The regulation and maintenance of IgE+ B cells in galactose-α-1,3-galactose allergy

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Abstract

The prevalence of allergic disease has increased at an alarming rate in the past few decades, affecting both children and adults. Despite numerous attempts to understand the mechanisms which drive sensitization to innocuous antigens, the factors that lead to the development of allergy remain unclear. Although a number of pharmacological agents have been developed to treat allergies, these treatments are largely aimed at reducing allergic symptoms. Additionally, the mechanisms which maintain allergic sensitization are not known, hampering the development of effective treatments for curing or even reducing allergic reactions. While it is clear that IgE+ B cells are vital for the pathogenesis of the allergic response, little is known about these cells. Here, we sought to elucidate the signals that drive the development of allergen specific IgE secreting B cells, as well as the mechanism by which these cells are maintained, in the context of galactose-alpha-1,3-galactose (alpha-gal), or red meat allergy.

Using mass cytometry (CyTOF) we have analyzed the expression of 23 cell surface markers in PBMCs from 19 alpha-gal-allergic patients and 20 non-allergic controls by CyTOF. Our data revealed previously unrecognized heterogeneity within traditional B cell subsets. Additionally, through the use of a novel bioinformatics pipeline, we identified a collection of B cell subsets, or clusters, which correlated with alpha-gal specific serum antibody titers. Functional analysis of these B cell clusters demonstrated that cells isolated from patients with red meat allergy preferentially secreted alpha-gal specific IgE following *in vitro* stimulation, implicating these novel B cell phenotypes in promoting IgE in alpha-gal allergy.

In addition to investigating circulating B cell subsets in patients with red meat allergy, we have developed a murine model of tick-induced IgE responses to investigate the signals that drive the development of the IgE response. Subcutaneous immunization with tick extract results in a robust, tick specific IgE response in C57BL/6 (B6) mice, however no significant induction in serum IgE resulted from intraperitoneal (I.P.) immunization with tick extract. The IgE response observed in mice immunized subcutaneously was accompanied by the concomitant induction of a germinal center (GC) response. This response is T cell dependent, as the inhibition of T cell help resulted in an attenuated IgE response. Utilizing MyD88 deficient mice, we have identified a requirement for MyD88 signaling in the production of IgE following subcutaneous immunization with tick extract, suggesting that toll-like receptor (TLR) signaling plays a role in the development of tick induced IgE responses. Additionally, through studies with MyD88 conditional knock out mice, we demonstrate that the requirement for MyD88 signaling is B cell intrinsic. Taken together, we have identified a role for MyD88 in the development of the IgE response to tick extract, and identified novel B cell subsets in red meat allergic patients that may play a role in the pathogenesis of food allergy, revealing new potential targets for effective treatment of prevention of allergic responses. The systems we have developed will provide tools for future investigations in the development and maintenance of IgE responses.

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List of Abbreviations

α-Gal: Galactose-α-1,3-Galactose

ACD: Acid Citrate Dextrose

αGT KO: Alpha-Galactosyl Transferase$^{-/-}$ Mouse

APCs: Antigen Presenting Cells

ASCs: Antibody-Secreting Cells

B6: C57BL/6

Bcl6: B-Cell Lymphoma 6

BLIMP1: B Lymphocyte-Induced Maturation Protein

BSA: Bovine Serum Albumin

CDR3: Complementarity-Determining Region 3

CyTOF: Mass Cytometry by Time-Of-Flight

DCs: Dendritic Cells

EGFR: Epidermal Growth Factor Receptor

EPIT: Epicutaneous Immunotherapy

EST: Expressed Sequence Tag

FDR: False Discovery Rate

GC: Germinal Center

GFP: Green Fluorescent Protein

H&E: Hematoxylin-And-Eosin

HDM: House Dust Mite

IFN: Interferon

IgH: Ig Heavy Chain

Ighγ1: Immunoglobulin Heavy Constant Gamma 1

IHC: Immunohistochemistry

IL: Interleukin

ILC2s: Type 2 Innate Lymphoid Cells

I.P.: Intraperitoneal

KLH: Keyhole Limpet Hemocyanin

OD: Optical Density

OIT: Oral Immunotherapy

OVA: Ovalbumin

NFIL3: Nuclear Factor, IL-3 Regulated

NP: 4-Hydroxy-3-Nitrophenylacetyl

Pax5: Paired Box Protein 5

PC: Plasma Cell

PBMC: Peripheral Blood Mononuclear Cells

Q-PCR: Quantitative Polymerase Chain Reaction

RMSF: Rocky Mountain Spotted Fever

SCIT: Subcutaneous Immunotherapy

SHM: Somatic Hypermutation

sIgE: Allergen-Specific IgE

SLE: Systemic Lupus Erythematosus

SLIT: Sublingual Immunotherapy

Tfh: T Follicular Helper

TLR: Toll-Like Receptors

t-SNE: T-Distributed Stochastic Neighbor Embedding

TSLP: Thymic Stromal Lymphopoietin

XBP1: X-box Binding Protein 1

CHAPTER 1: AN INTRODUCTION TO ALLERGY

Introduction

The frequency of allergic sensitization has risen at an astonishing rate in recent decades, reaching near epidemic levels. Hypersensitivity reactions to food affect 2-3% of adults and up to 8% of children in the United States alone [1, 2]. Despite the increasing prevalence, and potentially life-threatening symptoms of allergies, the cause of allergic sensitization remains unknown. Additionally, current treatments focus mainly on managing the symptoms of the allergic response. Few effective treatments exist that reduce allergic reactions, and currently there is no method to prevent the development of, or cure allergies. Increasing our understanding of the mechanisms that drive the development of allergies, as well as the immune cells that maintain sensitization to allergen is vital to identifying new targets to treat and prevent allergies.

Factors implicated in the development of allergies

Accurate estimates of the prevalence of allergy are difficult to measure. However, the data support the idea that rates of allergic sensitization are increasing [3]. Data released by the CDC in 2013 indicated that the prevalence of food allergies among children rose from 3.4% in 1997-1999, to 5.1% in 2009-2011 [4]. The reasons for this rapid increase in allergic sensitization are not clear, but one theory proposed to explain the increasing prevalence is the hygiene hypothesis. Originally coined by David Strachan in 1989, the hygiene hypothesis proposed that reduced microbial exposure due to declining family size and improving personal cleanliness and household amenities resulted in a loss of tolerance and the development of atopic disease [5]. Studies observing the change in frequency of allergy and hay fever in countries undergoing

urbanization (reviewed by [6]) support this hypothesis, however the current consensus in the literature is that changes in hygiene cannot be the sole cause of the increase in rates of allergy and asthma [7]. More recent studies have suggested that changes in microbiota composition, which is affected by reduced exposure to microbes and microbial products during early life, play a role in the development of allergic disease (reviewed [8]). Studies in children with food allergy revealed alterations in both microbial diversity and which species were most prevalent [9, 10].

In addition to evidence of a role for the microbiota in allergic sensitization, recent research points to a strong role for epithelial barrier disruption in the development of atopic disease, such as allergy, asthma, and atopic dermatitis. It is thought that disruption of the epithelial barrier may allow allergens and other irritants to penetrate the epithelia, come in contact with antigen presenting cells (APCs) and initiate an inflammatory response. A number of murine studies have shown that allergic sensitization to peanut can occur through the skin [11-13]. Defects in filaggrin, a protein which plays an important role in maintaining normal epithelial barrier function, are strongly associated with skin disorders including atopic dermatitis, eczema, and ichthyosis vulgaris in humans [14, 15], and these skin disorders have been linked to increased risk for the development of food allergy [3, 16, 17]. Indeed, a study by Brown and colleagues [18] found that mutations in the filaggrin gene confer a significant risk factor for peanut allergy. There is also evidence suggesting that increased use of antibiotics increased the risk of atopic dermatitis in children, and that exposure to endotoxin and the presence of household pets provided a protective effect[19], providing a link between the hygiene hypothesis and the role of skin barrier disruption in the development of atopic disease.

However, not all allergic individuals exhibit skin barrier disruption, or defects in filaggrin, indicating that other factors that drive the development of allergy remain unknown.

Therapies

Given the questions of what drives the initiation of allergies, or allergic sensitization, it is unsurprising that methods to prevent the development of allergy have not been successful. Furthermore, effective treatments to reduce the severity and/or cure allergies are also lacking. Traditionally, the main treatment option for food allergy has been allergen avoidance [20]. More recently, investigation into a number of methods of immunotherapy has begun. In these treatments allergic patients are given small, slowly escalating doses of allergen in order to modulate the immune response and develop tolerance to the allergen. Four main methods of immunotherapy are currently being pursued, with the goal of obtaining long-term unresponsiveness. These methods include epicutaneous immunotherapy (EPIT), where a patch containing the allergen is placed on the skin; oral immunotherapy (OIT), which consists of oral ingestion of powdered allergen; subcutaneous immunotherapy (SCIT), in which the patient receives injections of allergen extract; and sublingual immunotherapy (SLIT), where an extract of the allergen is placed under the tongue and then swallowed. The results of numerous trials on OIT, SLIT, and EPIT are reviewed by Burks et al. [21]. Of these, OIT shows the most promise for obtaining lasting unresponsiveness, but at the cost of high risk of adverse and even severe side effects, and long-term maintenance of desensitization has not been verified. On the other end of the spectrum, studies of EPIT have shown that the treatment is well

tolerated with only mild side effects, however very limited clinical desensitization was achieved. Similarly, SLIT treatment elicited limited side effects and few systemic reactions but only resulted in moderate desensitization. SCIT, commonly used for environmental allergies, has been limited in food allergy treatment due to the risk of severe systemic reactions [22]. The use of the monoclonal anti-IgE antibody Omalizumab, has shown some promise in reducing the occurrence of severe side effects and increasing the speed at which dose ramping can occur in OIT, but the efficacy of this treatment in maintaining long term desensitization has not been established [21].

Immunologic mechanisms of allergy

Although the specific factors that lead to sensitization have not been identified, a number of cells and cytokines that play a role in the allergic response have been identified. The allergic response is comprised of both innate and adaptive immune mediators. Initial encounter with allergen often occurs at the epithelial barrier, regardless of whether the allergen comes from the air (lung epithelia), food (gut epithelia), or an insect (skin epithelia). As such, epithelial cells and tissue resident innate immune cells are known to play an important role in inducing the allergic response (Fig. 1.1). Recognition of allergen, often through TLR signaling, drives production of the cytokines thymic stromal lymphopoietin (TSLP), interleukin (IL)-25, and IL-33 by epithelial cells [23]. These cytokines drive differentiation of downstream effector cells towards a phenotype which favors the development of an IgE response [24]. TSLP especially plays an important role in the initiation of the allergic response by activating basophils, which

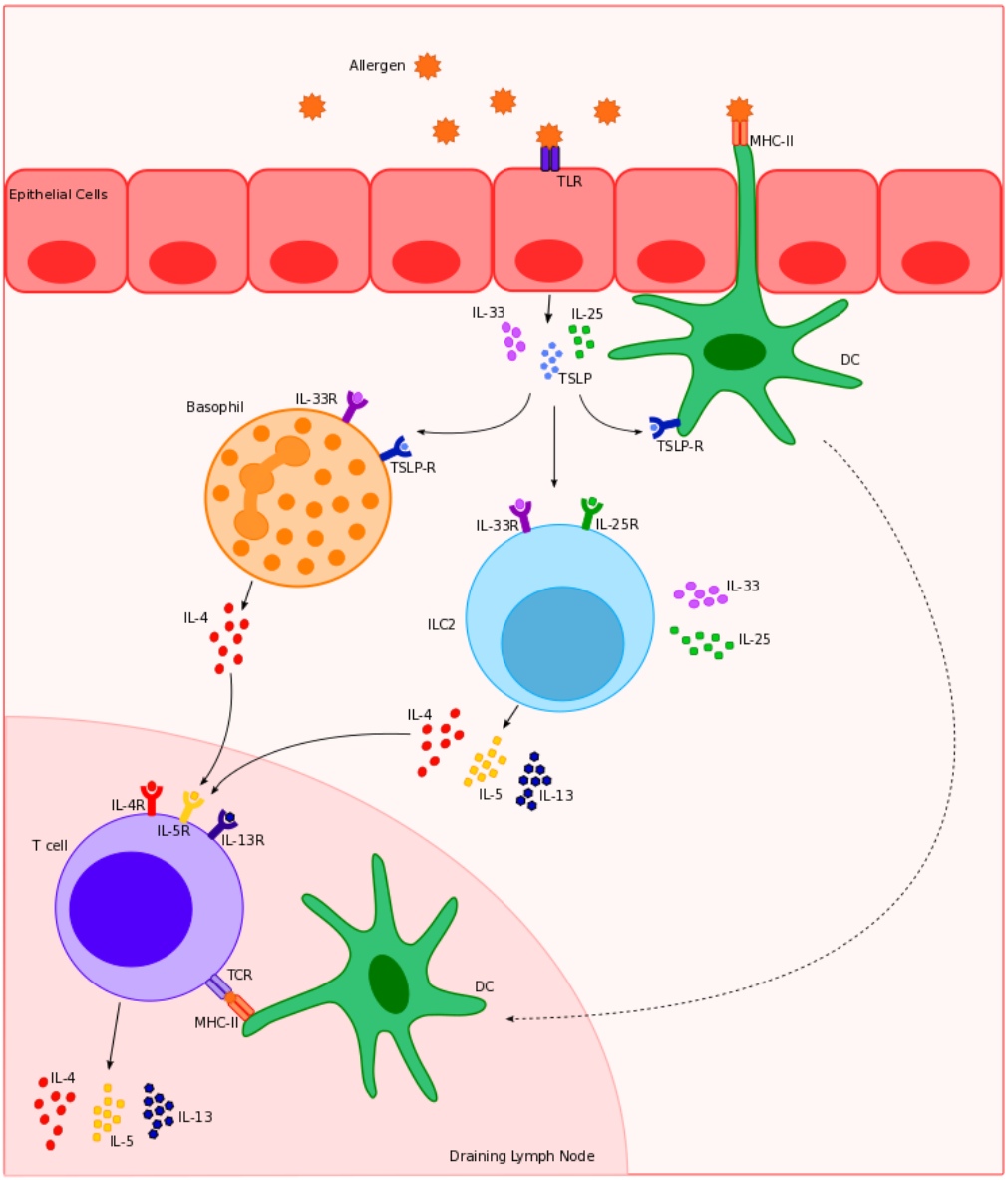

Figure 1.1 Innate immune mediators of T cell activation in allergy.
Epithelial cells are often the first cell to recognize allergen exposure. Upon activation these cells secrete IL-33, IL-25, and TSLP. These cytokines in turn activate tissue resident basophils, ILCs and dendritic cells which then generate pro-Th2 cytokines IL-4, IL-5, and IL-13. Together with signals provided by dendritic cells, IL-4, IL-5, and IL-13 will drive polarization of T cells to a Th2 phenotype.

then produce IL-4 and drive T cell polarization [25]. TSLP can also act directly on CD4 T cells to induce T cell differentiation into pro-allergic Th2 cells, likely through activation of Stat5 [26-28]. Another innate cell that is thought to play a key role in initiating the adaptive immune response to allergen are the more recently identified type 2 innate lymphoid cells (ILC2s). Like basophils, these cells are able to secrete cytokines that drive T cell polarization, including IL-4, IL-5, IL-13, IL-25, and IL-33 [29]. ILC2s can also potentially directly activate CD4 T cells through MHC-II, OX40L, CD80, and/or CD86 [30].

