *17*, 662-669.
Brunkow, M.E., Jeffery, E.W., Hjerrild, K.A., Paeper, B., Clark, L.B., Yasayko, S.A., Wilkinson, J.E., Galas, D., Ziegler, S.F., and Ramsdell, F. (2001). Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat Genet *27*, 68-73.
Bryce, P.J., Miller, M.L., Miyajima, I., Tsai, M., Galli, S.J., and Oettgen, H.C. (2005). Immune sensitization in the skin is enhanced by antigen-independent effects of IgE on mast cells. Novartis Found Symp *271*, 15-24; discussion 24-38, 95-19.
Burton, O.T., Logsdon, S.L., Zhou, J.S., Medina-Tamayo, J., Abdel-Gadir, A., Noval Rivas, M., Koleoglou, K.J., Chatila, T.A., Schneider, L.C., Rachid, R.*, et al.* (2014a). Oral immunotherapy induces IgG antibodies that act through FcgammaRIIb to suppress IgE-mediated hypersensitivity. J Allergy Clin Immunol *134*, 1310-1317 e1316.
Burton, O.T., Medina Tamayo, J., Stranks, A.J., Miller, S., Koleoglou, K.J., Weinberg, E.O., and Oettgen, H.C. (2018a). IgE promotes type 2 innate lymphoid cells in murine food allergy. Clin Exp Allergy *48*, 288-296.
Burton, O.T., Noval Rivas, M., Zhou, J.S., Logsdon, S.L., Darling, A.R., Koleoglou, K.J., Roers, A., Houshyar, H., Crackower, M.A., Chatila, T.A.*, et al.* (2014b). Immunoglobulin E signal inhibition during allergen ingestion leads to reversal of established food allergy and induction of regulatory T cells. Immunity *41*, 141-151.
Burton, O.T., Tamayo, J.M., Stranks, A.J., Koleoglou, K.J., and Oettgen, H.C. (2018b). Allergen-specific IgG antibody signaling through FcgammaRIIb promotes food tolerance. J Allergy Clin Immunol *141*, 189-201 e183.
Cahenzli, J., Koller, Y., Wyss, M., Geuking, M.B., and McCoy, K.D. (2013). Intestinal microbial diversity during early-life colonization shapes long-term IgE levels. Cell Host Microbe *14*, 559-570.
Cameron, L., Gounni, A.S., Frenkiel, S., Lavigne, F., Vercelli, D., and Hamid, Q. (2003). S epsilon S mu and S epsilon S gamma switch circles in human nasal mucosa following ex vivo allergen challenge: evidence for direct as well as sequential class switch recombination. J Immunol *171*, 3816-3822.
Cassani, B., Villablanca, E.J., Quintana, F.J., Love, P.E., Lacy-Hulbert, A., Blaner, W.S., Sparwasser, T., Snapper, S.B., Weiner, H.L., and Mora, J.R. (2011). Gut-tropic T cells that express integrin alpha4beta7 and CCR9 are required for induction of oral immune tolerance in mice. Gastroenterology *141*, 2109-2118.
Catanzaro, J.R., Strauss, J.D., Bielecka, A., Porto, A.F., Lobo, F.M., Urban, A., Schofield, W.B., and Palm, N.W. (2019). IgA-deficient humans exhibit gut microbiota dysbiosis despite secretion of compensatory IgM. Sci Rep *9*, 13574.
Caubet, J.C., Bencharitiwong, R., Moshier, E., Godbold, J.H., Sampson, H.A., and Nowak-Wegrzyn, A. (2012). Significance of ovomucoid- and ovalbumin-specific IgE/IgG(4) ratios in egg allergy. J Allergy Clin Immunol *129*, 739-747.

Cazac, B.B., and Roes, J. (2000). TGF-beta receptor controls B cell responsiveness and induction of IgA in vivo. Immunity *13*, 443-451.
Cerutti, A., and Rescigno, M. (2008). The biology of intestinal immunoglobulin A responses. Immunity *28*, 740-750.
Cerutti, A., Zan, H., Schaffer, A., Bergsagel, L., Harindranath, N., Max, E.E., and Casali, P. (1998). CD40 ligand and appropriate cytokines induce switching to IgG, IgA, and IgE and coordinated germinal center and plasmacytoid phenotypic differentiation in a human monoclonal IgM+IgD+ B cell line. J Immunol *160*, 2145-2157.
Chang, E., Kobayashi, R., Fujihashi, K., Komiya, M., and Kurita-Ochiai, T. (2021). Impaired salivary SIgA antibodies elicit oral dysbiosis and subsequent induction of alveolar bone loss. Inflamm Res *70*, 151-158.
Chang, J.C., Kuo, H.C., Hsu, T.Y., Ou, C.Y., Liu, C.A., Chuang, H., Liang, H.M., Huang, H.W., and Yang, K.D. (2013). Different genetic associations of the IgE production among fetus, infancy and childhood. PLoS One *8*, e70362.
Chen, K., Xu, W., Wilson, M., He, B., Miller, N.W., Bengten, E., Edholm, E.S., Santini, P.A., Rath, P., Chiu, A.*, et al.* (2009). Immunoglobulin D enhances immune surveillance by activating antimicrobial, proinflammatory and B cell-stimulating programs in basophils. Nat Immunol *10*, 889-898.
Cinicola, B.L., Pulvirenti, F., Capponi, M., Bonetti, M., Brindisi, G., Gori, A., De Castro, G., Anania, C., Duse, M., and Zicari, A.M. (2022). Selective IgA Deficiency and Allergy: A Fresh Look to an Old Story. Medicina (Kaunas) *58*.
Clement, R.L., Daccache, J., Mohammed, M.T., Diallo, A., Blazar, B.R., Kuchroo, V.K., Lovitch, S.B., Sharpe, A.H., and Sage, P.T. (2019). Follicular regulatory T cells control humoral and allergic immunity by restraining early B cell responses. Nat Immunol *20*, 1360-1371.
Cong, Y., Feng, T., Fujihashi, K., Schoeb, T.R., and Elson, C.O. (2009). A dominant, coordinated T regulatory cell-IgA response to the intestinal microbiota. Proc Natl Acad Sci U S A *106*, 19256-19261.
Conrad, M.L., Ferstl, R., Teich, R., Brand, S., Blumer, N., Yildirim, A.O., Patrascan, C.C., Hanuszkiewicz, A., Akira, S., Wagner, H.*, et al.* (2009). Maternal TLR signaling is required for prenatal asthma protection by the nonpathogenic microbe Acinetobacter lwoffii F78. J Exp Med *206*, 2869-2877.
Craig, S.W., and Cebra, J.J. (1971). Peyer's patches: an enriched source of precursors for IgA-producing immunocytes in the rabbit. J Exp Med *134*, 188-200.
de Sousa-Pereira, P., and Woof, J.M. (2019). IgA: Structure, Function, and Developability. Antibodies (Basel) *8*.
Debarry, J., Garn, H., Hanuszkiewicz, A., Dickgreber, N., Blumer, N., von Mutius, E., Bufe, A., Gatermann, S., Renz, H., Holst, O.*, et al.* (2007). Acinetobacter lwoffii and Lactococcus lactis strains isolated from farm cowsheds possess strong allergy-protective properties. J Allergy Clin Immunol *119*, 1514-1521.
Dolence, J.J., Kobayashi, T., Iijima, K., Krempski, J., Drake, L.Y., Dent, A.L., and Kita, H. (2018). Airway exposure initiates peanut allergy by involving the IL-1 pathway and T follicular helper cells in mice. J Allergy Clin Immunol *142*, 1144-1158 e1148.
EAACI (2015). The European Academy of Allergy and Clinical Immunology (EAACI) Advocacy Manifesto Tackling the Allergy Crisis in Europe—Concerted Policy Action Needed. .

Eberl, C., Ring, D., Munch, P.C., Beutler, M., Basic, M., Slack, E.C., Schwarzer, M., Srutkova, D., Lange, A., Frick, J.S.*, et al.* (2019). Reproducible Colonization of Germ-Free Mice With the Oligo-Mouse-Microbiota in Different Animal Facilities. Front Microbiol *10*, 2999.
Eberl, G., and Littman, D.R. (2004). Thymic origin of intestinal alphabeta T cells revealed by fate mapping of RORgammat+ cells. Science *305*, 248-251.
El Ansari, Y.S., Kanagaratham, C., Lewis, O.L., and Oettgen, H.C. (2020). IgE and mast cells: The endogenous adjuvant. Adv Immunol *148*, 93-153.
Elesela, S., Arzola-Martinez, L., Rasky, A., Ptaschinski, C., Hogan, S.P., and Lukacs, N.W. (2023). Mucosal IgA immune complex induces immunomodulatory responses in allergic airway and intestinal Th2 disease. J Allergy Clin Immunol.
Erazo, A., Kutchukhidze, N., Leung, M., Christ, A.P., Urban, J.F., Jr., Curotto de Lafaille, M.A., and Lafaille, J.J. (2007). Unique maturation program of the IgE response in vivo. Immunity *26*, 191-203.
Erkocoglu, M., Metin, A., Kaya, A., Ozcan, C., Akan, A., Civelek, E., Capanoglu, M., Ginis, T., and Kocabas, C.N. (2017). Allergic and autoimmune disorders in families with selective IgA deficiency. Turk J Med Sci *47*, 592-598.
Esterhazy, D., Loschko, J., London, M., Jove, V., Oliveira, T.Y., and Mucida, D. (2016). Classical dendritic cells are required for dietary antigen-mediated induction of peripheral T(reg) cells and tolerance. Nat Immunol *17*, 545-555.
Fadlallah, J., El Kafsi, H., Sterlin, D., Juste, C., Parizot, C., Dorgham, K., Autaa, G., Gouas, D., Almeida, M., Lepage, P.*, et al.* (2018). Microbial ecology perturbation in human IgA deficiency. Sci Transl Med *10*.
Fagarasan, S., and Honjo, T. (2003). Intestinal IgA synthesis: regulation of front-line body defences. Nat Rev Immunol *3*, 63-72.
Fagarasan, S., Kinoshita, K., Muramatsu, M., Ikuta, K., and Honjo, T. (2001). In situ class switching and differentiation to IgA-producing cells in the gut lamina propria. Nature *413*, 639-643.
Fageras, M., Tomicic, S., Voor, T., Bjorksten, B., and Jenmalm, M.C. (2011). Slow salivary secretory IgA maturation may relate to low microbial pressure and allergic symptoms in sensitized children. Pediatr Res *70*, 572-577.
Francis, J.N., James, L.K., Paraskevopoulos, G., Wong, C., Calderon, M.A., Durham, S.R., and Till, S.J. (2008). Grass pollen immunotherapy: IL-10 induction and suppression of late responses precedes IgG4 inhibitory antibody activity. J Allergy Clin Immunol *121*, 1120-1125 e1122.
Friman, V., Nowrouzian, F., Adlerberth, I., and Wold, A.E. (2002). Increased frequency of intestinal Escherichia coli carrying genes for S fimbriae and haemolysin in IgA-deficient individuals. Microb Pathog *32*, 35-42.
Frossard, C.P., Hauser, C., and Eigenmann, P.A. (2004). Antigen-specific secretory IgA antibodies in the gut are decreased in a mouse model of food allergy. J Allergy Clin Immunol *114*, 377-382.
Fu, W., Liu, X., Lin, X., Feng, H., Sun, L., Li, S., Chen, H., Tang, H., Lu, L., Jin, W.*, et al.* (2018). Deficiency in T follicular regulatory cells promotes autoimmunity. J Exp Med *215*, 815-825.
Galli, S.J., and Tsai, M. (2010). Mast cells in allergy and infection: versatile effector and regulatory cells in innate and adaptive immunity. Eur J Immunol *40*, 1843-1851.

Ghably, J., Saleh, H., Vyas, H., Peiris, E., Misra, N., and Krishnaswamy, G. (2015). Paul Ehrlich's mastzellen: a historical perspective of relevant developments in mast cell biology. Methods Mol Biol *1220*, 3-10.
Gimpel, A.K., Maccataio, A., Unterweger, H., Sokolova, M.V., Schett, G., and Steffen, U. (2021). IgA Complexes Induce Neutrophil Extracellular Trap Formation More Potently Than IgG Complexes. Front Immunol *12*, 761816.
Gonzalez-Figueroa, P., Roco, J.A., Papa, I., Nunez Villacis, L., Stanley, M., Linterman, M.A., Dent, A., Canete, P.F., and Vinuesa, C.G. (2021). Follicular regulatory T cells produce neuritin to regulate B cells. Cell *184*, 1775-1789 e1719.
Gowthaman, U., Chen, J.S., Zhang, B., Flynn, W.F., Lu, Y., Song, W., Joseph, J., Gertie, J.A., Xu, L., Collet, M.A.*, et al.* (2019). Identification of a T follicular helper cell subset that drives anaphylactic IgE. Science *365*.
Gri, G., Piconese, S., Frossi, B., Manfroi, V., Merluzzi, S., Tripodo, C., Viola, A., Odom, S., Rivera, J., Colombo, M.P.*, et al.* (2008). CD4+CD25+ regulatory T cells suppress mast cell degranulation and allergic responses through OX40-OX40L interaction. Immunity *29*, 771-781.
Gualdi, G., Lougaris, V., Baronio, M., Vitali, M., Tampella, G., Moratto, D., Tanghetti, P., Monari, P., Calzavara-Pinton, P., and Plebani, A. (2015). Burden of Skin Disease in Selective IgA Deficiency and Common Variable Immunodeficiency. J Investig Allergol Clin Immunol *25*, 369-371.
Hachem, C.E., Marschall, P., Hener, P., Karnam, A., Bonam, S.R., Meyer, P., Flatter, E., Birling, M.C., Bayry, J., and Li, M. (2023). IL-3 produced by T cells is crucial for basophil extravasation in hapten-induced allergic contact dermatitis. Front Immunol *14*, 1151468.
Hadis, U., Wahl, B., Schulz, O., Hardtke-Wolenski, M., Schippers, A., Wagner, N., Muller, W., Sparwasser, T., Forster, R., and Pabst, O. (2011). Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. Immunity *34*, 237-246.
Hagner, S., Harb, H., Zhao, M., Stein, K., Holst, O., Ege, M.J., Mayer, M., Matthes, J., Bauer, J., von Mutius, E.*, et al.* (2013). Farm-derived Gram-positive bacterium Staphylococcus sciuri W620 prevents asthma phenotype in HDM- and OVA-exposed mice. Allergy *68*, 322-329.
Hoh, R.A., Joshi, S.A., Lee, J.Y., Martin, B.A., Varma, S., Kwok, S., Nielsen, S.C.A., Nejad, P., Haraguchi, E., Dixit, P.S.*, et al.* (2020). Origins and clonal convergence of gastrointestinal IgE(+) B cells in human peanut allergy. Sci Immunol *5*.
Hong, X., Hao, K., Ladd-Acosta, C., Hansen, K.D., Tsai, H.J., Liu, X., Xu, X., Thornton, T.A., Caruso, D., Keet, C.A.*, et al.* (2015). Genome-wide association study identifies peanut allergy-specific loci and evidence of epigenetic mediation in US children. Nat Commun *6*, 6304.
Hoskinson, C., Dai, D.L.Y., Del Bel, K.L., Becker, A.B., Moraes, T.J., Mandhane, P.J., Finlay, B.B., Simons, E., Kozyrskyj, A.L., Azad, M.B.*, et al.* (2023). Delayed gut microbiota maturation in the first year of life is a hallmark of pediatric allergic disease. Nat Commun *14*, 4785.
Hua, X., Goedert, J.J., Pu, A., Yu, G., and Shi, J. (2016). Allergy associations with the adult fecal microbiota: Analysis of the American Gut Project. EBioMedicine *3*, 172-179.
Huber, B. (2006). [100 years of allergy: Clemens von Pirquet - his idea of allergy and its immanent concept of disease]. Wien Klin Wochenschr *118*, 573-579.

Iikura, M., Yamaguchi, M., Fujisawa, T., Miyamasu, M., Takaishi, T., Morita, Y., Iwase, T., Moro, I., Yamamoto, K., and Hirai, K. (1998). Secretory IgA induces degranulation of IL-3-primed basophils. J Immunol *161*, 1510-1515.
Iype, J., and Fux, M. (2021). Basophils Orchestrating Eosinophils' Chemotaxis and Function in Allergic Inflammation. Cells *10*.
Jutel, M., Jaeger, L., Suck, R., Meyer, H., Fiebig, H., and Cromwell, O. (2005). Allergen-specific immunotherapy with recombinant grass pollen allergens. J Allergy Clin Immunol *116*, 608-613.
Kalb, B., Marenholz, I., Jeanrenaud, A., Meixner, L., Arnau-Soler, A., Rosillo-Salazar, O.D., Ghauri, A., Cibin, P., Blumchen, K., Schlags, R.*, et al.* (2022). Filaggrin loss-of-function mutations are associated with persistence of egg and milk allergy. J Allergy Clin Immunol *150*, 1125-1134.
Kang, H.S., Chin, R.K., Wang, Y., Yu, P., Wang, J., Newell, K.A., and Fu, Y.X. (2002). Signaling via LTbetaR on the lamina propria stromal cells of the gut is required for IgA production. Nat Immunol *3*, 576-582.
Karlsson, M.R., Rugtveit, J., and Brandtzaeg, P. (2004). Allergen-responsive CD4+CD25+ regulatory T cells in children who have outgrown cow's milk allergy. J Exp Med *199*, 1679-1688.
Keet, C.A., Wood, R.A., and Matsui, E.C. (2012). Limitations of reliance on specific IgE for epidemiologic surveillance of food allergy. J Allergy Clin Immunol *130*, 1207-1209 e1210.
Kobayashi, T., Iijima, K., Dent, A.L., and Kita, H. (2017). Follicular helper T cells mediate IgE antibody response to airborne allergens. J Allergy Clin Immunol *139*, 300-313 e307.
Krombach, J.W., Kampe, S., Keller, C.A., and Wright, P.M. (2004). Pharaoh Menes' death after an anaphylactic reaction--the end of a myth. Allergy *59*, 1234-1235.
Kukkonen, K., Kuitunen, M., Haahtela, T., Korpela, R., Poussa, T., and Savilahti, E. (2010). High intestinal IgA associates with reduced risk of IgE-associated allergic diseases. Pediatr Allergy Immunol *21*, 67-73.
Kwon, D.I., Park, E.S., Kim, M., Choi, Y.H., Lee, M.S., Joo, S.H., Kang, Y.W., Lee, M., Jo, S.B., Lee, S.W.*, et al.* (2022). Homeostatic serum IgE is secreted by plasma cells in the thymus and enhances mast cell survival. Nat Commun *13*, 1418.
Laitinen, T., Rasanen, M., Kaprio, J., Koskenvuo, M., and Laitinen, L.A. (1998). Importance of genetic factors in adolescent asthma: a population-based twin-family study. Am J Respir Crit Care Med *157*, 1073-1078.
Lamkhioued, B., Gounni, A.S., Gruart, V., Pierce, A., Capron, A., and Capron, M. (1995). Human eosinophils express a receptor for secretory component. Role in secretory IgA-dependent activation. Eur J Immunol *25*, 117-125.
Larsen, F.S., Holm, N.V., and Henningsen, K. (1986). Atopic dermatitis. A genetic-epidemiologic study in a population-based twin sample. J Am Acad Dermatol *15*, 487-494.
Latcham, F., Merino, F., Lang, A., Garvey, J., Thomson, M.A., Walker-Smith, J.A., Davies, S.E., Phillips, A.D., and Murch, S.H. (2003). A consistent pattern of minor immunodeficiency and subtle enteropathy in children with multiple food allergy. J Pediatr *143*, 39-47.
Ludviksson, B.R., Arason, G.J., Thorarensen, O., Ardal, B., and Valdimarsson, H. (2005). Allergic diseases and asthma in relation to serum immunoglobulins and salivary