In addition to basophils and ILC2s, dendritic cells (DCs) also play a key role in initiating the adaptive immune response to allergen. DCs present antigen to T cells and provide costimulatory signals that are vital to activating these T cells. Tissue resident DCs provide constant surveillance of both the gut [31] and airways [32] through cellular processes that extend between epithelial cells and into the lumen. Upon antigen binding, DCs then traffic to draining lymph nodes, where they present antigen to, and activate T cells [33, 34]. The signals provided by DCs play a key role in T helper cell skewing. Production of the cytokine IL-12 by DCs favors differentiation of Th1 cells [35, 36], while the DC-derived signals that drive Th2 differentiation are not well understood [37], however, TSLP appears to be important in activating DCs to drive Th2 differentiation [38, 39].

Signals from epithelial cells, DCs and other innate lymphoid cells converge to activate T cells and drive T cell polarization, initiating the adaptive immune response to allergen. Activated helper T cells can be divided into a number of different populations based on their effector function. Originally, helper T cells were split into two groups: Th1

cells, which produce IFNy and are thought to mediate the adaptive response to intracellular pathogens [40], and Th2 cells, which secrete IL-4, IL-5 [41], and IL-13 [42] and drive the immune response to parasites and helminths [43], as well as allergens. More recently, a number of other T helper cell subsets have been identified, including Th17 cells and Th9 cells, which were originally considered to be Th2 cells and thought to play a role in allergic responses [40]. Th2 cells play a key role in inducing CSR to IgE in cognate B cells, through production of IL-4, IL-13 and CD40L expression, and mice deficient in IL-4 or IL-13 exhibit an impaired IgE response [44]. Allergen specific IgE produced by these B cells then binds to the FcεRI on mast cells and basophils, driving the synthesis and release of histamine, leukotrienes and other mediators of the allergic response, leading to the well-known symptoms of allergy [45]. Despite a large body of work elucidating the mechanisms promoting the Th2 response to allergen, the mechanisms that drive the development of an IgE+ B cell response to allergen are less well defined, including the signals that drive the development of an IgE response to innocuous antigen in some individuals, but not in others, whether IgE+ B cells are generated through direct or sequential switching, and the cellular source of IgE memory, which will be discussed in further detail below. However, the efficacy of anti-IgE therapy in treating allergic asthmatics highlights the potential benefits of understanding more about the IgE B cell response.

Mechanisms of class switching to IgE

The first major question that remains to be elucidated in the mechanisms driving the development of IgE B cells is the pathway by which allergen specific IgE is

generated. Unlike other isotypes, observations in both humans and mice suggest that sequential switching, in which IgM+ B cells undergo CSR to an IgG (most often IgG1) expressing intermediate before undergoing further CSR to produce IgE as opposed to a single CSR event from IgM+ to IgE+ (Fig. 1.2), may be the dominant way in which pathogenic, allergen specific IgE is generated. A number of studies examined the sequences of Sμ-Sε switch regions in allergic individuals and found that these regions contained Sγ switch region remnants, suggesting that IgE was generated through sequential switching [46-50]. Additionally, Looney et al. [46] identified common lineages in IgG1 and IgE B cells when analyzing the repertoire of the rearranged immunoglobulin gene variable (V) regions, supporting the argument that IgE was generated through a sequential switching pathway. However, studies analyzing somatic hypermutation (SHM) and examining the correlation between different Ig isotypes in patients with allergies to house dust mite (HDM) and the fungus *Alternaria alternata* suggest that IgE was derived through direct switching to IgE [51]. To further complicate matters, biopsies of nasal and bronchial mucosa of patients with allergic rhinitis and asthma, respectively, provided evidence of both direct and sequential switching to IgE occurring in the tissue [48, 52, 53]. Limitations in human studies, including low frequency of IgE+ cells in the circulation and inability to assess CSR *in vivo* following immunization, have so far prevented the resolution of these conflicting data and elucidation of the contribution of direct and sequential switching in driving production of pathogenic IgE. However, understanding the pathway by which IgE is generated will be vital for designing treatment strategies to target the source of allergen specific IgE.

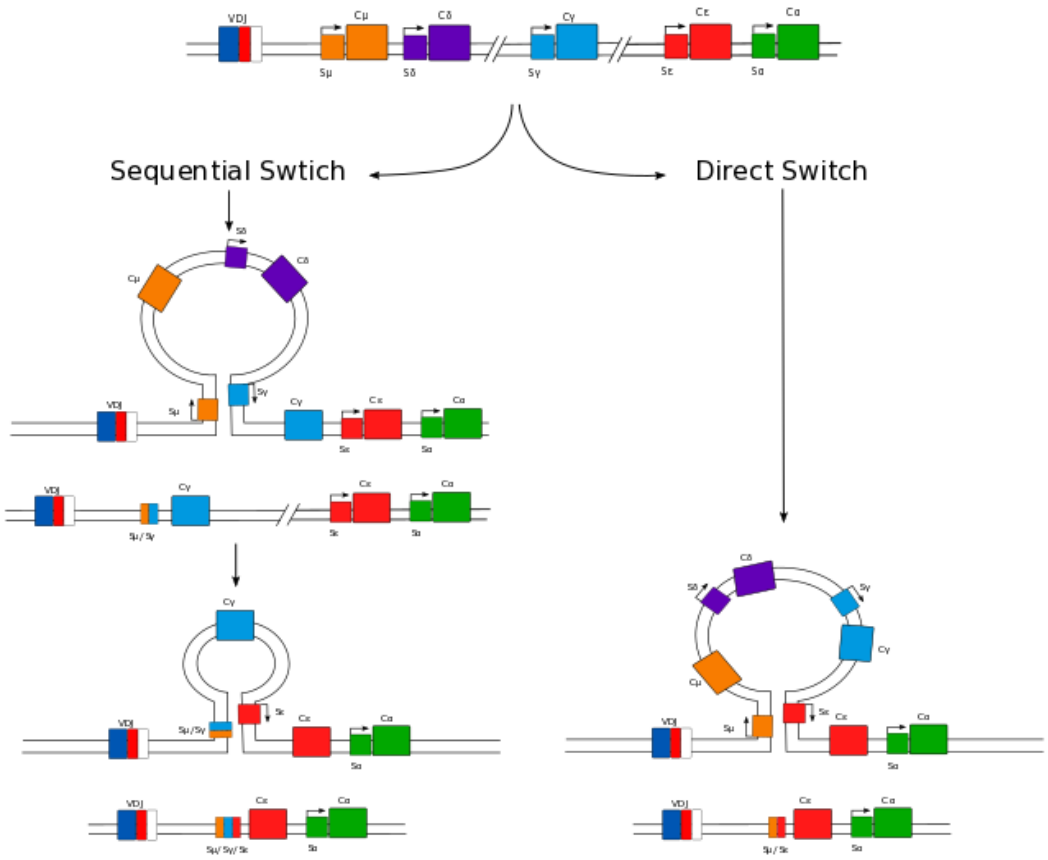

Figure 1.2 Direct vs Sequential Switching to IgE.
IgE can be generated through two different methods of class switch recombination. In the sequential switching pathway B cells undergo multiple CSR events to generate an IgE+ B cell. An initial switching event produces an IgG+ B cell, followed by another switch resulting in an IgE+ B cell. A portion of IgE+ B cells generated through sequential switching will retain a fragment of the γ switch region. In the more traditional direct pathway a single recombination event occurs, generating an IgE+ B cell.

Given the inherent difficulties and limitations in human studies, many groups have turned to mouse models to study the contribution of direct versus sequential switching to IgE. Similar to the methods used in the human studies noted above, Xiong et al. [54] detected Sγ1 fragments within Sμ-Sε switch regions in mice. This study found that the frequency of Sμ-Sε switch regions containing Sγ1 fragments increased with increasing immunization, as did the affinity of IgE antibodies for antigen, suggesting that sequential switching was necessary for the production of IgE+ B cells with high affinity for antigen. This conclusion was further supported by reduced levels of somatic hypermutation and affinity maturation of IgE antibodies produced by mice which are unable to produce IgG1 These data seem to conflict with an earlier study by Jung, Siebenkotten, and Radbruch [55], in which mice with impaired switching to IgG1 exhibited no defect in class switching to IgE during the primary or secondary response to the parasitic worm, *Nippostrongylus brasiliensis*, or NP-ovalbumin (NP-OVA). However, the affinity of the IgE generated in this study was not assessed, and further characterization of the IgE produced by these mice could resolve the discrepancy between these two studies.

Other recent work has attempted to tease apart the contribution of direct versus sequential class switching to IgE production by directly tracking IgE+ B cells. Previously, this was hampered by difficulties in detecting IgE+ cells *in vivo* due the low frequency of IgE+ cells compared to other isotypes [56], as well as the potential for identifying false positive cells due to IgE binding to the low affinity IgE receptor (CD23) on non-IgE+ B cells [57, 58]. To overcome these difficulties, several groups have developed IgE reporter mice to study class switching to, and maintenance of IgE+ cells.

Talay and colleges generated a bicistronic IgE-Green Fluorescent Protein (GFP) mouse in which GFP was inserted into the IgE encoding locus downstream of the exon that encodes the cytoplasmic domain of membrane bound IgE [59]. In contrast to the work of Xiong et al.[54], Talay and colleges [59] suggest that IgE+ B cells are generated through a direct switch pathway in IgE-GFP mice following either immunization with NP-OVA or infection with *N. brasiliensis*. This conclusion was based off of the observation that GFP+ IgE+ B cells were detected in the GC of mice 7 days after infection with *N. brasiliensis*, similar to the kinetics observed for IgG1+ B cells. Similar kinetics between the development of IgG1+ and IgE+ B cells were also observed following immunization with NP-OVA. Despite the potential utility of the IgE-GFP mice in studying IgE class switching, the data are conflicting. While differing routes of immunization or differences in the immunogen that was used my account for the differences observed in these models, further work is needed to reconcile these conflicting reports.

IgE reporter mice have also been widely used to assess the location of IgE class switching, as well as the source of IgE memory in murine models. Some groups hypothesize that CSR to IgE^+ is unlikely to occur in germinal centers (GC) due to high levels of Bcl6 expression in GC B cells, which antagonizes Stat6 binding, a key transcription factor for IgE expression [60, 61]. Data from the IgE-GFP mice indicated that IgE+ B cells were indeed found within GCs, in contrast to earlier work by Erazo et al. [62] which found that IgE+ B cells were localized outside the GC and exhibit plasma cell (PC) like phenotype. The ability to visualize the localization of IgE+ B cells in the lymph node should readily answer the question of whether or not IgE+ B cells undergo CSR and affinity maturation within the GC, however the conclusion is not so clear cut.

Data from another recently developed IgE reporter mouse, the Verigem mouse, differ from the findings of Talay et al.[59]. In Verigem mice the final exon of membrane bound IgE is linked to the yellow fluorescent protein, Venus, and translation of the IgE locus in these mice also results in translation of a separate Venus protein [63]. Following immunization with either *N. brasiliensis* or NP-KLH with the adjuvant alum, IgE+ B cells were detected in GCs of Verigem mice, but these cells upregulated Blimp-1 and exited the GC more rapidly than other isotypes and expressed a PC-like phenotype. In contrast to the conclusions of Yang, Sullivan, and Allen [63], He and colleagues[64] suggest that these IgE+ GC B cells rapidly undergo apoptosis after migrating from the GC rather than further differentiating into PCs. Data from the CεGFP IgE reporter mouse, which contains an IRES-GFP cassette inserted into the 3' UTR of the membrane encoding Cε gene, also suggests that IgE+ B cells are generated within GCs, but that these cells rapidly exit the GC [64]. Given the conflicting results of these studies, the anatomical location of CSR to IgE, and whether these cells are generated through direct or sequential switching remain unclear. New models will likely be required to resolve these questions.

IgE memory

Debate about the location and the pathways by which IgE+ B cells are generated (Fig. 1.3) further complicate efforts to resolve the mechanisms by which IgE memory is maintained after allergic sensitization. Understanding how allergic memory is maintained will be key to developing effective therapies for treating individuals who have already become sensitized. In most antibody responses, isotype switched memory B cells develop