immunoglobulin A in pre-school children: a follow-up community-based study. Clin Exp Allergy *35*, 64-69.
Ludviksson, B.R., Eiriksson, T.H., Ardal, B., Sigfusson, A., and Valdimarsson, H. (1992). Correlation between serum immunoglobulin A concentrations and allergic manifestations in infants. J Pediatr *121*, 23-27.
Macpherson, A.J., and Uhr, T. (2004). Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science *303*, 1662-1665.
Maeta, A., Katahira, R., Matsushima, M., Onishi, H., Nakamura, Y., and Takahashi, K. (2018). Stepwise oral immunotherapy for 10 days in an egg-white allergy mouse model did not ameliorate the severity of allergy but induced the production of allergen-specific IgA. Biosci Biotechnol Biochem *82*, 2176-2179.
Magen, E., Masalha, A., Waitman, D.A., Kahan, N., Viner, I., Klassov, L., and Vardy, D. (2017). Prevalence of dermatologic diseases among patients with selective immunoglobulin A deficiency. Allergy Asthma Proc *38*, 70-77.
Malbec, O., and Daeron, M. (2007). The mast cell IgG receptors and their roles in tissue inflammation. Immunol Rev *217*, 206-221.
Marenholz, I., Nickel, R., Ruschendorf, F., Schulz, F., Esparza-Gordillo, J., Kerscher, T., Gruber, C., Lau, S., Worm, M., Keil, T.*, et al.* (2006). Filaggrin loss-of-function mutations predispose to phenotypes involved in the atopic march. J Allergy Clin Immunol *118*, 866-871.
Mbawuike, I.N., Pacheco, S., Acuna, C.L., Switzer, K.C., Zhang, Y., and Harriman, G.R. (1999). Mucosal immunity to influenza without IgA: an IgA knockout mouse model. J Immunol *162*, 2530-2537.
McCoy, K.D., Harris, N.L., Diener, P., Hatak, S., Odermatt, B., Hangartner, L., Senn, B.M., Marsland, B.J., Geuking, M.B., Hengartner, H.*, et al.* (2006). Natural IgE production in the absence of MHC Class II cognate help. Immunity *24*, 329-339.
Mirpuri, J., Raetz, M., Sturge, C.R., Wilhelm, C.L., Benson, A., Savani, R.C., Hooper, L.V., and Yarovinsky, F. (2014). Proteobacteria-specific IgA regulates maturation of the intestinal microbiota. Gut Microbes *5*, 28-39.
Mkaddem, S.B., Christou, I., Rossato, E., Berthelot, L., Lehuen, A., and Monteiro, R.C. (2014). IgA, IgA receptors, and their anti-inflammatory properties. Curr Top Microbiol Immunol *382*, 221-235.
Moll, J.M., Myers, P.N., Zhang, C., Eriksen, C., Wolf, J., Appelberg, K.S., Lindberg, G., Bahl, M.I., Zhao, H., Pan-Hammarstrom, Q.*, et al.* (2021). Gut Microbiota Perturbation in IgA Deficiency Is Influenced by IgA-Autoantibody Status. Gastroenterology *160*, 2423-2434 e2425.
Morawska, I., Kurkowska, S., Bebnowska, D., Hrynkiewicz, R., Becht, R., Michalski, A., Piwowarska-Bilska, H., Birkenfeld, B., Zaluska-Ogryzek, K., Grywalska, E.*, et al.* (2021). The Epidemiology and Clinical Presentations of Atopic Diseases in Selective IgA Deficiency. J Clin Med *10*.
Moro, K., Yamada, T., Tanabe, M., Takeuchi, T., Ikawa, T., Kawamoto, H., Furusawa, J., Ohtani, M., Fujii, H., and Koyasu, S. (2010). Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. Nature *463*, 540-544.
Mosmann, T.R., and Coffman, R.L. (1987). Two types of mouse helper T-cell clone Implications for immune regulation. Immunol Today *8*, 223-227.

Murthy, A.K., Dubose, C.N., Banas, J.A., Coalson, J.J., and Arulanandam, B.P. (2006). Contribution of polymeric immunoglobulin receptor to regulation of intestinal inflammation in dextran sulfate sodium-induced colitis. J Gastroenterol Hepatol *21*, 1372-1380.
Nagaishi, T., Watabe, T., Kotake, K., Kumazawa, T., Aida, T., Tanaka, K., Ono, R., Ishino, F., Usami, T., Miura, T.*, et al.* (2022). Immunoglobulin A-specific deficiency induces spontaneous inflammation specifically in the ileum. Gut *71*, 487-496.
Nagashima, K., Sawa, S., Nitta, T., Tsutsumi, M., Okamura, T., Penninger, J.M., Nakashima, T., and Takayanagi, H. (2017). Identification of subepithelial mesenchymal cells that induce IgA and diversify gut microbiota. Nat Immunol *18*, 675-682.
Neill, D.R., Wong, S.H., Bellosi, A., Flynn, R.J., Daly, M., Langford, T.K., Bucks, C., Kane, C.M., Fallon, P.G., Pannell, R.*, et al.* (2010). Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. Nature *464*, 1367-1370.
Ng, A., and Boersma, P. (2023). Diagnosed Allergic Conditions in Adults: United States, 2021, U.S.D.o.H.a.H. Services, ed.
Nieves, D.S., Phipps, R.P., Pollock, S.J., Ochs, H.D., Zhu, Q., Scott, G.A., Ryan, C.K., Kobayashi, I., Rossi, T.M., and Goldsmith, L.A. (2004). Dermatologic and immunologic findings in the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome. Arch Dermatol *140*, 466-472.
Noon, L. (1953). Prophylactic inoculation against hay fever. Int Arch Allergy Appl Immunol *4*, 285-288.
Noval Rivas, M., Burton, O.T., Oettgen, H.C., and Chatila, T. (2016). IL-4 production by group 2 innate lymphoid cells promotes food allergy by blocking regulatory T-cell function. J Allergy Clin Immunol *138*, 801-811 e809.
Noval Rivas, M., Burton, O.T., Wise, P., Charbonnier, L.M., Georgiev, P., Oettgen, H.C., Rachid, R., and Chatila, T.A. (2015). Regulatory T cell reprogramming toward a Th2-cell-like lineage impairs oral tolerance and promotes food allergy. Immunity *42*, 512-523.
Nowak-Wegrzyn, A., Szajewska, H., and Lack, G. (2017). Food allergy and the gut. Nat Rev Gastroenterol Hepatol *14*, 241-257.
Obata, K., Mukai, K., Tsujimura, Y., Ishiwata, K., Kawano, Y., Minegishi, Y., Watanabe, N., and Karasuyama, H. (2007). Basophils are essential initiators of a novel type of chronic allergic inflammation. Blood *110*, 913-920.
Ober, C., and Yao, T.C. (2011). The genetics of asthma and allergic disease: a 21st century perspective. Immunol Rev *242*, 10-30.
Oefner, C.M., Winkler, A., Hess, C., Lorenz, A.K., Holecska, V., Huxdorf, M., Schommartz, T., Petzold, D., Bitterling, J., Schoen, A.L.*, et al.* (2012). Tolerance induction with T cell-dependent protein antigens induces regulatory sialylated IgGs. J Allergy Clin Immunol *129*, 1647-1655 e1613.
Oettgen, H.C. (2016). Fifty years later: Emerging functions of IgE antibodies in host defense, immune regulation, and allergic diseases. J Allergy Clin Immunol *137*, 1631-1645.
Osawa, R., Akiyama, M., and Shimizu, H. (2011). Filaggrin gene defects and the risk of developing allergic disorders. Allergol Int *60*, 1-9.
Ozcan, E., Notarangelo, L.D., and Geha, R.S. (2008). Primary immune deficiencies with aberrant IgE production. J Allergy Clin Immunol *122*, 1054-1062; quiz 1063-1054.
Palosuo, K., Karisola, P., Savinko, T., Fyhrquist, N., Alenius, H., and Makela, M.J. (2021). A Randomized, Open-Label Trial of Hen's Egg Oral Immunotherapy: Efficacy and

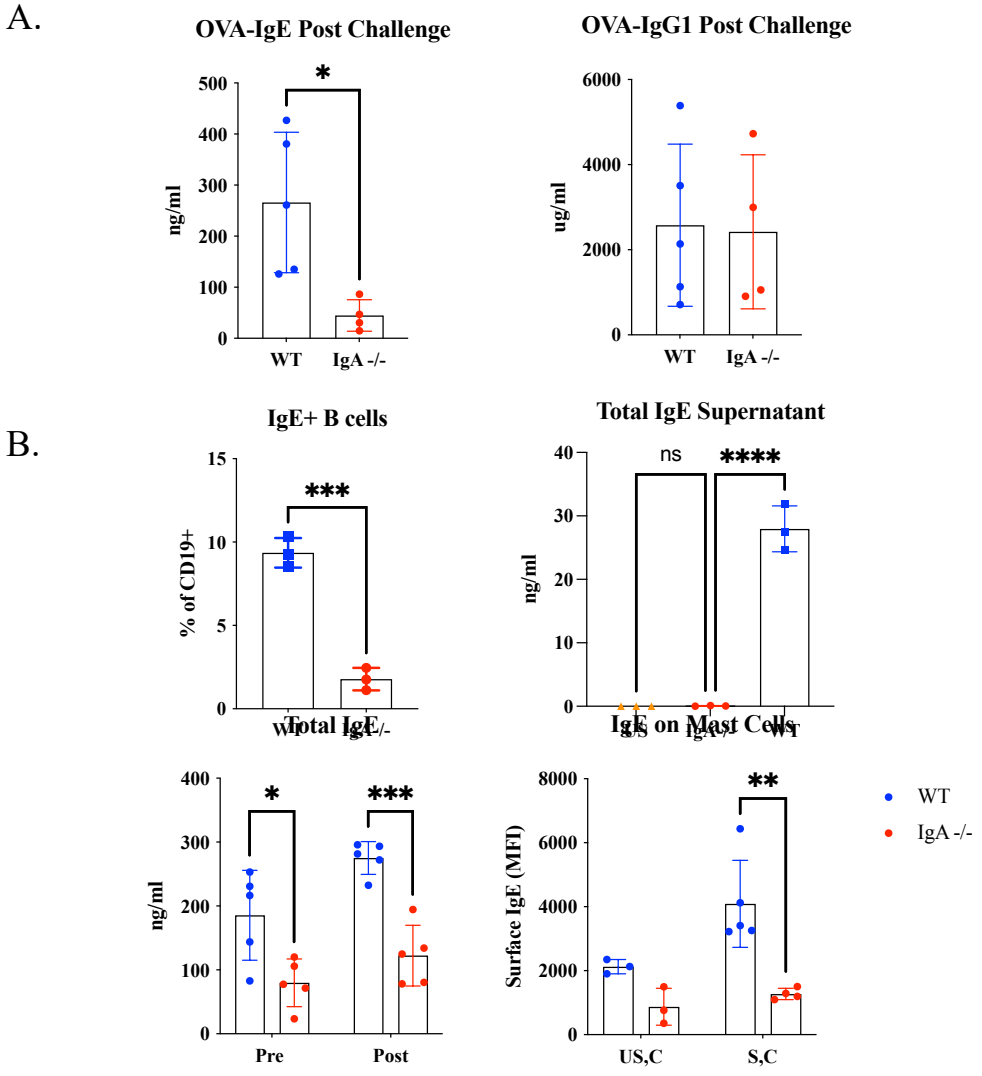
Humoral Immune Responses in 50 Children. J Allergy Clin Immunol Pract *9*, 1892-1901 e1891.
Perner, C., Flayer, C.H., Zhu, X., Aderhold, P.A., Dewan, Z.N.A., Voisin, T., Camire, R.B., Chow, O.A., Chiu, I.M., and Sokol, C.L. (2020). Substance P Release by Sensory Neurons Triggers Dendritic Cell Migration and Initiates the Type-2 Immune Response to Allergens. Immunity *53*, 1063-1077 e1067.
Pitlick, M.M., and Pongdee, T. (2022). Combining Biologics Targeting Eosinophils (IL-5/IL-5R), IgE, and IL-4/IL-13 in Allergic and Inflammatory Diseases. World Allergy Organ J *15*, 100707.
Poto, R., Gambardella, A.R., Marone, G., Schroeder, J.T., Mattei, F., Schiavoni, G., and Varricchi, G. (2022). Basophils from allergy to cancer. Front Immunol *13*, 1056838.
Prausnitz, C., and Kustner, H. (1921). Studien uber die ueberempfinclichkeit. Zentralbl Bakteriol *86*.
Qamar, N., Fishbein, A.B., Erickson, K.A., Cai, M., Szychlinski, C., Bryce, P.J., Schleimer, R.P., Fuleihan, R.L., and Singh, A.M. (2015). Naturally occurring tolerance acquisition to foods in previously allergic children is characterized by antigen specificity and associated with increased subsets of regulatory T cells. Clin Exp Allergy *45*, 1663-1672.
Ramachandran, M., and Aronson, J.K. (2011). John Bostock's first description of hayfever. J R Soc Med *104*, 237-240.
Rasanen, M., Laitinen, T., Kaprio, J., Koskenvuo, M., and Laitinen, L.A. (1998). Hay fever--a Finnish nationwide study of adolescent twins and their parents. Allergy *53*, 885-890.
Reikvam, D.H., Derrien, M., Islam, R., Erofeev, A., Grcic, V., Sandvik, A., Gaustad, P., Meza-Zepeda, L.A., Jahnsen, F.L., Smidt, H.*, et al.* (2012). Epithelial-microbial crosstalk in polymeric Ig receptor deficient mice. Eur J Immunol *42*, 2959-2970.
Reisinger, J., Horak, F., Pauli, G., van Hage, M., Cromwell, O., Konig, F., Valenta, R., and Niederberger, V. (2005). Allergen-specific nasal IgG antibodies induced by vaccination with genetically modified allergens are associated with reduced nasal allergen sensitivity. J Allergy Clin Immunol *116*, 347-354.
Reljic, R., Crawford, C., Challacombe, S., and Ivanyi, J. (2004). Mouse monoclonal IgA binds to the galectin-3/Mac-2 lectin from mouse macrophage cell lines. Immunol Lett *93*, 51-56.
Renz, H., and Skevaki, C. (2021). Early life microbial exposures and allergy risks: opportunities for prevention. Nat Rev Immunol *21*, 177-191.
Ruiter, B., Knol, E.F., van Neerven, R.J., Garssen, J., Bruijnzeel-Koomen, C.A., Knulst, A.C., and van Hoffen, E. (2007). Maintenance of tolerance to cow's milk in atopic individuals is characterized by high levels of specific immunoglobulin G4. Clin Exp Allergy *37*, 1103-1110.
Ryffel, B., Le Hir, M., Muller, M., and Eugster, H.P. (1998). Correction of the TNF-LT alpha-deficient phenotype by bone marrow transplantation. Dev Immunol *6*, 253-260.
Sandin, A., Bjorksten, B., Bottcher, M.F., Englund, E., Jenmalm, M.C., and Braback, L. (2011). High salivary secretory IgA antibody levels are associated with less late-onset wheezing in IgE-sensitized infants. Pediatr Allergy Immunol *22*, 477-481.
Santos, A.F., James, L.K., Kwok, M., McKendry, R.T., Anagnostou, K., Clark, A.T., and Lack, G. (2020). Peanut oral immunotherapy induces blocking antibodies but does not