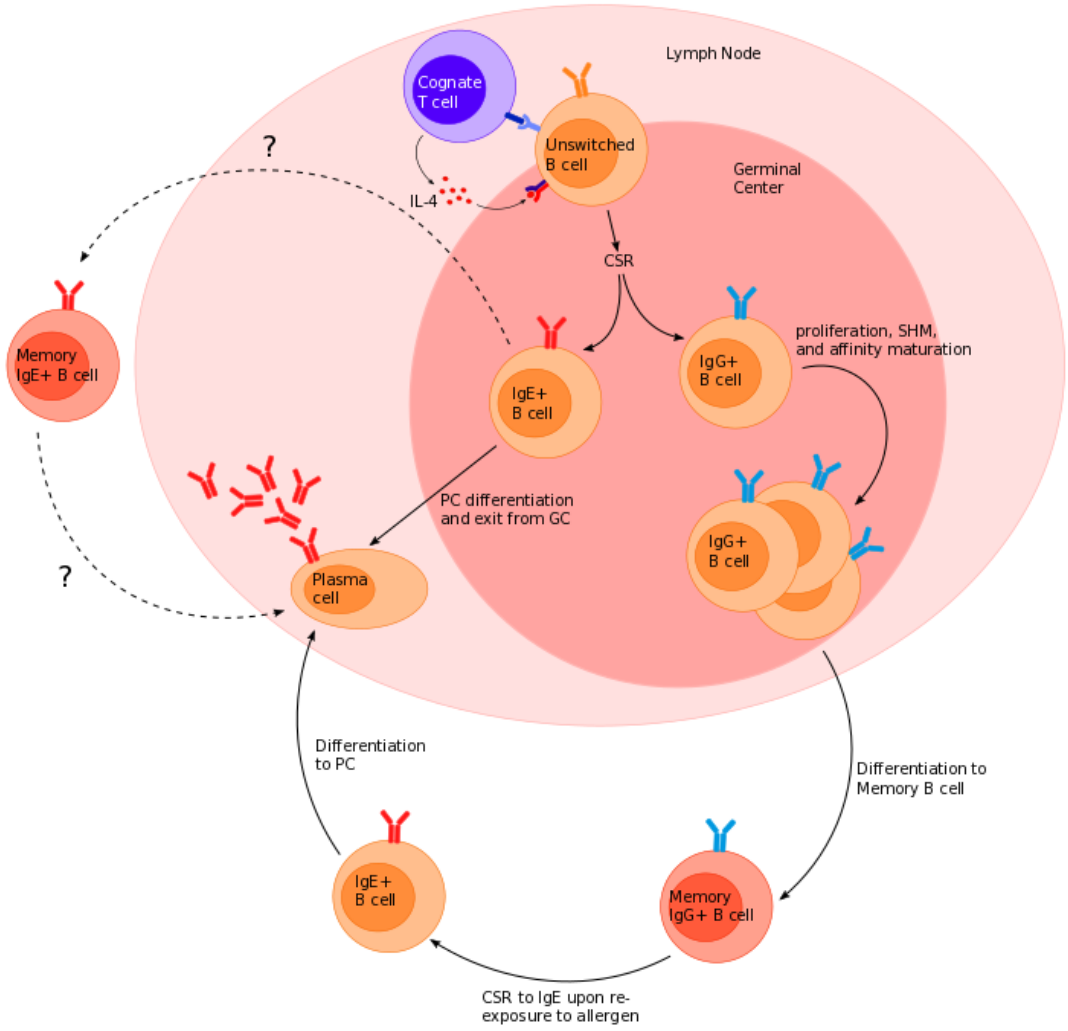

Figure 1.3 Possible mechanisms for the generation of allergic memory.
Current evidence suggests that IgE+ B cells rapidly exit the germinal center and give rise to short lived plasma cells. The ability of IgE+ B cells generated through direct switching to differentiate into long lived memory B cells is unclear. In contrast, IgG+ GC B cells remain in the germinal center and undergo multiple rounds of proliferation, somatic hypermutation, and affinity maturation, resulting in high affinity allergen specific B cells. These cells can then differentiate into memory B cells, and may undergo further CSR upon re-stimulation to generate high affinity IgE+ cells.

in the GC following CSR, affinity maturation, and expansion of allergen specific B cells [45]. However, given the evidence suggesting that IgE+ B cells only transiently localize to GCs, rapidly differentiate to PCs or undergo apoptosis after exit from the GC, and have low rates of affinity maturation, it seems unlikely that IgE memory arises from the conventional pathway. Some studies have suggested that memory IgG1 B cells that undergo a secondary CSR event serve as the reservoir for IgE memory[64, 65]. Adoptive transfer experiments have frequently been employed to further investigate the contribution of IgE+ vs IgG1+ B cells to IgE memory. B cell deficient μMT mice that received IgE+ B cells from *N. Brasiliensis* infected IgE-GFP mice exhibited significantly higher serum IgE titers following infection with *N. Brasiliensis* compared to mice that received IgE- B cells from uninfected IgE-GFP mice [59]. This data suggests that IgE+ B cells are the source of IgE memory. However, this experiment transferred bulk IgE- cells as opposed to specifically sorting out IgG1+ cells, and therefore the frequency of memory IgG1 cells transferred to recipients may have been insufficient to mount an IgE memory response. Memory B cells from mice with a monoclonal B cell receptor for influenza hemagglutinin (TBmc mice) were also transferred into naïve recipients to assess whether IgE+ or IgG1+ B cells were the main source of allergic memory. In these experiments He et al. transferred either total memory B cells, or IgE depleted memory B cells from OVA-HA immunized recipients into naïve recipients before challenge with OVA [64]. No difference was observed in HA-specific IgE titers between the two groups, suggesting that IgE- memory B cells that undergo a secondary CSR event to generate IgE+ B cells are a source of IgE memory.

While less work has been done to investigate the source of IgE memory in humans, sequencing of the Ig heavy chain (IgH) region of both allergic and non-allergic subjects to compare variable gene segments and complementarity-determining region 3 (CDR3) in order to identify clonally related lineages suggest that most IgE+ cells are derived from high affinity IgG+ cells [46], supporting the conclusions of He and colleagues [64]. However, a population of unswitched, IgM+ memory B cells that have undergone somatic hypermutation have been described in humans [66-68]. These IgM+CD27+ cells are long lived [69], and rapidly differentiate into Ig secreting cells following stimulation [70, 71]. While IgM+ memory B cells have not previously been implicated in the allergic response, our data from human subjects suggests that unswitched B cells may be important for mediating α-gal allergy.

Another key aspect of allergic memory that remains unclear is the development of long-lived IgE PCs. Long-lived PCs have been detected in both humans and mice, and play a vital role in maintaining immunity [72]. These cells are maintained in survival niches, often in the bone marrow, continuously secreting protective antibodies for years [73]. However, pathogenic long-lived PCs can also drive disease pathogenesis, as is the case in systemic lupus erythematosus (SLE) [74]. As with IgE+ GC and memory B cells, the study of long-lived IgE+ PCs has been complicated by the challenges associated with identifying IgE+ cells. However, there is evidence to suggest that long-lived IgE+ PCs develop in the context of allergy. Low levels of allergen specific IgE is chronically found in the serum of allergic individuals, even when patients have not been re-exposed to allergen for months or years [75]. Additionally, transfer of peanut allergy from donor to recipient following bone marrow transplant in humans has been

reported multiple times [76-78], suggesting that the bone marrow of allergic patients contains long-lived, allergen specific IgE+ PCs. As an alternative to long-lived IgE PCs, it's possible that chronic allergen specific IgE secretion is driven by memory IgG+ B cells that undergo another CSR event and differentiate to PCs, and sequencing of Ig heavy chain V-region to identify clonally related lineages of B cells in healthy and allergic subjects suggested that most IgE+ cells arise from high affinity IgG precursors [46].

Murine studies have also been employed to investigate IgE+ long-lived PCs, as the bone marrow and other lymphoid structures can be probed for these cells. A study by Luger and colleagues [75] suggested that long-lived IgE+ PCs were generated during the allergic response to OVA. IgE+ PCs were detected in the bone marrow and spleen of immunized mice 29 days after challenge, far beyond when short-lived PCs in the draining lymph node have declined. These cells were resistant to treatment with cyclophosphamide, indicating that these cells had exited the cell cycle, a characteristic of long-lived PCs [79]. However, due to the non-physiological route of sensitization and the use of adjuvants in conjunction with allergen, these conclusions have not been widely accepted in the allergy field. Experiments in IgE-GFP mice also suggested that long-lived IgE+ PCs are generated during the allergic response and that these cells localize to the bone marrow [59]. In contrast, only short-lived IgE+ PCs, found in the draining lymph node, were detected in Verigem mice following challenge with allergen [63]. Further studies are required to determine the source of chronic allergen specific IgE secretion. The use of different genetic modifications of the IgE loci, adjuvants, and non-physiologic routes of sensitization in the murine studies detailed above may account for the

conflicting conclusions about the pathway by which IgE+ B cells develop and the maintenance of allergic memory. Therefore, the development of new murine models of allergy could help to answer the questions that still remain about IgE+ B cells.

α-Gal allergy

Allergic reactions against the oligosaccharide galactose-α-1,3-galactose (α-gal) were first recognized in the US following the approval of Cetuximab, a mouse-human chimeric antibody specific for Epidermal Growth Factor Receptor (EGFR) for treatment of a variety of cancers [80]. Clinical trials of Cetuximab indicated a low risk of hypersensitivity responses, and when reactions did occur the severity was mild [80, 81]. However, when use of Cetuximab increased a high frequency of hypersensitivity reactions was observed in patients in the southeastern US. Observations at the University of North Carolina revealed that severe (grade 3 or 4) reactions occurred in approximately 22% of patients, far higher than the frequency of 3% observed nationally [82]. Analysis of pre-treatment serum revealed that individuals who experienced reactions had pre-existing IgE that bound to Cetuximab, and further work determined that these antibodies were specific to α-gal, an oligosaccharide found on the murine portion of the antibody [83]. Interestingly, α-gal-specific IgE was also found in a subset of the control subjects from Tennessee, but not in those from Boston or California [83].

At the time that Cetuximab infusions were found to induce severe hypersensitivity responses in a subset of patients, numerous case reports of urticaria, angioedema or anaphylaxis with no clear cause came to the attention of physicians [84]. These cases occurred in the same region as the severe Cetuximab reactions. In some cases, patients

suggested that the responses seemed to occur several hours after consuming red meat, such as beef, pork, lamb, or venison. Identifying the cause of these responses was initially challenging, as a delay between consumption and reaction is unusual in the context of food allergy [84]. Additionally, in many cases patients who experienced these responses had a history of consuming red meat with no adverse reaction for decades [85], unlike most food allergies that arise early in life. Skin prick tests with beef, pork, or lamb extract also showed no significant response, suggesting that red meat was not the culprit. However, intradermal testing with the meat extracts elicited strong positive results and food challenges confirmed that these responses were caused by consumption of red meat [84]. Further work revealed that α-gal-specific IgE was driving the allergic response to red meat in these individuals [84]. Similar responses to red meat have also been reported in Australia [86], France [87, 88], Germany [89], Spain [90], Sweden [91, 92], and Japan [93]. The unusual nature of the allergic response to α-gal has made diagnosis of α-gal allergy challenging, and anecdotal evidence suggests that it is not usual for patients to have experienced multiple allergic reactions to red meat before α-gal-specific IgE titers are assessed and the allergy is identified.

The driving factor for development of α-gal-specific IgE in the US was initially unclear. Both Cetuximab hypersensitivity and red meat allergy were restricted to the same geographical area, mainly North Carolina, Virginia, Tennessee, Arkansas, and part of Missouri [82], suggesting that an environmental influence may exist. Interestingly, this region overlapped with the area that had the highest incidence of Rocky Mountain Spotted Fever (RMSF) [94], of which the tick *Amblyomma americanum* (lone star tick) is a major vector. Interestingly, three individuals investigating the α-gal story developed red

meat allergy and they had all recently received tick bites [94]. Comparison of serum obtained before and after tick bites in these individuals revealed a significant increase in α-gal-specific IgE titers. A link between tick bites and red meat allergy had already been identified in Australia [86], providing support to the theory that tick bites could drive production of α-gal-specific IgE. Further investigation revealed that a large contingent of patients in the US with red meat allergy also had a history of tick bites. Analysis of serum from these patients revealed a significant correlation between IgE to α-gal and IgE to *A. americanum* proteins [94, 95]. More recently α-gal has been found in the gut [92] and saliva [96] of ticks linked to red meat allergy in other countries, and tick bites were sufficient to induce α-gal-specific IgE titers in α-gal deficient mice [96]. In the context of a tick bite, exposure to α-gal would occur through the skin. Given the current evidence for the skin as an important site for allergic sensitization, as outlined above, these data further support the hypothesis that tick bites drive the development of red meat allergy.

Studies of α-gal first began decades ago in the transplant field. α-gal is present on the tissues and cells of all non-primate mammals. Humans and primates lack functional alpha-1,3-galactosyltransferase, the enzyme required to generate α-gal, and do not produce the oligosaccharide [97, 98]. Interestingly, all immunocompetent humans naturally produce IgG antibodies that are able to bind to α-gal [99], and this is thought to be driven by exposure to α-gal expressed by microbes in the gastrointestinal tract [100]. These anti-Gal IgG antibodies are capable of driving rejection of tissue grafts from other mammalian species, and therefore α-gal was originally identified as a major barrier to

xenotransplantation [101]. What drives the transition from anti-Gal IgG to α-gal-specific IgE in the context of red meat allergy is currently unknown.

We propose that α-gal allergy may serve as an ideal system to study the development and maintenance of the IgE+ B cells which drive pathogenesis of allergic disease. Unlike other allergies, in which the sensitization event(s) have not been identified, the evidence clearly points to tick bites as initiating the development of α-gal-specific IgE. Knowledge of the initiating event provides a previously unavailable opportunity to study the signals that drive sensitization to allergen. Additionally, although α-gal allergy has been documented in children [102], the allergy frequently develops in adults, which eases acquisition of larger sample volumes for human studies. Because of the role of α-gal in preventing xenotransplantation, a number of α-gal-deficient animals have been developed and are available for the development of new allergy models. Although a variety of murine models of allergy have already been established, these models generally rely on the use of adjuvants or non-physiologically relevant routes of sensitization, as detailed above. Differences in sensitization methods may explain the conflicting results obtained by murine allergy studies to this point, and these results may not be indicative of what happens in allergic humans. However, our work, as well as that of Araujo and colleagues[96], indicates that no additional adjuvants are required to develop and IgE response to tick extract. Therefore, the results of murine studies of tick-borne allergy may more accurately represent the immunological mechanisms that occur in α-gal allergic humans.

Project Rationale

The rising prevalence of allergic disease, combined with the potential for life-threatening reactions in allergic individuals makes the development of effective treatments for the prevention of allergic reactions a pressing public health issue. While previous studies have provided some understanding of the immunologic mechanisms of allergies, our lack of knowledge of the signals that drive the development of an IgE response to innocuous antigen, and how allergic memory is maintain has impeded the development of more effective therapies for treatment and prevention of the allergic response. The purpose of this work is to identify the B cell subsets which mediate allergic pathogenies in the context of red meat allergy, to develop a new, physiologically relevant model system for the study of the IgE response, and to improve our understanding of the mechanisms by which an IgE response is induced. We hypothesize that a novel class switched B cell population(s) can be found in allergic individuals that mediate allergic pathogenesis. Additionally, we theorize that tick derived proteins include molecules that activate the immune response, providing an adjuvant effect that leads to the development of an IgE response against alpha-gal. The results of these studies will improve our understanding of the immunologic mechanisms of the IgE response and provide new targets for the treatment of allergy.

CHAPTER 2: AN INTEGRATED FRAMEWORK USING HIGH-DIMENSIONAL MASS CYTOMETRY AND FLUORESCENT FLOW CYTOMETRY IDENTIFIES DISCRETE B CELL SUBSETS IN PATIENTS WITH RED MEAT ALLERGY

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Abstract

Background: B cells play a critical role in the development and maintenance of food allergy by producing allergen-specific IgE. Despite the importance of B cells in IgE-mediated food allergy, the identity of sIgE-producing human B cells and how IgE is regulated are poorly understood.

Objective: To identify the immunophenotypes of circulating B cells associated with the production of galactose-alpha-1,3-galactose specific IgE production in patients with red meat allergy.

Methods: B cells in PBMC samples obtained from 19 adults with physician-diagnosed red meat allergy and 20 non-meat allergic healthy controls were assessed by mass cytometry along with a bioinformatics analysis pipeline to identify discrete B cell phenotypes that associated with serum sIgE. Fluorescent flow cytometry was then applied to sort purify discrete B cell subsets, and B cells were functionally evaluated on an individual cell level for the production of sIgE by ELISPOT.

Results: Discrete B cell phenotypes abundant in meat allergic subjects, but not in non-meat allergic controls, were found in peripheral blood that do not share typical characteristics of classical isotype-switched memory B cells that express high levels of CD27. These B cell subsets shared higher IgD and lower IgM expression levels coupled with CXCR4, CCR6 and CD25 expression. *In vitro* polyclonal stimulation of purified B cell subsets from meat allergic subjects demonstrated that these subsets were enriched for cells induced to secrete sIgE.

Conclusions and Clinical Relevance: Circulating B cells display increased abundance of discrete B cell subsets in meat allergic subjects. This observation, coupled with the

capacity of individual B cell subsets to produce sIgE following activation, implicates these novel B cell phenotypes in promoting IgE in meat allergy.

Introduction

Red meat allergy, also known as α-gal syndrome, is among a minority of food allergies that pose a serious acute health risk through induction of IgE-mediated anaphylactic reactions. This novel form of food allergy develops in adults worldwide and is thought to result from tick bites through mechanisms that remain unknown [84, 86, 87, 89, 90, 92, 94, 103, 104]. Allergic reactions in patients following consumption of red meat are driven by allergen-specific IgE (sIgE) against the oligosaccharide galactose-α-1,3-galactose (α-gal) [84], which is present in the tissues of all non-primate mammals [97, 98]. Despite the importance of IgE in the pathogenesis of allergic diseases, the identity of sIgE-producing human B cells and their frequency are poorly understood. The reason for this is because B cells that express IgE are found at very low frequencies and that serum IgE binds to Fc receptors for IgE on the surface of B cells [105-109]. Moreover, there has been a lack of robust assays that allow for comprehensive immunophenotyping of B cells within complex biological samples. Although some studies have described IgE-expressing B cells in the blood of allergic and healthy individuals [110-112], the contribution of such cells to IgE responses is unclear. These observations underscore a need to evaluate IgE-producing B cells with greater resolution to determine their clinical relevance in allergic diseases.

Here, we sought to interrogate the phenotypes of circulating B cells in patients with food allergy to red meat. The study was designed to sample B cells in peripheral blood of patients actively avoiding meat who had positive α-gal sIgE titers and histories of delayed urticaria after eating mammalian meat. Using mass cytometry with a bioinformatics analysis pipeline and traditional fluorescent-based flow cytometric cell

sorting approaches, we aimed to determine whether discrete B cell subsets could be identified in meat allergic subjects that associated with α-gal sIgE production. Mass cytometry by time-of-flight (CyTOF) combines antibodies labeled with metal isotopes with mass spectrometry, which allows for single-cell analysis of more than 40 parameters simultaneously with minimal interference from signal overlap between channels that are encountered with highly-multiparametric flow cytometry [113-115]. Application of the computational algorithms t-SNE, an automated clustering tool [116, 117], and flowType, which defines all possible cell subsets that correlate with a clinical parameter [118, 119], to CyTOF datasets led to identification of discrete B cell subsets whose abundance correlated with α-gal sIgE titers in blood of patients. Our analytical approach also facilitated the transition from CyTOF to fluorescence-based cell sorting, enabling functional examination of cultured B cell subsets that cannot be achieved with mass cytometry since cells are vaporized. Testing the capacity of these rare B cell subsets to secrete antibody following *in vitro* stimulation demonstrated that such cells produced α-gal sIgE in patients with red meat allergy. Our findings support a novel B cell signature in meat allergic subjects that associates with α-gal sIgE production, which may play a role in the pathogenesis of this food allergy.

Materials and methods

Human subjects

All participants were adults (ages 23-77) and included patients with physician-diagnosed red meat allergy with α-gal sIgE positive titers > 0.35 IU/mL, and non-meat allergic healthy controls with no α-gal sIgE antibodies (Table S2.1). Inclusion criteria for meat

allergic subjects included histories of delayed urticaria after meat consumption. All meat allergic subjects were actively avoiding meat. Non-meat allergic controls had no history of reactions to mammalian meat by self-report, no history of other food allergies or anaphylaxis, and had normal total IgE levels for age. Subjects were non-pregnant without a history of chronic illness. All subjects were recruited through the UVA Allergic Diseases Clinic, or else by advertisement. All studies were approved by the University of Virginia Institutional Review Board under protocols #13166 and #13298, and all study participants gave written informed consent.

Total and α-gal specific IgE

Total and α-gal IgE antibodies in blood serum were measured using the ImmunoCAP system (Phadia US, Portage, Mich) with the ImmunoCAP 250 instrument, according to the manufacturer's instructions. The streptavidin CAP technique was used to measure IgE levels to α-gal as described previously [83, 120], in which 5 μg of biotinylated cetuximab, a chimeric mouse-human IgG1 mAb against epidermal growth factor receptor that contains α-gal moieties on the Fab portion of the cetuximab heavy chain that are recognized by IgE specific for α-gal, was added to each streptavidin-coated CAP before serum was added. Results were calculated as kUA/L. α-gal sIgE antibody-secreting cells were quantified by ELISPOT. Briefly, ELISPOT plates were coated overnight at 4°C with 10 μg/mL unlabeled cetuximab mAb. Plates were blocked with 10% FBS in RPMI, washed and samples were added in appropriate dilutions for 18 h. After washing, biotin-conjugated mouse anti-human IgE antibody (BioLegend, San Diego, Calif) was added overnight. Plates were washed and HRP-avidin (SouthernBiotech) was added for 24 h.

AEC substrate solution was used to obtain coloration, and washing plates with water stopped the reaction. The number of antibody-secreting spots was quantified using a dual-axis light-dissecting microscope and confirmed using an Immunospot Microanalyzer (Cellular Technology Limited, Shaker Heights, OH). The numbers of IgE ASCs per million cells were calculated.

Cell preparation and culture

Peripheral blood mononuclear cells (PBMC) of 14 meat allergic subjects and 10 non-meat allergic controls were isolated from venous blood in collection tubes with acid citrate dextrose (ACD) by density-gradient centrifugation on Ficoll-Paque PREMIUM media (GE Healthcare, Pittsburgh, Penn), and washed twice in PBS. Cells were either frozen in FBS (ThermoFisher Scientific, Rockford, Ill) with 20% DMSO achieving > 95% cell viability, or freshly enriched for B cells by negative selection using the Human Pan B cell isolation kit (Miltenyi Biotech) and sort purified. B cells were cultured in RPMI (ThermoFisher Scientific) with 15% FBS, L-glutamine-penicillin-streptomycin (Sigma Aldrich, St. Louis, MO), beta mercaptoethanol (Sigma-Aldrich), and 20 ng/mL recombinant human IL-4 (Peprotech, Rocky Hill, NJ). In 96-well plates, cells were cultured in the presence of 12.5 ng/mL PMA (EMD Millipore Corp, Billerica, Mass) and 500 ng/mL ionomycin (EMD Chemicals, San Diego, Calif) at 37°C in an atmosphere of 5% CO_2. Cells were collected at day 8 to quantify α-gal sIgE-secreting cells by ELISPOT.

Mass cytometry data acquisition and analysis

Thawed PBMCs from 19 meat allergic subjects and 20 non-meat allergic controls were washed in PBS with 5% FBS and incubated with 5 μM Cell-ID Cisplatin (Fluidigm, San Francisco, Calif) to determine viability, as previously described [121]. Mean PBMC viability after thawing was 94%, as determined by trypan blue exclusion. Patient samples were obtained over a period of two years, with initial samples analyzed prior to the availability of barcoding reagents. Therefore, barcoding was not used in cell preparation and analysis to maintain consistency across all samples. For cell surface marker analysis, 3×10^6 live cells were first incubated with Fc block to prevent non-specific binding, washed, and stained in 100 μL PBS with 5% FBS and Ab cocktail at room temperature for 30 min. All of the antibodies used in the panel (Table S2.2) were purchased pre-conjugated to metals (Fluidigm) and concentrations optimized through the Mass Cytometry Antibody Bank services provided by the UVA Flow Cytometry Core Facility. Surface phenotypes between freshly isolated and cryopreserved PBMCs were compared and confirmed to be equivalent. Cells were washed twice in PBS with 5% FBS and then stained with Cell-ID Intercalator-Ir (Fluidigm) in Maxpar Fix and Perm Buffer (Fluidigm) at 4°C overnight. Samples were washed once in PBS with 5% FBS, once in Maxpar H_2O, resuspended in Maxpar H_2O, and then collected on a CyTOF 2 instrument (Fluidigm). For quality control, the acquisition rate was maintained under 400 events/s, and events were normalized using bead standards to minimize batch effects, as previously described [122]. FCS files containing the normalized single-cell data were uploaded to Cytobank for analysis. All data sets used here are publicly available at http://www.cytobank.org.

Negative staining for cisplatin and positive staining for iridium identified cells, and doublets were excluded by higher DNA content and longer event length. Human immune cell subsets were identified using standard markers as reported previously [123, 124]. Total $CD4^+$ T cells were identified by gating on $CD3^+CD20^-CD4^+$ cells. Total $CD8^+$ T cells were identified by gating on $CD3^+CD20^-CD8^+$ cells. NK cells were identified by gating on $CD3^-CD20^-CD14^-CD56^+$ cells. DCs were identified by gating on $CD3^-CD20^-CD56^-CD14^-$HLA-DR^+ cells. Monocytes were identified by gating on $CD3^-CD20^-CD14^+$ cells. Total B cells were identified by gating on $CD20^+$ $CD3^-$ cells, and were analyzed using the data analysis platforms viSNE and flowType. viSNE is a CyTOF analysis tool that uses the t-distributed stochastic neighbor embedding (t-SNE) algorithm to analyze and display high-dimensional data on a two-dimensional map, with cells colored according to frequency or expression of a marker (available at http://www.cytobank.org) [125]. The Bioconductor package flowType is a method that defines all possible cell subpopulations within a sample based on a certain combination of marker expression levels from all parameters measured (available at http://www.bioconductor.org). The heterogeneity of B cell populations was first measured using t-SNE inferred clustering by viSNE. FlowType was used on the arsinh transformed data (cofactor 5) to partition the cells into a immunophenotype hierarchy as described previously [126]. To identify immunophenotypes of B cells found to be differentially abundant between allergic and control subjects, a generalized linear model was fitted to the immunophenotyped hierarchy, using a quasi-likelihood framework in edgeR to estimate the dispersion of cell counts as described previously [126, 127]. Immunophenotypes enriched in allergic subjects and having adjusted *P*-values better than a false discovery rate (FDR) using the

Benjamini-Hochberg method of 5% were plotted in a heat map. The code to perform this set of analyses is available on the cyttools github at https://github.com/bc2zb/cyttools.

Flow cytometry and cell sorting

For flow cytometric analysis, PBMCs were stained with fluorescent-labeled surface markers (Table S2.3), followed by the live/dead stain AQUA (Invitrogen). Cells were acquired on an Invitrogen Attune NxT, or sorted with a Becton Dickinson Influx Cell Sorter, and analysis was performed using FlowJo software version 9.3.3 (TreeStar Inc., Ashland, Ore). Dead cells and doublets were excluded from the analysis and cell population gates were determined using fluorescence-minus-one controls.

Statistics

Data are presented as the mean ± SD. Statistical analyses were performed using GraphPad Prism, version 6 (GraphPad Software, La Jolla, Calif). Statistics were performed with the Fisher exact test, and data with a 2-sided P-value < 0.05 was considered significant. Pearson's correlation coefficient was used to determine the strength of association between 2 variables. The Mann-Whitney U nonparametric test was used to compare differences between allergic and control groups for the frequencies of α-gal sIgE ASCs in cultures of sorted B cells.

Results

Meat allergic subjects and healthy controls show equivalent distribution of major circulating immune cell populations

We first established a CyTOF staining panel for testing the cellular heterogeneity within PBMCs isolated from venous blood. This panel contained 23 leukocyte markers that allowed us to identify all of the major immune cell populations, and to profile B cells with greater dimensionality by incorporating various markers based on prior indications of their utility in polychromatic flow cytometry from the literature (Table S2.2). CyTOF was performed on cryopreserved PBMC samples obtained from the blood of meat allergic patients with a range of positive α-gal sIgE titers and healthy controls (Fig. 2.1A). Characteristics of subject groups are summarized in Supplementary Table 2.1. After data normalization, we initially assessed the frequencies of major immune cell populations ($CD4^+$ and $CD8^+$ T cells, $CD56^+$ NK cells, HLA-DR^+ DCs, $CD14^+$ monocytes, and total $CD20^+$ B cells), identified by manual gating on standard individual markers. No significant differences in the frequencies of T cells, B cells, NK cells, and DCs between allergic and control samples were observed (Fig. 2.1B). Monocytes from allergic subjects were found at higher frequencies compared with controls. We further compared the frequencies of the major mature B cell populations found in peripheral blood when manually gated on the standard phenotypic markers CD3, CD20, IgD, and CD27. No significant differences in the frequencies of naïve B cells ($CD3^-CD20^+IgD^+CD27^-$), unswitched memory B cells ($CD3^-CD20^+IgD^+CD27^+$) and switched memory B cells ($CD3^-CD20^+IgD^-CD27^+$) were observed in meat allergic subjects compared with healthy controls (Fig. 2.1C). There was also no significant difference in the frequency of the double negative B cell population (IgD^-CD27^-), which includes switched memory B cells [128, 129]. Interestingly, this B cell population has been shown

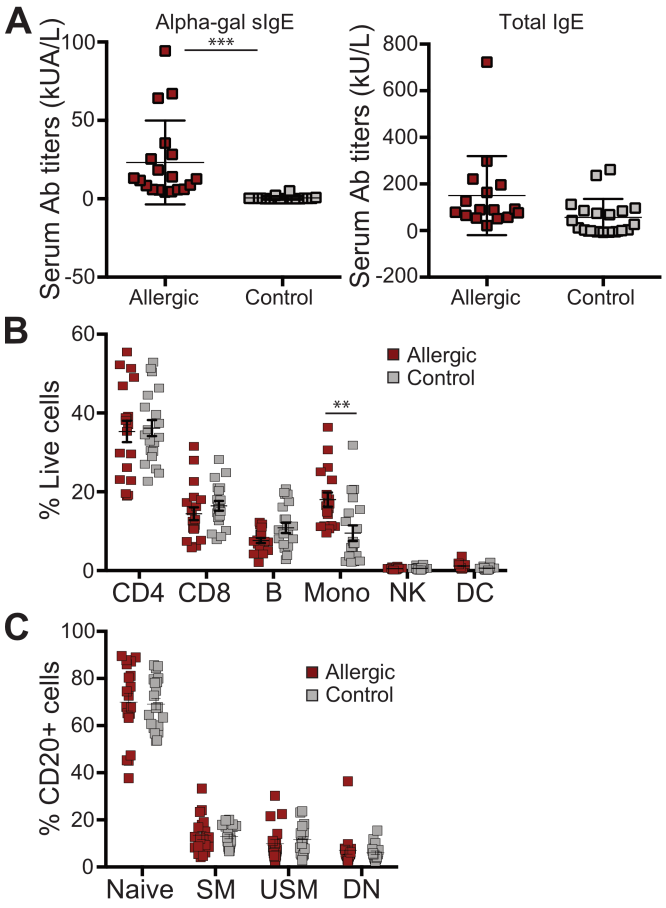


Figure 2.1 Frequencies of circulating immune cell populations in meat allergic and control subjects.