change the functional characteristics of peanut-specific IgE. J Allergy Clin Immunol *145*, 440-443 e445.
Savilahti, E.M., Kuitunen, M., Savilahti, E., and Makela, M.J. (2014). Specific antibodies in oral immunotherapy for cow's milk allergy: kinetics and prediction of clinical outcome. Int Arch Allergy Immunol *164*, 32-39.
Savilahti, E.M., Rantanen, V., Lin, J.S., Karinen, S., Saarinen, K.M., Goldis, M., Makela, M.J., Hautaniemi, S., Savilahti, E., and Sampson, H.A. (2010). Early recovery from cow's milk allergy is associated with decreasing IgE and increasing IgG4 binding to cow's milk epitopes. J Allergy Clin Immunol *125*, 1315-1321 e1319.
Savilahti, E.M., and Savilahti, E. (2013). Development of natural tolerance and induced desensitization in cow's milk allergy. Pediatr Allergy Immunol *24*, 114-121.
Schulten, V., Tripple, V., Seumois, G., Qian, Y., Scheuermann, R.H., Fu, Z., Locci, M., Rosales, S., Vijayanand, P., Sette, A.*, et al.* (2018). Allergen-specific immunotherapy modulates the balance of circulating Tfh and Tfr cells. J Allergy Clin Immunol *141*, 775-777 e776.
Schwartz, C., Khan, A.R., Floudas, A., Saunders, S.P., Hams, E., Rodewald, H.R., McKenzie, A.N.J., and Fallon, P.G. (2017). ILC2s regulate adaptive Th2 cell functions via PD-L1 checkpoint control. J Exp Med *214*, 2507-2521.
Seidl, K.J., Manis, J.P., Bottaro, A., Zhang, J., Davidson, L., Kisselgof, A., Oettgen, H., and Alt, F.W. (1999). Position-dependent inhibition of class-switch recombination by PGK-neor cassettes inserted into the immunoglobulin heavy chain constant region locus. Proc Natl Acad Sci U S A *96*, 3000-3005.
Shahin, R.Y., Ali, F.H.A., Melek, N.A.N., Elateef, I.A.A., and Attia, M.Y. (2020). Study of selective immunoglobulin A deficiency among Egyptian patients with food allergy. Cent Eur J Immunol *45*, 184-188.
Shamji, M.H., Larson, D., Eifan, A., Scadding, G.W., Qin, T., Lawson, K., Sever, M.L., Macfarlane, E., Layhadi, J.A., Wurtzen, P.A.*, et al.* (2021). Differential induction of allergen-specific IgA responses following timothy grass subcutaneous and sublingual immunotherapy. J Allergy Clin Immunol *148*, 1061-1071 e1011.
Shamji, M.H., Sharif, H., Layhadi, J.A., Zhu, R., Kishore, U., and Renz, H. (2022). Diverse immune mechanisms of allergen immunotherapy for allergic rhinitis with and without asthma. J Allergy Clin Immunol *149*, 791-801.
Shan, M., Carrillo, J., Yeste, A., Gutzeit, C., Segura-Garzon, D., Walland, A.C., Pybus, M., Grasset, E.K., Yeiser, J.R., Matthews, D.B.*, et al.* (2018). Secreted IgD Amplifies Humoral T Helper 2 Cell Responses by Binding Basophils via Galectin-9 and CD44. Immunity *49*, 709-724 e708.
Shreffler, W.G., Wanich, N., Moloney, M., Nowak-Wegrzyn, A., and Sampson, H.A. (2009). Association of allergen-specific regulatory T cells with the onset of clinical tolerance to milk protein. J Allergy Clin Immunol *123*, 43-52 e47.
Sicherer, S.H., Furlong, T.J., Maes, H.H., Desnick, R.J., Sampson, H.A., and Gelb, B.D. (2000). Genetics of peanut allergy: a twin study. J Allergy Clin Immunol *106*, 53-56.
Siraganian, R.P., de Castro, R.O., Barbu, E.A., and Zhang, J. (2010). Mast cell signaling: the role of protein tyrosine kinase Syk, its activation and screening methods for new pathway participants. FEBS Lett *584*, 4933-4940.

Steffen, U., Koeleman, C.A., Sokolova, M.V., Bang, H., Kleyer, A., Rech, J., Unterweger, H., Schicht, M., Garreis, F., Hahn, J.*, et al.* (2020). IgA subclasses have different effector functions associated with distinct glycosylation profiles. Nat Commun *11*, 120.
Stefka, A.T., Feehley, T., Tripathi, P., Qiu, J., McCoy, K., Mazmanian, S.K., Tjota, M.Y., Seo, G.Y., Cao, S., Theriault, B.R.*, et al.* (2014). Commensal bacteria protect against food allergen sensitization. Proc Natl Acad Sci U S A *111*, 13145-13150.
Stone, K.D., Prussin, C., and Metcalfe, D.D. (2010). IgE, mast cells, basophils, and eosinophils. J Allergy Clin Immunol *125*, S73-80.
Strachan, D.P. (1989). Hay fever, hygiene, and household size. BMJ *299*, 1259-1260.
Strait, R.T., Mahler, A., Hogan, S., Khodoun, M., Shibuya, A., and Finkelman, F.D. (2011). Ingested allergens must be absorbed systemically to induce systemic anaphylaxis. J Allergy Clin Immunol *127*, 982-989 e981.
Sugimoto, M., Kamemura, N., Nagao, M., Irahara, M., Kagami, S., Fujisawa, T., and Kido, H. (2016). Differential response in allergen-specific IgE, IgGs, and IgA levels for predicting outcome of oral immunotherapy. Pediatr Allergy Immunol *27*, 276-282.
Tan, D., Yin, W., Guan, F., Zeng, W., Lee, P., Candotti, F., James, L.K., Saraiva Camara, N.O., Haeryfar, S.M.M., Chen, Y.*, et al.* (2022). B cell-T cell interplay in immune regulation: A focus on follicular regulatory T and regulatory B cell functions. Front Cell Dev Biol *10*, 991840.
Tezuka, H., Abe, Y., Asano, J., Sato, T., Liu, J., Iwata, M., and Ohteki, T. (2011). Prominent role for plasmacytoid dendritic cells in mucosal T cell-independent IgA induction. Immunity *34*, 247-257.
Tsai, M., Mukai, K., Chinthrajah, R.S., Nadeau, K.C., and Galli, S.J. (2020). Sustained successful peanut oral immunotherapy associated with low basophil activation and peanut-specific IgE. J Allergy Clin Immunol *145*, 885-896 e886.
Tsai, M., Starkl, P., Marichal, T., and Galli, S.J. (2015). Testing the 'toxin hypothesis of allergy': mast cells, IgE, and innate and acquired immune responses to venoms. Curr Opin Immunol *36*, 80-87.
Urm, S.H., Yun, H.D., Fenta, Y.A., Yoo, K.H., Abraham, R.S., Hagan, J., and Juhn, Y.J. (2013). Asthma and risk of selective IgA deficiency or common variable immunodeficiency: a population-based case-control study. Mayo Clin Proc *88*, 813-821.
van Asperen, P.P., Gleeson, M., Kemp, A.S., Cripps, A.W., Geraghty, S.B., Mellis, C.M., and Clancy, R.L. (1985). The relationship between atopy and salivary IgA deficiency in infancy. Clin Exp Immunol *62*, 753-757.
van den Oord, R.A., and Sheikh, A. (2009). Filaggrin gene defects and risk of developing allergic sensitisation and allergic disorders: systematic review and meta-analysis. BMJ *339*, b2433.
Vinuesa, C.G., Fagarasan, S., and Dong, C. (2013). New territory for T follicular helper cells. Immunity *39*, 417-420.
Vogel, K., Blumer, N., Korthals, M., Mittelstadt, J., Garn, H., Ege, M., von Mutius, E., Gatermann, S., Bufe, A., Goldmann, T.*, et al.* (2008). Animal shed Bacillus licheniformis spores possess allergy-protective as well as inflammatory properties. J Allergy Clin Immunol *122*, 307-312, 312 e301-308.
Wagenaar, L., Bol-Schoenmakers, M., Giustarini, G., Garssen, J., Smit, J.J., and Pieters, R.H.H. (2019). Mouse strain differences in response to oral immunotherapy for peanut allergy. Immun Inflamm Dis *7*, 41-51.