(**A**) Serum alpha-gal sIgE and total IgE titers in meat allergic (n = 19) and non-meat allergic controls (n = 20), ***$P < 0.001$. (**B**) $CD4^+$ and $CD8^+$ T cells, natural killer cells (NK), dendritic cells (DC), monocytes (Mono), and total B cells expressed as proportion of live $CD45^+$ leukocytes, as well as (**C**) naïve B cells, immunoglobulin unswitched (USM) and switched (SM) memory B cells, and double negative (DN) B cells expressed as proportion of total $CD20^+$ B cells of subjects were quantified by manual gating.

to contain $IgE^{+}CD27^{-}$ memory B cells that are enriched in the blood of children with food allergy [130]. Flow cytometric analysis using fluorescent-labeled CD3, CD20, IgD, and CD27 confirmed these results (Fig S2.1).

Comparable results were also observed when B cells were automatically gated using the cloud-based Astrolabe platform designed for mass cytometry data (astrolabediagnostics.com). Using machine learning, Astrolabe identifies cell subsets based on curation of phenotypic signatures reported in the literature and performs differential abundance analysis. Moreover, this platform allows for the stratification of cellular subsets based on expression of markers that correlate with clinical parameters, including disease states. No significant differences in the frequencies of 7 curated B cell subsets, representing naïve, memory B cells and double negative B cells based on IgD and CD27 expression, were found between subject groups (Fig. 2.2A-B). Taken together, these findings support the view that defining major immune non-B cell and B cell populations based solely on conventional immune parameters shows no difference in cell frequencies of meat allergic subjects, and does not account for potential cellular heterogeneity within these populations.

Distinct B cell phenotypes are observed in meat allergic subjects

Having established that the standard B cell markers used to delineate total, naïve and memory B cells did not reveal changes in cell numbers from meat allergic and healthy subjects, we applied the t-SNE dimensionality reduction approach, viSNE [116, 117] to the CyTOF datasets. Analysis of total $CD20^{+}$ B cells using viSNE revealed heterogeneity both within the major mature B cell populations and between meat allergic and control

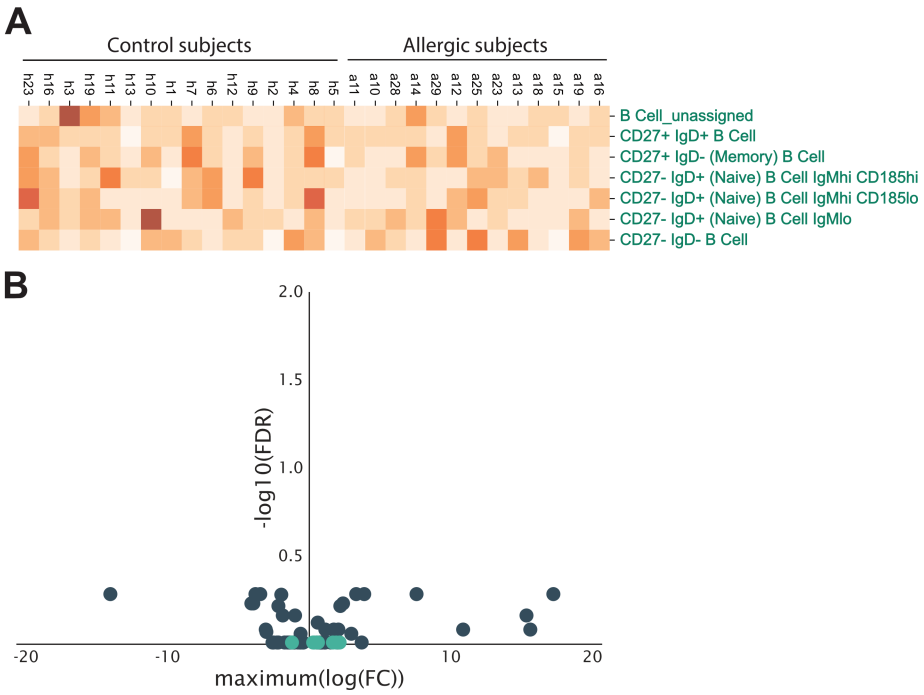


Figure 2.2 Comparable frequencies of curated B cell populations defined by automated gating in meat allergic and control subjects.
(**A**) Heatmap of the relative frequency of 7 different B cell populations in meat allergic (n = 19) and non-meat allergic control (n = 20) subjects. B cell populations were assigned by the Astrolabe Diagnostics analysis platform. (**B**) Differential abundance analysis of the frequencies of B cell populations (light blue dots) compared to non-B cell immune cell populations (dark blue dots) shows no difference between allergic and control subjects.

subjects (Fig. 2.3A, Fig. S2.2). In particular, both naïve and unswitched memory B cells were divided into multiple islands that were spatially separated, indicating phenotypic heterogeneity within these B cell populations that has not been appreciated previously. Although the location of each B cell population within the viSNE plots were similar between allergic and control subjects, there were marked differences in the clustering patterns of cells within each island. To prevent variability among subjects of each group from obscuring differences between meat allergic and control cohorts, the datasets from each subject group were concatenated and total $CD20^+$ B cells were analyzed by viSNE to determine whether the differences observed previously in individual subjects (Fig. 2.3A, Fig. S2.2) were maintained across each group. Results confirmed that notable differences in the distribution of naïve and unswitched memory B cells between meat allergic subjects and controls were observed (Fig. 2.3B). Moreover, coloring the t-SNE plots to show IgD and CD27 expression levels revealed substantial differences in the clustering pattern of IgD^+ B cells and smaller differences in the clustering pattern of $CD27^+$ B cells between meat allergic and control subjects (Fig. 2.3C). Differences in the distribution of B cell populations between meat allergic and control subjects were also found using SPADE [131], a computational tool that clusters cells into biologically relevant subpopulations based on the intensity of each marker (Fig. S2.3). Given that the frequencies of total naïve B cells and unswitched memory B cells were not different between the subject groups (Fig. 2.1C), these findings establish the capacity of our CyTOF panel to detect phenotypic changes of B cells within these cell populations when expression of all 23 markers are simultaneously analyzed.

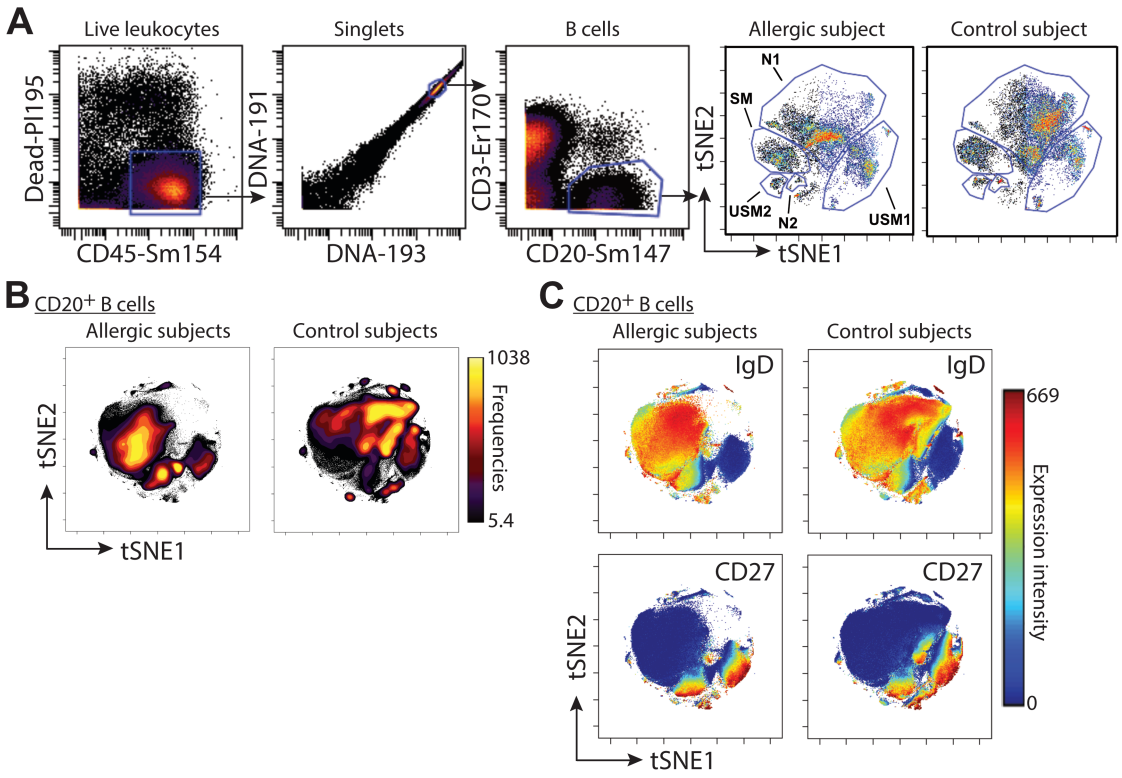


Figure 2.3 viSNE analysis of CyTOF data shows phenotypic changes of B cells in meat allergic subjects.

(**A**) Gating strategy for viSNE analysis on CD20^{+} B cells. Representative data from 1 meat allergic and 1 control subject showing heterogeneous clusters of naïve B cells (N1, N2), unswitched memory B cells (USM1, USM2) and switched memory B cells (SM) based on IgD and CD27 expression. (**B** and **C**) viSNE plots of concatenated datasets from meat allergic (n = 19) and non-meat allergic (n = 20) control subjects. Plots are colored by (**B**) cell frequency, or by (**C**) IgD and CD27 expression levels.

Markers within the CyTOF panel are identified that define B cell clusters associated with α-gal sIgE in meat allergic subjects

To determine the identity of markers that distinguished B cell phenotypes associated with red meat allergy subjects, we analyzed the CyTOF data using the flowType algorithm [118, 119]. This computational approach first uses automated gating to define all possible cell subsets within a dataset based on integrated marker expression levels, and then assesses the relationship between each subset and the clinical outcome. Results demonstrated that approximately 500,000 immunophenotypes of B cells were identified within the CyTOF panel by flowType (Fig. S2.4A). These immunophenotypes result from further division of traditional B cell populations into related subpopulations. To identify the immunophenotypes of B cells found to be differentially abundant between meat allergic and control subjects, a generalized linear model was fitted to the data, using a quasi-likelihood framework to estimate the dispersion of cell counts [126, 127]. The frequencies of 710 B cell immunophenotypes (hereafter, referred to as subsets) with adjusted *P*-values better than a FDR of 5% were found significantly enriched in meat allergic subjects compared to control subjects, and are plotted in a heatmap (Fig. 2.4A). These B cell subsets varied in frequency between allergic subjects but were observed in those subjects with positive α-gal sIgE titers. However, a correlation with these B cell subsets and the range of α-gal sIgE titers was not apparent. No significant correlation between the frequencies of these B cell subsets and total IgE titers, patient age, or gender in meat allergic subjects was found (data not shown), suggesting that the abundance of discrete B cell subsets is linked to positive α-gal sIgE in meat allergic subjects.

Expression levels of 11 of the 23 markers in the CyTOF panel were used by

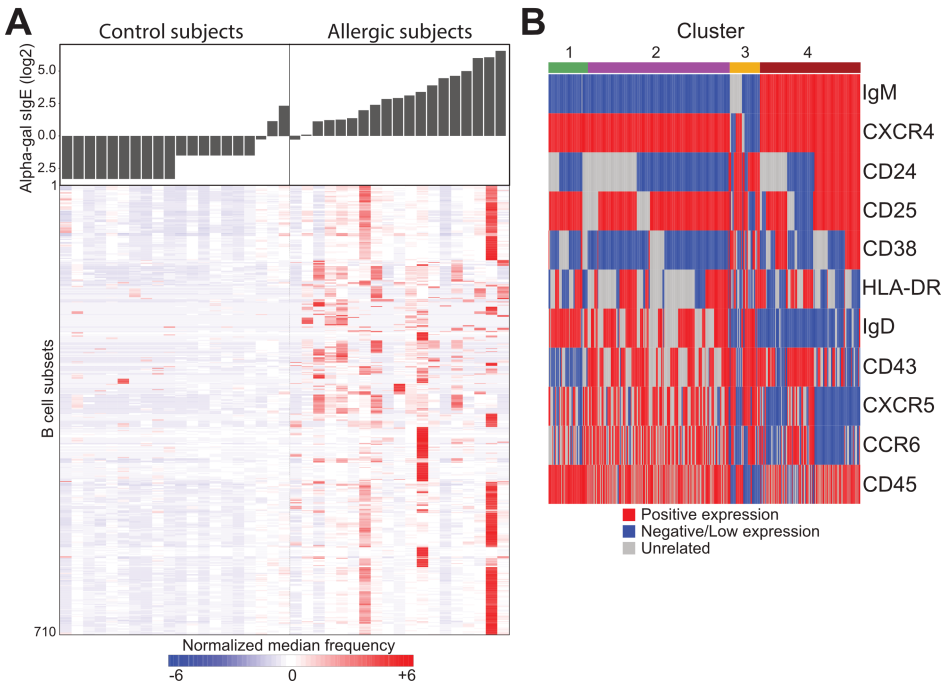


Figure 2.4 FlowType analysis identifies discrete B cell immunophenotypes that associate with alpha-gal sIgE in meat allergic subjects.
(**A**) Heat map resulting from flowType analysis of CyTOF datasets. Each row represents a phenotypically distinct B cell subset based on marker expression levels, with its relative frequency in each subject indicated by the color scale. Corresponding serum alpha-gal sIgE for each subject is shown above the heat map. (**B**) Heat map showing the expression of 11 markers within the CyTOF panel needed to minimally define B cell subsets abundant in meat allergic subjects. Expression levels of the indicated marker are assigned to each row, with each column representing a distinct B cell subset. B cell subsets were assigned to 4 main clusters based on the degree of shared marker expression. FlowType analysis was completed by Dr. Brain Capaldo.

flowType to define these B cell subsets. These subsets were assigned into four main clusters based on similar expression patterns of the 11 markers (Fig. 2.4B). Cluster 1 comprised B cell subsets that shared negative expression of IgM, CD24, CD38, and CD43, variable expression of HLA-DR, and shared positive expression of IgD, CD45, CXCR4, and CD25. Cluster 2 comprised phenotypically related B cell subsets that shared negative or low expression of IgM, CD38 and CD24, but generally shared positive expression of IgD, CXCR4, CCR6, and CD43. Cluster 3 comprised B cells that shared negative or low expression of IgM and CXCR4 and positive expression of CD24, CD25 and IgD. Cluster 4 comprised B cell subsets that shared positive expression of IgM and CXCR4 but were heterogeneous in their expression levels of other markers. Interestingly, expression levels of the classical memory B cell marker CD27 did not discriminate the four clusters of B cell subsets from the meat allergic subjects using flowType, with some B cells expressing CD27 and some B cells lacking CD27 expression (Fig. S2.4B). Membrane IgG and IgE expression was not detectable on B cells of any cluster identified by flowType (data not shown). Together, these results demonstrated that the B cell signature associated with red meat allergy is rare compared to the overall number of B cell immunophenotypes identified by our CyTOF panel, and does not show features of conventional human memory B cells, as measured by high expression of CD27 and intermediate antibody isotypes.