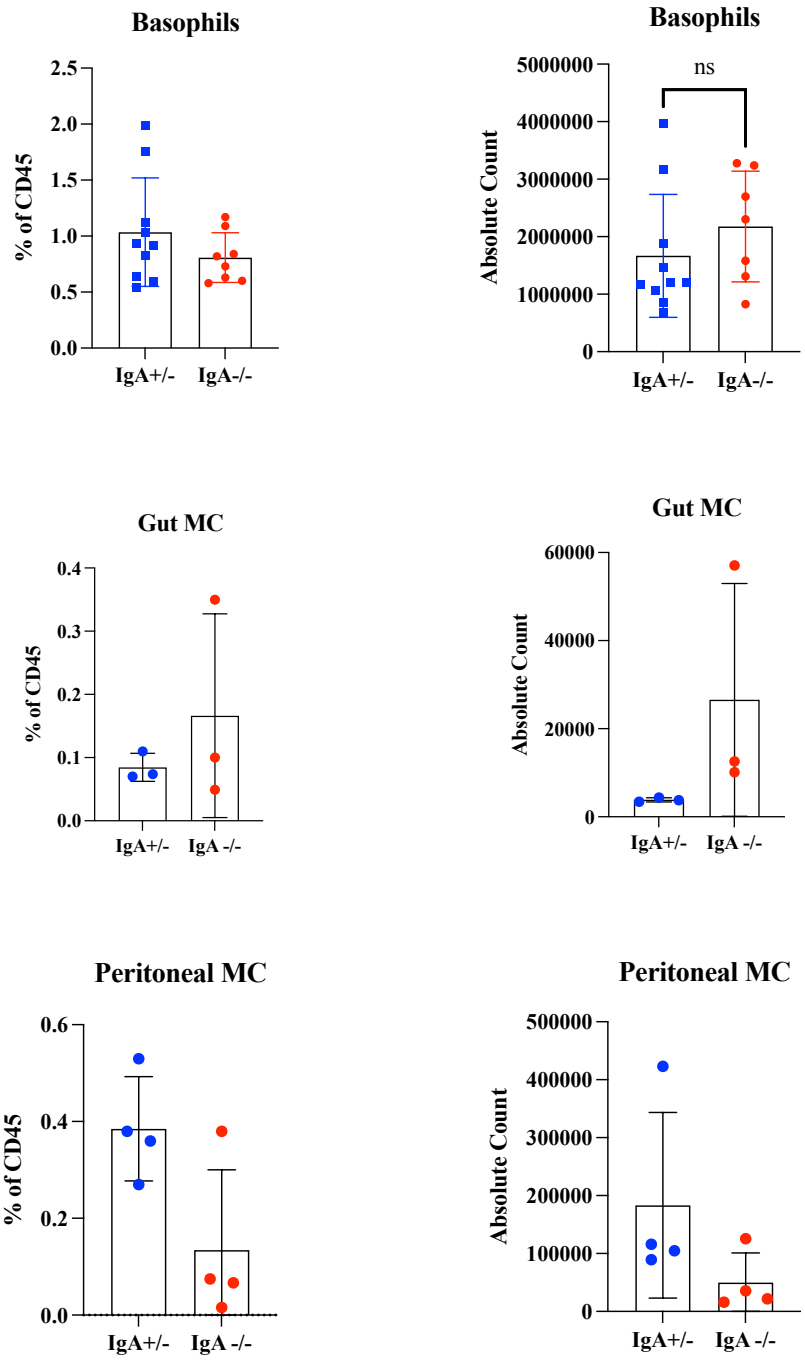
Walker, M.T., Green, J.E., Ferrie, R.P., Queener, A.M., Kaplan, M.H., and Cook-Mills, J.M. (2018). Mechanism for initiation of food allergy: Dependence on skin barrier mutations and environmental allergen costimulation. J Allergy Clin Immunol *141*, 1711-1725 e1719.
Wang, W., Yao, T., Zhang, T., Quan, M., Wang, C., Wang, C., Zhang, L., Tang, X., Jian, S., and Song, H. (2020). Selective immunoglobulin A deficiency (SIgAD) primarily leads to recurrent infections and autoimmune diseases: A retrospective study of Chinese patients in the past 40 years. Genes Dis *7*, 115-121.
Wijburg, O.L., Uren, T.K., Simpfendorfer, K., Johansen, F.E., Brandtzaeg, P., and Strugnell, R.A. (2006). Innate secretory antibodies protect against natural Salmonella typhimurium infection. J Exp Med *203*, 21-26.
Wines, B.D., and Hogarth, P.M. (2006). IgA receptors in health and disease. Tissue Antigens *68*, 103-114.
Winters, A., Bahnson, H.T., Ruczinski, I., Boorgula, M.P., Malley, C., Keramati, A.R., Chavan, S., Larson, D., Cerosaletti, K., Sayre, P.H.*, et al.* (2019). The MALT1 locus and peanut avoidance in the risk for peanut allergy. J Allergy Clin Immunol *143*, 2326-2329.
Wright, B.L., Kulis, M., Orgel, K.A., Burks, A.W., Dawson, P., Henning, A.K., Jones, S.M., Wood, R.A., Sicherer, S.H., Lindblad, R.W.*, et al.* (2016). Component-resolved analysis of IgA, IgE, and IgG4 during egg OIT identifies markers associated with sustained unresponsiveness. Allergy *71*, 1552-1560.
Wyss, M., Brown, K., Thomson, C.A., Koegler, M., Terra, F., Fan, V., Ronchi, F., Bihan, D., Lewis, I., Geuking, M.B.*, et al.* (2019). Using Precisely Defined in vivo Microbiotas to Understand Microbial Regulation of IgE. Front Immunol *10*, 3107.
Xiong, H., Dolpady, J., Wabl, M., Curotto de Lafaille, M.A., and Lafaille, J.J. (2012). Sequential class switching is required for the generation of high affinity IgE antibodies. J Exp Med *209*, 353-364.
Yamamoto, M., Kweon, M.N., Rennert, P.D., Hiroi, T., Fujihashi, K., McGhee, J.R., and Kiyono, H. (2004). Role of gut-associated lymphoreticular tissues in antigen-specific intestinal IgA immunity. J Immunol *173*, 762-769.
Yin, X., Chen, S., and Eisenbarth, S.C. (2021). Dendritic Cell Regulation of T Helper Cells. Annu Rev Immunol *39*, 759-790.
Yu, W., Freeland, D.M.H., and Nadeau, K.C. (2016). Food allergy: immune mechanisms, diagnosis and immunotherapy. Nat Rev Immunol *16*, 751-765.
Zhang, B., Liu, E., Gertie, J.A., Joseph, J., Xu, L., Pinker, E.Y., Waizman, D.A., Catanzaro, J., Hamza, K.H., Lahl, K.*, et al.* (2020). Divergent T follicular helper cell requirement for IgA and IgE production to peanut during allergic sensitization. Sci Immunol *5*.
Zhang, T., Franklin, A., Boboila, C., McQuay, A., Gallagher, M.P., Manis, J.P., Khamlichi, A.A., and Alt, F.W. (2010). Downstream class switching leads to IgE antibody production by B lymphocytes lacking IgM switch regions. Proc Natl Acad Sci U S A *107*, 3040-3045.
Zhu, Z., Lee, P.H., Chaffin, M.D., Chung, W., Loh, P.R., Lu, Q., Christiani, D.C., and Liang, L. (2018). A genome-wide cross-trait analysis from UK Biobank highlights the shared genetic architecture of asthma and allergic diseases. Nat Genet *50*, 857-864.
Zivkovic, J., Lipej, M., Banic, I., Bulat Lokas, S., Nogalo, B., Lulic Jurjevic, R., and Turkalj, M. (2019). Respiratory and allergic disorders in children with severe and partial immunoglobulin A immunodeficiency. Scand J Immunol *90*, e12828.

8. Supplementary Data



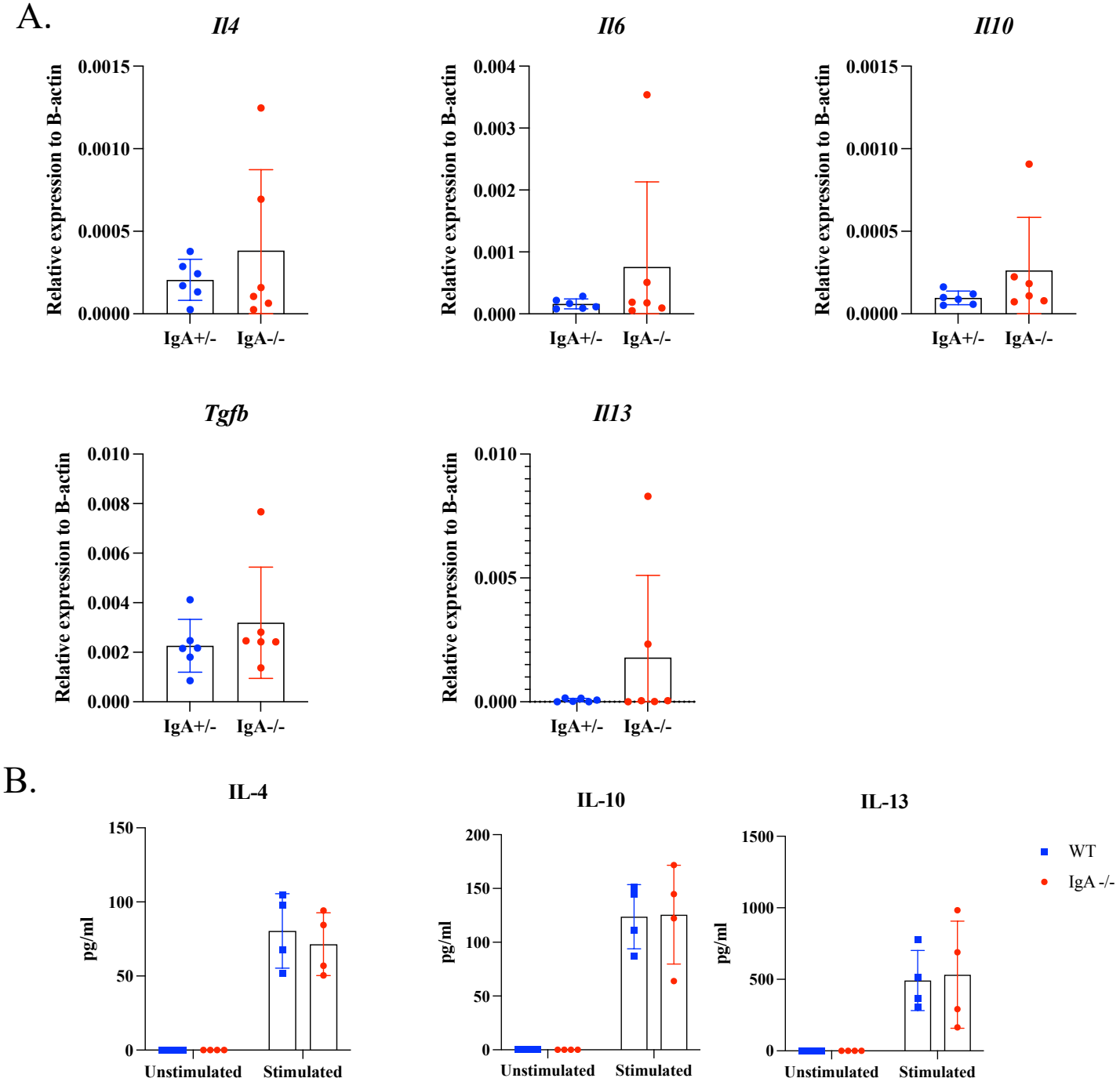
Supplementary Figure 1. IgE class switching in IgA$^{-/-}$ mice made in the 1990's

(A) Mice were sensitized with two OVA/alum injections (*i.p.*) two weeks apart, and then challenged (i.g) with OVA. Total serum IgE levels, and MFI of IgE on intestinal mast cells (left). Serum OVA-IgE and OVA-IgG1 levels after challenge (right). (B) Splenocytes from WT and IgA$^{-/-}$ mice were stimulated with anti-CD40 and IL-4. Bar graph showing mean percentage of CD19^{+} IgE^{+} cells after stimulation (left). Total IgE levels in the supernatant after 7 days of stimulated splenocytes (right). Data shown mean ± SEM are from one of three independent experiments. *P <.05, **P<.01, ***P<0.001. US,C : unsensitized and challenged mice. S,C : sensitized and challenged mice.