B cells from clusters identified by flowType are capable of producing α-gal sIgE following in vitro stimulation

To evaluate and compare the functional properties of B cells associated with red

meat allergy, we designed a fluorescent cytometry panel based on the markers used by flowType to group B cell subsets into the four main clusters (Supplementary Table 3). This approach allowed us to sort live B cells within each cluster to test their capacity to secrete α-gal sIgE *in vitro* following stimulation (Fig. 2.5A). We tested the ability for polyclonal stimulation of B cells to induce α-gal sIgE production to preclude suppositions of the activation requirements for these novel B cell phenotypes. The structural conformation of α-gal has not been determined, nor is it clear if and how the structural conformation affects IgE binding and B cell activation [95]. After stimulation with PMA, ionomycin and IL-4, we first assessed α-gal sIgE in culture supernatants by ImmunoCAP. However, given the small number of cells of each B cell cluster from subjects and the short half-life of soluble IgE the amount of IgE antibodies contained in supernatants was below the level of detection (data not shown). We therefore employed the ELISPOT assay as a sensitive method to enumerate α-gal sIgE antibody-secreting cells (ASCs) from the B cell cultures (Fig. 2.5B-C). Results demonstrated that α-gal sIgE-secreting B cells following *in vitro* stimulation were detected in 9 of 14 meat allergic subjects, with α-gal sIgE ASCs from the same individual found in Clusters 2 and 3 of four patients, Clusters 1 and 3 in one patient, Clusters 1, 2 and 3 in one patient, and in Cluster 2 only of three patients. In contrast, α-gal sIgE-secreting B cells following stimulation were detected in Clusters 1 and 3 from one control subject, with no ASCs found in Cluster 2. Analysis of Cluster 4 revealed that three allergic patients but no control subjects had α-gal sIgE ASCs following stimulation. These findings demonstrated that Clusters 2 and 3, which contain rare subsets of B cells with higher IgD

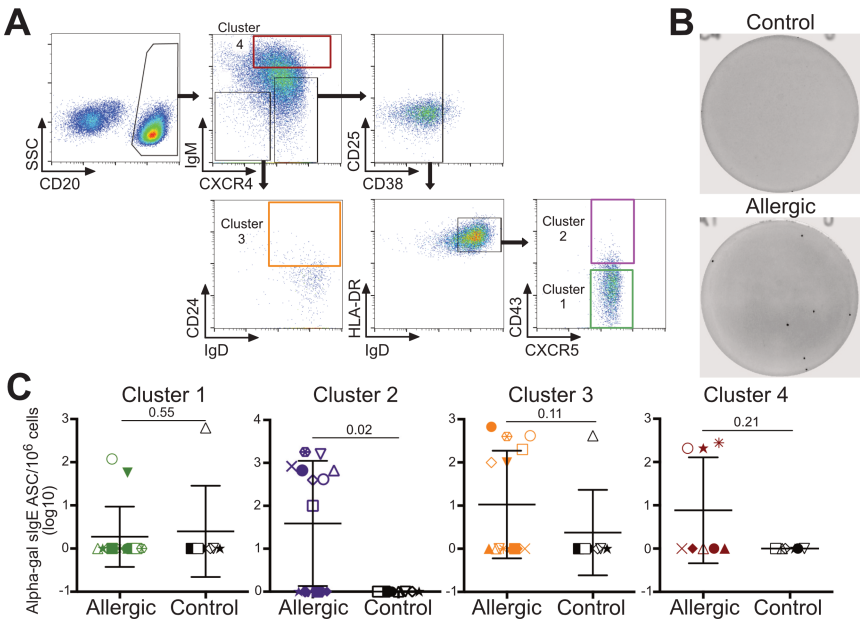


Figure 2.5 Polyclonal stimulation of B cells sorted from B cell clusters identified by flowType are capable of producing alpha-gal sIgE in meat allergic subjects.
(**A**) Gating strategy for sort purification of B cell subsets grouped into 4 main clusters, with each cluster indicated by a colored gate. (**B** and **C**) B cells of each cluster from meat allergic (n = 14) and non-meat allergic control (n = 10) subjects were cultured 8 days and analyzed for alpha-gal sIgE antibody-secreting cells (ASC) by ELISPOT. (**B**) Representative ELISPOT assay images from 1 allergic and 1 control subject. (**C**) Frequencies of alpha-gal sIgE ASCs of each B cell cluster after culture expressed as the number of ASCs per million cells. *P*-values are shown in each panel.

and lower IgM expression levels coupled with CXCR4, CCR6 and CD25 expression, are enriched for cells that are able to differentiate into α-gal sIgE producing B cells following *in vitro* polyclonal activation.

Discussion

Using an integrated framework that combined mass cytometry with a bioinformatics pipeline, we have demonstrated that a novel B cell signature is associated with meat allergic subjects. We have taken advantage of the algorithms, viSNE and flowType, to identify the minimal number of markers within our CyTOF panel that defined increased abundance of novel B cell phenotypes in PBMCs of meat allergic subjects. Eleven surface markers defined 710 phenotypically-related but discrete subsets of B cells in meat allergic subjects, which can be grouped into four main clusters based on shared expression levels. Moreover, we confirmed these findings using fluorescent flow cytometry that enabled subsequent functional analysis of purified B cells contained within each cluster to secrete α-gal sIgE following *in vitro* stimulation.

Our findings demonstrated that the B cell subsets capable of secreting sIgE following activation are found within the naïve population and do not share typical characteristics of classical isotype-switched memory B cells that express high levels CD27 [66, 70]. These cells may represent a novel population of memory B cells lacking CD27 expression found in the peripheral circulation [128, 129] that are prone to differentiate into IgE-secreting cells specific for α-gal in meat allergic patients. Early work in human adults identified peripheral blood memory B cells that express IgM with or without IgD [66, 67, 132], or express IgD only [133, 134]; these cells show

somatically mutated IgV genes suggesting the involvement of T cell help for their development. Interestingly, given the cutaneous route of sensitization implicated in the development of meat allergy, memory B cells displaying low to negative CD27 expression have been described in human tissues near epithelial surfaces [135-137]. Further work will be required to assess the antigen experience state of the B cell subsets we have identified, including analysis of the IgV genes.

Several questions remain regarding the mechanisms of α-gal sIgE production from the B cell subsets in meat allergic subjects. First, although it is not feasible to functionally interrogate each B cell subset within an assigned cluster, further work is needed to explore the composition of potentially functionally distinct subsets to determine whether one exhibits a propensity for α-gal sIgE production following stimulation. Moreover, it will be necessary to elucidate how these B cells respond to T cell-dependent signals as well as T cell-independent signals, as activation of B cells responding to carbohydrate antigens such as α-gal is thought to be independent of cognate T cell help. Finally, an important question in the study of food allergy is whether sIgE-producing B cells traffic to the gut and mediate local IgE responses to digested allergens. Notably, B cell subsets within Clusters 2 and 3 of meat allergic subjects shared high expression levels of the chemokine receptors CXCR4 and CCR6, which have been implicated in regulating mucosal immunity and driving intestinal inflammation during disease pathogenesis [39, 138]. Additional work is needed to better understand the contribution of these chemokine receptors on B cells for trafficking to the gut mucosa in food allergy.

Increasing our understanding of the B cells that maintain allergic sensitization is critical for identifying new targets to treat and prevent allergies. Additionally, detection of novel B cell immunophenotypes associated with red meat allergy may serve as a biomarker to identify individuals who have, or will become sensitized to red meat, as well as identifying affected individuals who may develop severe allergic reactions such as anaphylaxis. Longitudinal studies assessing the frequency of these B cell subset in the circulation of individuals with red meat allergy, and those who are at high risk of obtaining bites from the lone star tick—and thus developing the allergy—would be useful in determining the utility of these B cell subsets as biomarkers for diagnosis.

Supporting Information

Supplemental Table S2.1: Clinical characteristics of human subjects

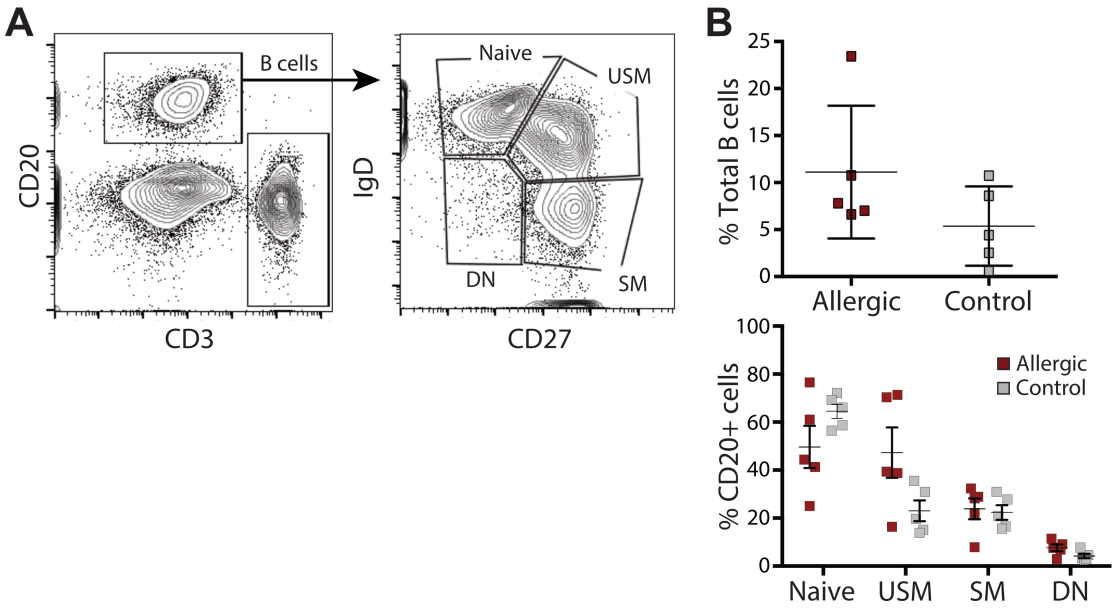
	Allergic	**Non-allergic**
Total mass cytometry samples	19	20
Male (%)	10 (53)	10 (50)
Female (%)	9 (47)	10 (50)
Total cell culture samples	14	10
Male (%)	10 (71)	6 (60)
Female (%)	4 (29)	4 (40)
Age (mean ± SEM)	59.8 ± 2.4	43.2 ± 2.7
Sensitized to peanut (%)	0	0
Total IgE (kU/L) (mean ± SEM)	361.4 ± 128.2	66.6 ± 17.2
Alpha-gal sIgE (kUA/L) (mean ± SEM)	69.8 ± 42.3	0.58 ± 0.25

Supplemental Table S2.2: Mass cytometry staining panel

Antibody	Surface Molecule	Clone	Manufacturer	Label	Dilution
CD3	CD3	UCHT1	DVS Sciences	170 Er	200
CD4	CD4	SK3	DVS Sciences	174 Yb	200
CD8	CD8	SK1	DVS Sciences	168 Er	200
CD14	CD14	M5E2	DVS Sciences	151 Eu	100
CD16	FcγRIII	3G8	DVS Sciences	148 Nd	100
CD20	B lymphocyte antigen	2H7	DVS Sciences	147 Sm	200
CD24	Heat stable antigen	ML4	DVS Sciences	169 Tm	100
CD27	CD27	L128	DVS Sciences	158 Gd	100
CD38	cyclic ADP ribose hydrolase	HIT2	DVS Sciences	167 Er	100
CD43	Leukosialin	84-3C1	DVS Sciences	150 Nd	100
CD56	NCAM	CM33B	DVS Sciences	176 Yb	100
CD86	B7-2	IT2.2	DVS Sciences	156 GDd	100
CD127	IL7R-α	AO19D5	DVS Sciences	165Ho	100
CD138	Syndecan 1	DL-101	DVS Sciences	145 Nd	100
CD184	CXCR4	12G5	DVS Sciences	175 Lu	100
CD185	CXCR5	51505	DVS Sciences	171 Yb	100
CD196	CCR6	G034E3	DVS Sciences	141 Pr	100
HLA-DR	HLA-DR	L243	DVS Sciences	143 Nd	100
IgD	IgD	IA6-2	DVS Sciences	146 Nd	100
IgM	IgM	MHM-88	DVS Sciences	172 Yb	100
CD45	PTPRC	HI30	DVS Sciences	154 Sm	400
CD25	IL2R-α chain	2A3	DVS Sciences	149 Sm	100
CD11b	ITGAM	ICRF44	DVS Sciences	144Nd	100

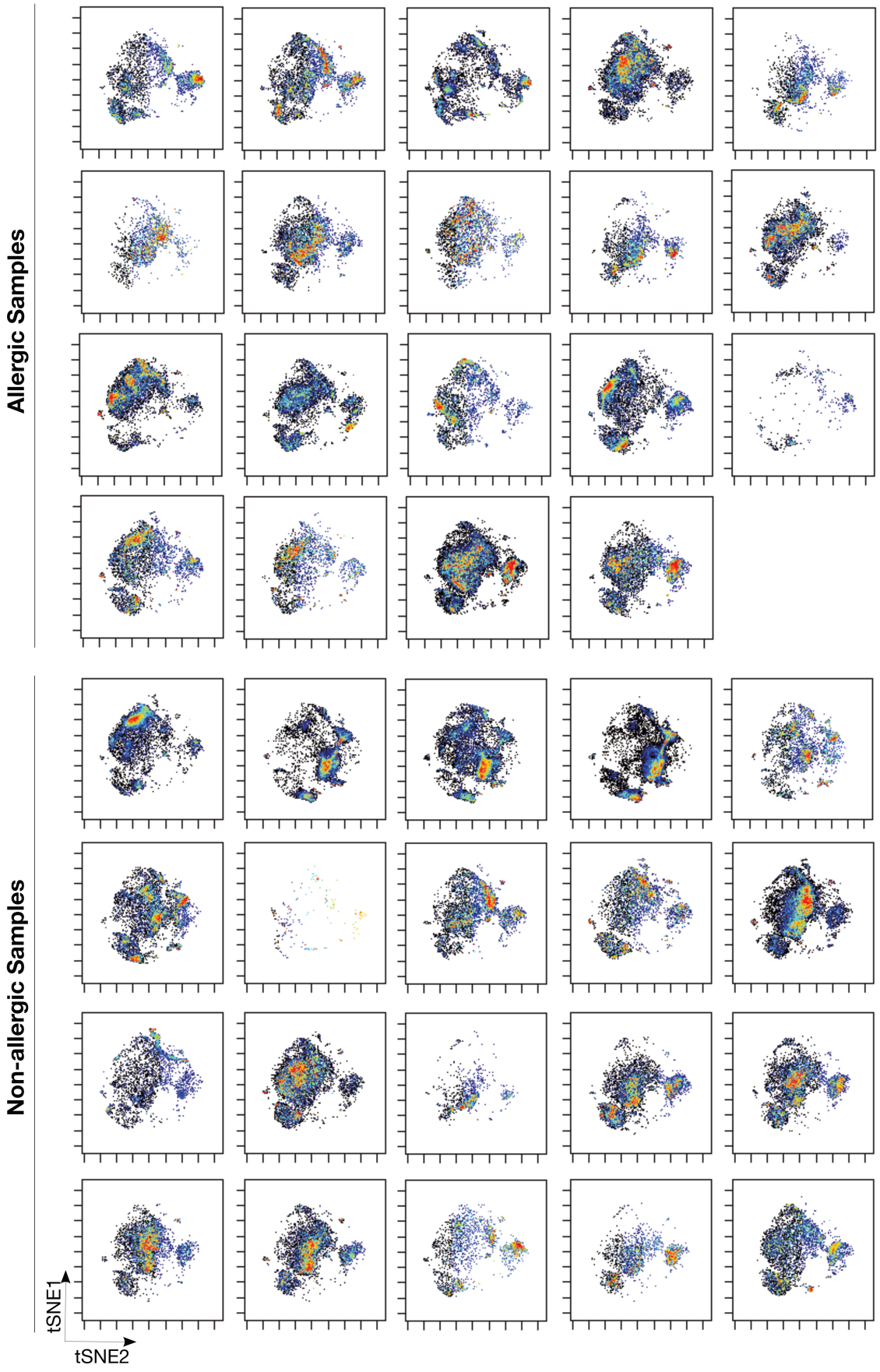
Supplemental Table S2.3: Flow cytometry staining panel

Antibody	Clone	Manufacturer	Label	Dilution
CD20	2H7	BioLegend	FITC	100
CXCR4	12G5	eBioscience	PE	50
CD43	HIT-3A	BioLegend	APC	50
CXCR5	MU5UBEE	eBioscience	PECy7	50
CD24	ML5	BioLegend	PerCPCy5.5	50
CD38	HIT2	Invitrogen	PE-Texas Red	50
IgD	IA6-2	BioLegend	APCCy7	100
IgM	SA-DA4	eBioscience	Biotin	100
HLA-DR	L243	BioLegend	BV510	20
CD25	BC96	BioLegend	BV711	20
Streptavidin	--	eBioscience	EF450	200
live/dead	--	Invitrogen	Aqua	200



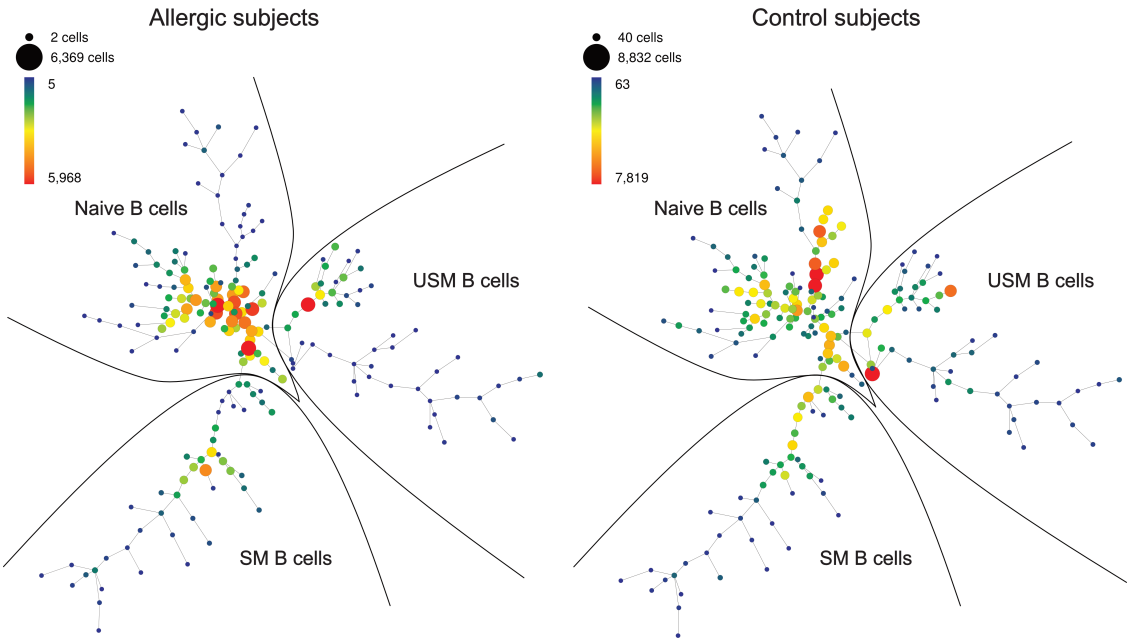
Supplemental Figure S2.1 Distribution of major B cell populations assessed by flow cytometry.

(**A** and **B**) Mature B cells were measured by fluorescent flow cytometry. Bar plots represent mean ± SD of the indicated cell population (n = 5 in each group), which were not statistically significant between groups. USM, immunoglobulin-unswitched memory B cells; SM, switched memory B cells; DN, $IgD^{-}CD27^{-}$ double negative B cells.

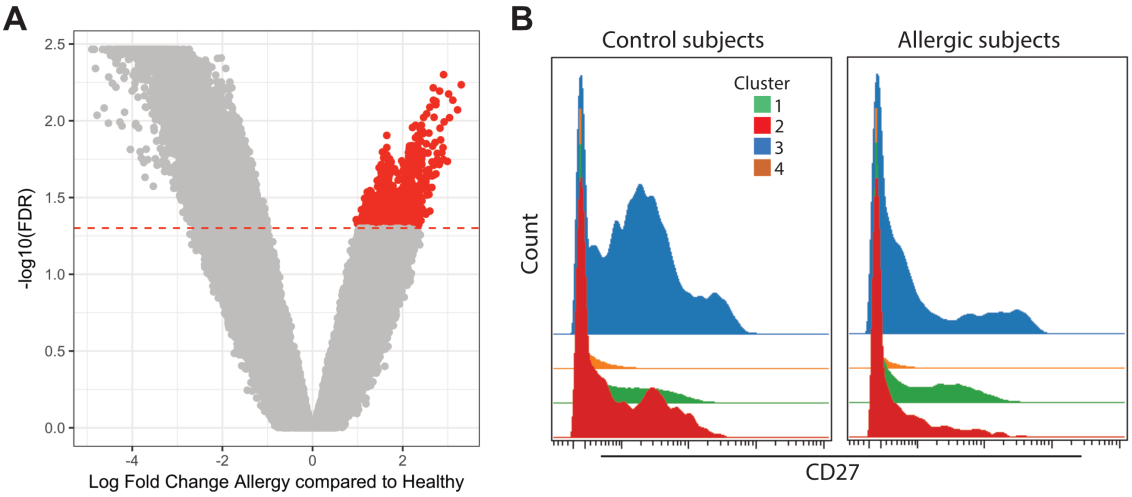

Allergic Samples
Non-allergic Samples
tSNE1
tSNE2

Supplemental Figure S2.2 viSNE maps of $CD20^+$ B cells from individual data sets of meat allergic and control subjects.

PBMCs from 20 non-meat allergic controls and 19 meat allergic subjects were stained and acquired by CyTOF. viSNE maps of B cells gated on live, singlet, $CD20^+CD3^-$ cells from individual subjects are shown. Plots are colored by cell frequency.



Supplemental Figure S2.3 SPADE analysis of CyTOF data shows phenotypic changes of B cells in meat allergic subjects.
SPADE analysis of concatenated data sets of each subject group is shown. Each circular node represents a phenotypically similar population of B cells, with the relationship between nodes reflecting the most similar phenotypes to adjacent nodes. Node size and color represent the number of cells. Naïve, $CD27^-$ B cells; USM, $CD27^+IgD^+$ immunoglobulin-unswitched memory B cells; SM, $CD27^+IgD^-$ switched memory B cells.



Supplemental Figure S2.4 Assessment of changes in B cell subsets between allergic and control subjects.

(**A**) Volcano plot of log2 fold-change versus –log10 FDR-corrected P-value for all B cell immunophenotypes in 19 allergic and 20 control subjects measured by CyTOF. Red horizontal line denotes the 0.05 selected FDR-corrected P-value cutoff. Red dots represent B cell subsets expressed at significantly higher frequencies in allergic patients compared to controls. Grey dots represent no changes in B cell subset frequencies (below red line), or B cell subsets expressed at significantly higher frequencies in controls compared to allergic subjects. (**B**) Histograms showing CD27 expression levels on the indicated B cell clusters measured by CyTOF.

CHAPTER 3: CUTANEOUS EXPOSURE TO CLINICALLY RELEVANT LONE STAR TICKS PROMOTE IGE PRODUCTION AND HYPERSENSITIVITY THROUGH CD4$^+$ T CELL- AND MYD88-DEPENDENT PATHWAYS IN MICE

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Abstract

Tick-borne allergies are a growing public health concern and have been associated with the induction of IgE-mediated food allergy to red meat. However, despite the increasing prevalence of tick bite-induced allergies, the mechanisms by which cutaneous exposure to ticks leads to sensitization and the production of IgE antibodies are poorly understood. To address this question, an in vivo approach was used to characterize the IgE response to lone star tick proteins administered through the skin of mice. The results demonstrated that tick sensitization and challenge induced robust production of IgE antibodies and supported a role for IgE-mediated hypersensitivity reactions in sensitized animals following oral administration of meat. Induction of IgE responses were dependent on cognate CD4+ T cell help during sensitization but not challenge with cutaneous tick exposure. In addition, IgE production was dependent on B cell intrinsic MyD88 expression, suggesting an important role for TLR signaling in B cells to induce IgE responses to tick proteins. This model of tick-induced IgE responses could be used to study the factors within tick bites that cause allergies and to investigate how sensitization to food antigens occurs through the skin that leads to IgE production.

Introduction

Food allergy is an adverse immune response to a given food and is a growing public health concern that can carry a high risk of life-threatening allergic reactions [2, 139, 140]. It is estimated that IgE-mediated food allergy affects 2-10% of the general population [141]. There is no treatment to prevent or cure food allergies and thus for most patients the only management is avoidance of foods [142]. IgE antibodies are produced by B cells as a result of antigen exposure through skin, gut, or respiratory tract. Food antigens are then processed by antigen presenting cells and presented to $CD4^+$ T cells that provide help to B cells leading to the production of specific IgE antibodies. Upon re-exposure of antigen, a more rapid and greater IgE response ensues. In view of the essential role of B cells in IgE-mediated hypersensitivity, interference with their sensitization to foods is considered a potential new therapeutic strategy in food allergy.

Red meat allergy is among a minority of food allergies that pose a serious acute health risk that can induce severe cutaneous, gastrointestinal and respiratory reactions [86, 143]. This novel form of food allergy develops worldwide in adults who have tolerated meat consumption for years and is postulated to result from tick bites through mechanisms that remain unknown [86, 87, 89-91, 94, 143-145]. In the United States, bites from lone star ticks can induce IgE antibodies to tick proteins and is the primary tick species associated with red meat allergy. Allergic reactions in affected individuals following meat consumption are mediated by IgE antibodies specific for galactose-alpha-1,3-galactose (α-gal), a blood group antigen of nonprimate mammals and therefore present in common dietary meat such as beef, pork and lamb [97, 98]. α-gal is also contained within the intestinal tract and saliva of ticks [86, 92]. Thus, an emerging

thought is that exposure to tick bites promotes cutaneous sensitization to tick antigens such as α-gal leading to the development of IgE-mediated food allergy to meat digested through the oral route. However, the underlying mechanisms by which skin exposure to ticks leads to the development and maintenance of IgE production has not been investigated, and there currently is not a clinically relevant animal model of IgE-mediated hypersensitivity induced by ticks.

Here, we have developed a new mouse model to study IgE responses to cutaneous tick exposure by administering lone star tick antigens through the skin of mice. We demonstrate that tick sensitization and challenge induces specific IgE and IgG1 antibody production in healthy mice, and elicits hypersensitivity reactions in sensitized animals following oral administration of meat. Upon tick exposure, mice depleted of $CD4^+$ T cells exhibited specific IgG1 but not IgE production compared with controls. Inhibition of T cell help by blocking CD40 ligand (CD154) during the sensitization phase or the challenge phase revealed that cognate T cell help is required for sensitization but not for recall to produce specific IgE and IgG1 antibody responses. Finally, we show that sensitization to tick exposure requires MyD88 signaling and this requirement is B cell intrinsic. Collectively, our findings identify $CD4^+$ T cells and MyD88 signaling pathways as important underlying mechanisms for the induction of IgE responses to cutaneous tick exposure and establish an in vivo model that can be used to investigate how IgE is regulated in tick borne-induced allergies.

Materials and Methods

Mice

WT C57BL/6J, MyD88$^{-/-}$, MyD88^{fl}, Cγ1-cre mice (all on the C57BL/6J background), and BALB/c mice, were obtained from The Jackson Laboratory (Bar Harbor, ME). The α-galactosyl transferase$^{-/-}$ mouse (αGT KO) was a generous gift of Dr. Uri Galili (Rush University, Chicago, IL). All of the mice were maintained at the University of Virginia, under specific pathogen-free conditions and used according to the regulations and standard guidelines of the Institutional Animal Care and Use Committee. All experiments were conducted with 8-10-week old female mice unless otherwise noted.

Tick Extract Preparation

Extract from lone star ticks was prepared from larval, pathogen-free Amblyomma americanum purchased from the Oklahoma State Tick Rearing Facility (Stillwater, OK), as previously described under endotoxin-free conditions [94, 146]. Briefly, ticks were flash frozen, homogenized into powder form and reconstituted in a borate buffered saline, pH 8.0, overnight at 4°C in the presence of protease inhibitors. Proteins were extracted by defatting the saline solution with ethyl ether and separated by centrifugation at 3000 rpm for 5 minutes. The aqueous layer containing proteins was collected for analysis.