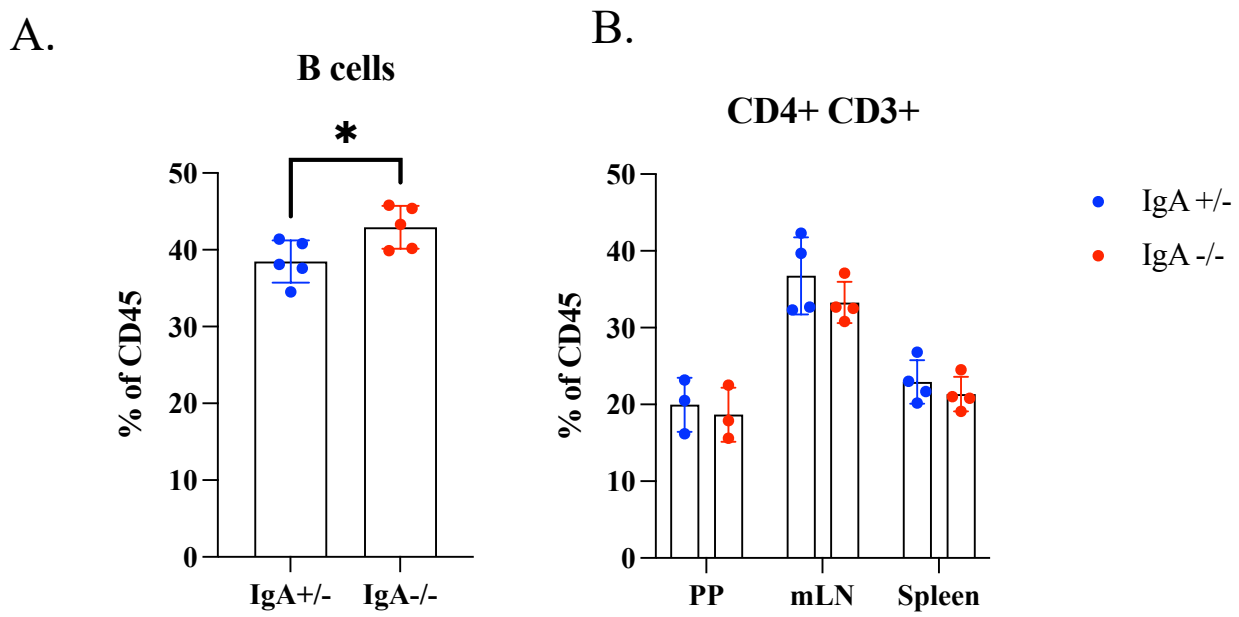
Supplementary Figure 2. Flow cytometric evaluation of blood basophil frequencies, gut mast cells and peritoneal mast cells (MC) in IgA+/- and IgA$^{-/-}$ mice.

Blood basophils (CD49b^{+} IgE^{+}), gut and peritoneal mast cells (c-kit^{+} IgE^{+}) as a percentage of CD45 (left) with absolute counts (right). Data shown is representative of two independent experiments, n= 3-10 mice/group.



Supplementary Figure 3. Flow cytometric evaluation of mast cells and blood basophil frequencies in IgA+/- and IgA$^{-/-}$ mice.

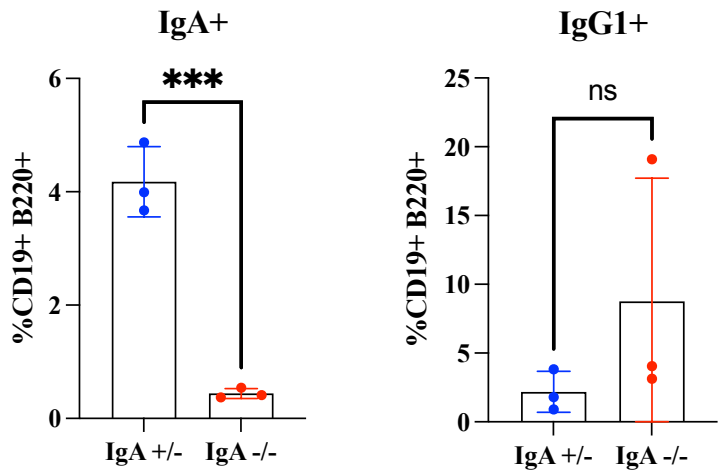
(A)Relative gene expression of *IL4, IL6, IL-10, TGFB,* and *IL13* from the small intestine of IgA$^{+/-}$ and IgA$^{-/-}$ mice. (B) IL-4, IL-10 and IL-13 levels in the supernatant after 3-day stimulation of splenocytes with PMA and ionomycin. Data shown is from one experiment,



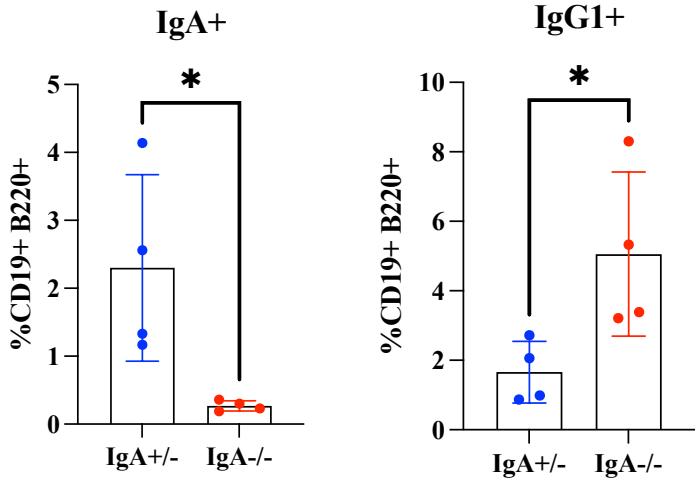
Supplementary Figure 4. Flow cytometric evaluation B cells in the blood, and CD4+ T cells in the PP, mLN, and spleen of IgA+/- and IgA$^{-/-}$ mice.

(A) Frequency of CD19^{+} B cells in the blood of IgA+/- and IgA$^{-/-}$ mice. (B) Frequency of CD4^{+} CD3^{+} T cells in the PP, mLN, and spleen. Data shown is one experiment representative of 3 independent experiments, n= 3-5 mice/group *P <.05

SI

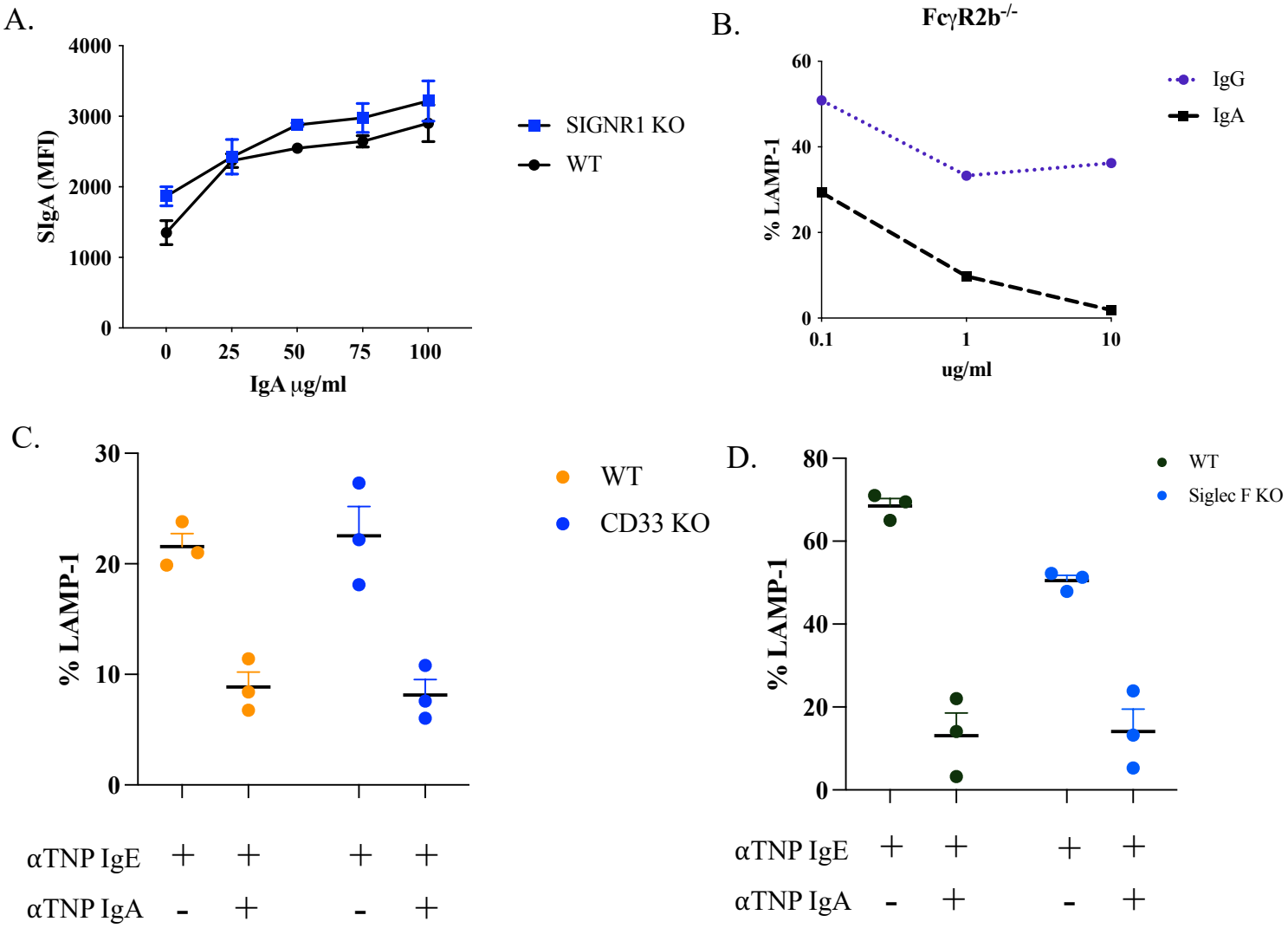


mLN



Supplementary Figure 5. Flow cytometric evaluation of IgA$^+$ and IgG1$^+$ B cells in the gut and mLN of IgA+/- and IgA$^{-/-}$ mice.

(A) Frequency of IgA$^+$ CD19$^+$ B220$^+$ B cells, and IgG1$^+$ CD19$^+$ B220$^+$ B cells in the small intestine (SI) (upper panels), and mLN (lower panels). P <.05, ***P<0.001



Supplementary Figure 6. IgA binding/inhibition effects on either SIGNR1, FcγR2b, CD33, or Siglec F KO BMMC's

(A)Mean fluorescence intensity (MFI) of BMMCs stained with anti-IgA after incubation with anti-TNP IgA in WT and SIGNR1 KO BMMC's. (B) Percent degranulation of LAMP-1 induction in IgE sensitized BMMCs incubated with anti-TNP IgA, or anti-TNP IgG in FcyR2B KO BMMC's (C) Percent degranulation of LAMP-1 induction in IgE sensitized BMMCs incubated with or without anti-TNP IgA in CD33 KO BMMC's (D) Percent degranulation of LAMP-1 induction in IgE sensitized BMMCs incubated with or without anti-TNP IgA in Siglec F KO BMMC's

9. Summary

Background: Mast cells have long been implicated in the pathogenesis of IgE-mediated allergies. They express the high-affinity IgE receptor FCεR1 on their surface. Crosslinking of IgE antibodies with their respective polyvalent antigen triggers a signaling cascade that results in their degranulation, releasing an array of preformed inflammatory mediators, as well as the production of inflammatory cytokines. To counteract activating signals, mast cells also express inhibitory receptors such as FcγRIIb, the IgG inhibitory receptor that inhibits IgE-mediated cell activation. While our lab and others have shown a vital role for IgG in suppressing IgE induced allergic mechanisms, the role of typically non inflammatory antibodies such as IgA in regulating mast cell function is unknown. Furthermore, IgA antibodies protective roles at mucosal surfaces is well studied in infectious disease, and autoimmunity, but unknown in the context of allergic disease, despite IgA deficiency being the most common immunodeficiency (Morawska et al., 2021). Increased susceptibility to atopy in IgA deficient patients (Aghamohammadi et al., 2009; Erkocoglu et al., 2017; Gualdi et al., 2015; Shahin et al., 2020), and reports correlating low IgA levels in the serum and saliva with increased incidence of allergy, led us to believe that IgA might be involved in the development of allergic disease.

Aims and Hypothesis: In our first project, we hypothesize, that similar to IgG, IgA will inhibit mast cell signaling, and degranulation and suppress generation of cytokines. As for Project 2, our working hypothesis posits that IgA plays a crucial role in dampening Th2 responses, and as a result, IgA-deficient mice are expected to exhibit a Th2-dominant phenotype at baseline.

Method and results: We utilized a range of experimental tools, such as murine cultured mast cells, peritoneal mast cells, human basophils, and monoclonal hapten-specific IgA, and IgA isolated from the sera of individuals who had undergone peanut OIT in Project 1. Our objective was to examine whether antigen-specific IgA antibodies have an inhibitory effect on mast cell and basophil activation mediated by FcεRI. We find that IgA binds to bone marrow-derived mast cells (BMMCs) and peritoneal mouse mast cells in a manner

dependent on calcium and sialic acid. Antigen-specific IgA also inhibits IgE-mediated mast cell degranulation by downregulating phospho-Syk, and thus suppressing the release of cytokines. Furthermore, we found that antigen-specific IgA, similar to IgG, can prevent passive systemic anaphylaxis in mice. Extending our studies to human effector cells of allergy, we found that antigen-induced activation of basophils from peanut allergic subjects was suppressed by peanut-specific IgA.

In Project 2 we compared the immunological phenotypes of IgA-deficient mice with their IgA-sufficient littermates. These investigations provided context regarding the influence of IgA in regulating commensalism, the biology of gastrointestinal T helper cells, and the development of B cells. IgA deficient mice exhibit hyperimmunoglobulinemia, with increased levels of IgE, and MMCP-1. The hyperimmunoglobulinemia was attributed to the dysregulated Tfh/Tfr responses in Peyer's Patches. We also observed a reduction in many CD4+ T helper cells in $IgA^{-/-}$ mice in the Peyer's Patches, and an increase in CD19+ B cells in the blood, mLN, and spleen. $IgA^{-/-}$ mice also had an altered microbiome compared to littermate controls, with an increase in the phyla Bacteroidetes, and a decrease in Firmicutes. There were differences in several other taxa including order, class, and family. The *Lachnospiraceae* family, was decreased in $IgA^{-/-}$ mice, while *Porphyromonadaceae* were increased compared to littermate controls. At the species level, both *Porphyromonadaceae*, and *Prevotella* were increased in $IgA^{-/-}$ mice as well.

Conclusion: Here we uncover an inhibitory role for IgA in mast cell degranulation. We conclude that IgA serves as a regulator of mast cell degranulation, including anaphylaxis, providing a new therapeutic potential for IgE-mediated allergic disease. Additionally, through the development of mice deficient in IgA but with an intact IgE locus, we have been able to investigate the consequences of IgA deficiency on immune equilibrium and systemic antibody responses in the context of allergic conditions. We conclude that the role of IgA in maintaining the gut microbiome plays a crucial role in regulating adaptive immune responses, particularly in the induction of IgE antibodies.

10. Verzeichnis der akademischen Lehrer

Meine akademischen Lehrer waren die Herren

Dr. Harald Renz
Philipps Marburg Universität

Dr. Hans Oettgen
Harvard Medical School/Boston Children’s Hospital

11. Acknowledgements

This thesis was done in the laboratory of Dr. Hans Oettgen at Boston Children's Hospital in collaboration with Professor Dr. Harald Renz at the Institute of Laboratory Medicine and Pathobiochemistry at the Philipps University of Marburg. I would like to express my extreme gratitude to Dr. Harald Renz for giving me the opportunity to be a PhD student under his supervision and guidance. His kind and encouraging nature has really helped me see this project to completion. Furthermore, this project would have not come to fruition without my co-supervisor Dr. Hans Oettgen. I credit everything I have learned about allergy and immunology to him. I was a master's student in his lab with big dreams, and he helped mold me into the scientist I am today. A lab is nothing without its members. Many thanks are given to my friend, and colleague, Dr. Cynthia Kanagaratham. She was my support system, and mentor, and I have thoroughly enjoyed my time in the lab much more with her presence. I also couldn't have done a lot of my work without the help of Brianna, Owen, Jenna, and Kameryn for putting up with long sac days. Thank you guys for your lighthearted and joyful spirits!

I would also like to thank Hani Harb, who encouraged me to do the PhD, and connected me with the program at Marburg.

Lastly, I must end this by thanking my family. My biggest support system. They were there with me through it all. My parents, Fawzi, Abeer, and Nadeen. Thanks for putting up with me. And Talal, my fiancé, thank you for pushing me towards the finish line.