Immunization

Mice were sensitized by s.c. injections in the flank with 50 μg of tick extract in 100 μl total volume on days 0 and 7. On day 31, tick-sensitized mice were challenged by s.c. injection in the flank with 50 μg of tick extract in 100 μl total volume and analyzed 4 days later. In experiments testing the adjuvant effects of tick extract, mice were s.c. injected with 50 μg tick extract plus 50 μg of the antigen, 4-hydroxy-3-nitrophenylacetyl

(NP) conjugated to keyhole limpet hemocyanin (KLH) at a ratio of 36 (Biosearch Technologies, Petaluma, CA) in 100 µl volume on days 0, 7 and 31. Mice serving as naïve controls were s.c. injected with PBS in 100 µl volume. Serum was obtained on day 35 unless otherwise indicated and antibody titers were measured by ELISA. Single cell suspensions prepared from inguinal lymph nodes and spleen were analyzed using flow cytometry.

Histology

Skin samples for hematoxylin-and-eosin (H&E) staining were obtained on day 35 and fixed in 10% formalin (Fisher Scientific) for 48 hours and then transferred into 70% ethanol. The samples were embedded in paraffin, and 5-µm sections were taken for H&E staining by the University of Virginia Histology Core. A qualified pathologist at the University of Virginia performed histologic examinations. Severity of dermal thickening, vasodilation/endotheliitis, nerve swelling/perineural and skeletal muscle infiltration, and leukocyte infiltration were each scored on a scale of 0 to 5 as follows: 0, normal; 1, minimal, focal infiltration; 2, mild infiltration; 3, moderate, multifocal infiltration, no fibrosis; 4, marked infiltration, fibrosis; 5, diffuse, severe infiltration, fibrosis. Scores for each pathological finding were combined to yield a composite score for each sample. Detection of mast cells in the skin of mice was performed by toluidine blue staining as previously described [147]. In brief, sections of tissues from mice were re-hydrated in xylene, xylene plus ethanol, and then sequentially in graded percentages of ethanol. Each section was then washed with PBS and stained with 0.1% toluidine blue dye for 30 minutes. Dehydration of slides was carried out and mounted. Mast cells were identified

by the presence of purple cells and counted by blinded observers for each section [148]. The number of mast cells was counted per unit area using a measuring eyepiece. Images were acquired using an Olympus BH2 (BHTU) Polarizing Trinocular microscope and analyzed using NIH Image J software (Bethesda, MD).

Basophil Activation

Naïve and immune mice were orally gavaged with 50 μg of beef thyroglobulin (Sigma-Aldrich, St. Louis, MO) in 100 μl of PBS. Approximately 100 μl of blood was collected 30, 60 and 90 minutes following oral gavage and was combined with an equal volume of heparin. Blood was then treated with ammonium chloride-Tris to lyse red blood cells. The remaining cells were fluorescently labeled and basophil activation was assessed by flow cytometry.

$CD4^+$ T Cell Depletion

Mice received s.c. injections with 50 μg lone star tick extract or PBS on days 0, 7 and 31. Mice administered tick extract were divided into two groups with one receiving i.p. injections of 250 μg anti-CD4 mAb (GK1.5 clone, Bio X Cell, West Lebanon, NH) and the other receiving 250 μg control rat IgG on days 27, 29 and 31. On day 35, serum was isolated for ELISA analysis and inguinal lymph node and spleen single cell suspensions were evaluated by flow cytometry.

MR1 Treatment

Mice sensitized and challenged with tick extract were randomly divided into three groups and treated with the CD40 ligand (CD154) mAb (MR1 clone; Bio X Cell, Lebanon, NH). The first group received i.p. injections of 500 μg MR1 on day 5 and 250 μg MR1 on days 7, 9, 11, and 13 following the initial s.c. injection of tick extract. The second group received a single i.p. injection of 500 μg MR1 on day 31 following the third s.c. injection of tick extract. The third group received i.p. injections of control hamster IgG on days 5, 7, 9, 11, 13 and 31.

TLR Screen

TLR ligand screening was performed by InvivoGen (San Diego, CA). Briefly, HEK293 cells engineered to express a single TLR and a SEAP-reporter plasmid that is induced upon activation of NF-κB and AP-1 were incubated with tick extract. TLR activation was assessed by SEAP expression, measured as absorbance at OD_{650} nm. Cells incubated with known TLR ligands served as the positive control and unstimulated cells served as the negative control.

Immunoglobulin ELISAs

Total serum IgE, tick-specific IgE and IgG1 titers, and NP hapten-specific antibody titers were determined by ELISA. For total serum titers, Costar high binding plates (Corning, Corning, NY) were coated with 5 μg/ml unlabeled anti-mouse IgE (Southern Biotech, Birmingham, AL). For antigen-specific ELISAs, high binding plates were coated with tick extract at a concentration of 5 μg/ml, or NP conjugated to Bovine Serum Albumin (BSA) at ratios of 32 or 4 (Biosearch Technologies) at a concentration of 5 μg/ml, as

previously described [149]. Serum was diluted 1:50 for IgE and 1:10,000 for IgG1, and serially titrated in two-fold increments. HRP-labeled anti-mouse IgE or IgG1 (Southern Biotech, applied according to manufacturer's instructions) served as the detection antibody and the assay was developed using TMB (BD Pharmingen), with 2N H_2SO_4 used as the stop solution. Plates were read at 450 nm using a BioTek plate reader. Total IgE antibody titers were quantified through a standard curve obtained using unlabeled IgE (Southern Biotech), while optical density (OD) values were compared for antigen-specific assays. All samples were assessed in duplicate.

Flow Cytometry

Single cell suspensions from pooled inguinal lymph nodes or spleen of each mouse were treated with erythrocyte lysis buffer (20 mM Tris/HCl, 155 mM NH_4Cl, pH = 7.2) and washed with PBS. Cell counts were determined using a Neubauer hemocytometer. Cells were stained with fluorescently labeled mAbs as previously described [150]. The following antibodies were used: B220-APCCy7 (RA3-6B2; BD Biosciences, San Jose, CA), CD4-FITC (RM4-5; BD Biosciences), CD95-Biotin (15A7; eBioscience), Streptavadin-PE (BioLegend, San Diego, CA), GL7-EF450 (GL-7; eBioscience), PD-1-PECy7 (RMP1-30; BioLegend), CXCR5-APC (L138D7; BioLegend), IgE-FITC (RME-1; BioLegend), FcεRI-FITC (MAR-1; BioLegend), CD200R-PE (OX-108; BioLegend), cKit-APC (2B8; eBioscience), CD45-APC (104; eBioscience) CD49b-PerCPCy5.5 (DX5; BioLegend), phosphorylated p38-PECy7 (pT180/pY182; BD Biosciences), CD63-APCCy7 (NVG-2; BioLegend), CD41-BV421 (WMReg30; BD Biosciences). Cell viability was determined using live/dead AQUA (Invitrogen, Carlsbad, CA) and doublets

were excluded based on forward scatter and pulse width. Samples were acquired on a CyAn ADP LX (Beckman Coulter, Brea, CA) or CytoFLEX (Beckman Coulter) and analyzed using FlowJo software version 10.1r7 (Treestar, Ashland, OR).

Statistical Analyses

All analyses were performed with Prism 5.0 software (GraphPad, San Diego, CA). Statistical significance comparing two experimental groups was determined using unpaired Student t-test with Welch's correction. Data are presented as mean ± SEM, unless otherwise stated. The threshold for significance was $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

Results

Cutaneous exposure of tick extract induces an IgE response in mice

To determine whether skin exposure to lone star ticks induces a systemic IgE response in mice, extract prepared from A. americanum larvae was administered s.c. to C57BL/6 mice. This approach was taken since larvae aggressively bite humans [151-153] and would expose the dermis and blood to tick proteins. Mice were sensitized with tick extract on days 0 and 7 and then challenged with tick extract on day 31 (Fig. 3.1A). The immune response to tick extract challenge was assessed on day 35. Results demonstrated that total IgE levels in serum of immunized mice increased significantly compared with naïve controls (Fig. 3.1B). Analysis of serum IgE titers over the course of tick immunization indicated that sensitization induced a small but not statistically significant increase in IgE after one week (day 7), which increased significantly after two weeks (day 14) following the second immunization (Fig. 3.1C). Levels of IgE were found reduced by day 31 nearly to the levels measured in naïve mice. Upon challenge, serum IgE rapidly increased within 4 days (day 35), surpassing the levels observed following the second immunization (Fig. 3.1C). IgE antibodies specific for A. americanum proteins correlate strongly with IgE antibodies to α-gal in human subjects with a history of tick bites [94]. Thus, we further assessed the specificity of IgE by ELISA and found that mice immunized with tick extract mounted a significant tick-specific IgE response compared to naïve controls (Fig. 3.1D). Moreover, tick-specific IgE was not detected from serum of mice immunized with the irrelevant house dust mite (HDM) allergen confirming the specificity of the assay. Balb/C mice sensitized and challenged with tick extract also showed greater total and tick-specific IgE serum levels compared to naïve controls,

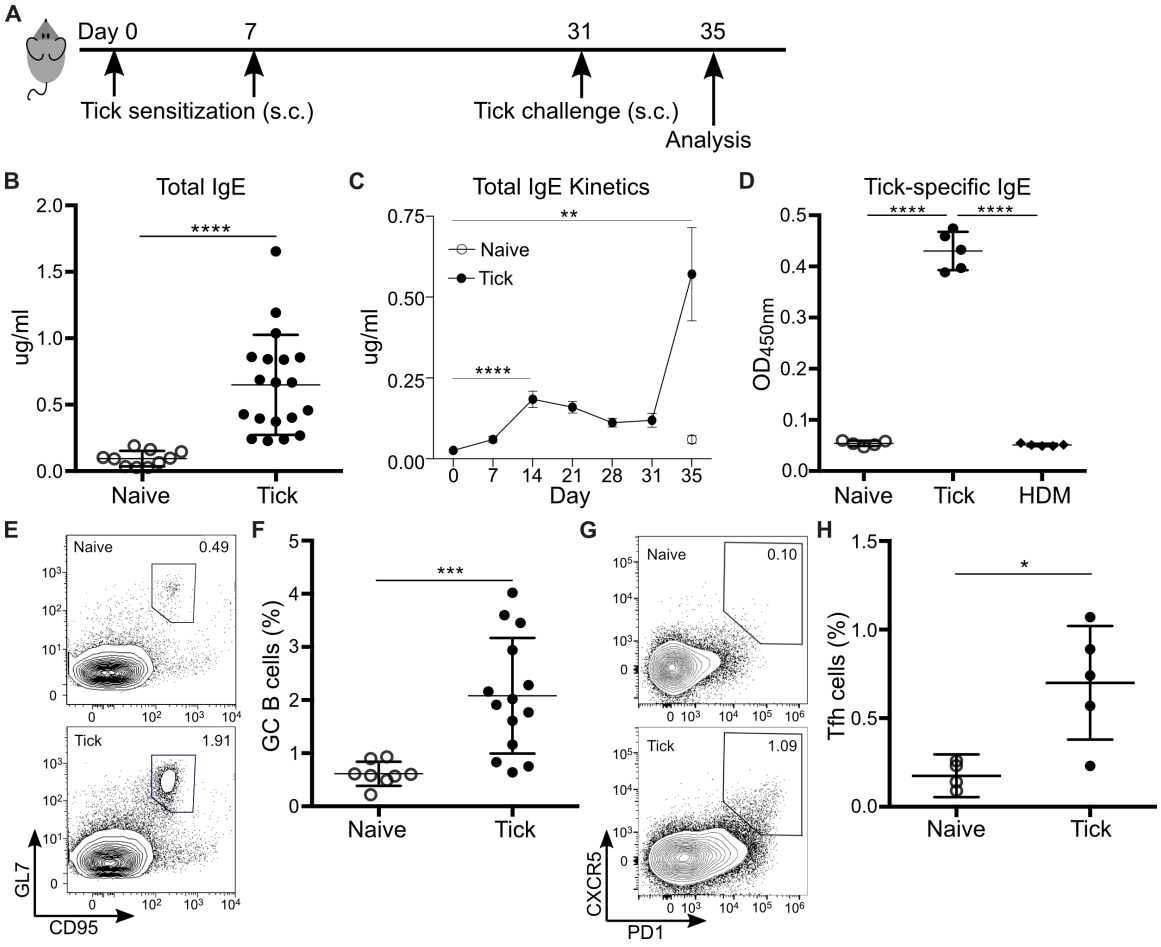


Figure 3.1 Cutaneous tick exposure induces an IgE response.
(**A**) Mice were injected subcutaneously with tick extract on days 0, 7 and 31, and analyzed on day 35. (**B** and **C**) Serum levels of total IgE and (**D**) tick-specific IgE were assessed by ELISA. House dust mite (HDM) extract was used to confirm specificity of the assay. (**E** and **F**) Percentages of GC B cells ($B220^{+}CD95^{+}GL7^{+}$) and (**G** and **H**) Tfh cells ($CD4^{+}PD\text{-}1^{+}CXCR5^{+}$) in inguinal lymph nodes of mice were quantified by flow cytometry. Representative contour plots showing the frequencies of (**E**) GC B cells and (**G**) Tfh cells. Each symbol represents an individual mouse and error bars show the mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

demonstrating that the IgE response induced by cutaneous tick exposure is not strain dependent (Fig. S3.1, A and B).

Previous studies showed that IgE production in secondary responses required $CD4^+$ T cell help [154, 155]. Thus, we evaluated the development of germinal center (GC) responses in the draining inguinal lymph nodes and non-draining spleens of mice. Challenge with tick extract induced a small, but significant increase in both GC B cells (Fig. 3.1, E and F) and T follicular helper (Tfh) cells (Fig. 3.1, G and H) in the inguinal lymph nodes. In contrast, no induction of either GC B cells or Tfh cells were observed in the spleen (data not shown), consistent with the route of subcutaneous exposure to antigen. Taken together, these data indicate that subcutaneous sensitization and challenge of healthy mice with lone star tick extract induces a strong IgE response.

Meat allergic patients experience local reactions to tick bites [86, 94, 156]. To determine whether cutaneous exposure to tick extract induced local inflammatory responses in mice, histopathological examination of skin sections taken at the site of immunization was performed. Skin sections from the site of tick extract injections showed the following four findings: dermal thickening; muscle fiber atrophy; mixed inflammatory cell infiltration including granulocytes, lymphocytes and plasma cells, some with Russell bodies; and mast cell infiltration (Fig. 3.2, A – E). In skin of mice injected with PBS, no such symptoms were evident. These findings were separately evaluated in a blinded manner using a scoring system and the combined pathology scores were quantified. Results demonstrated a greater pathology score in skin sections of mice immunized with tick extract compared with naïve, PBS-treated controls (Fig. 3.2, F). Finally, greater numbers of mast cells were found within the skin of mice sensitized and

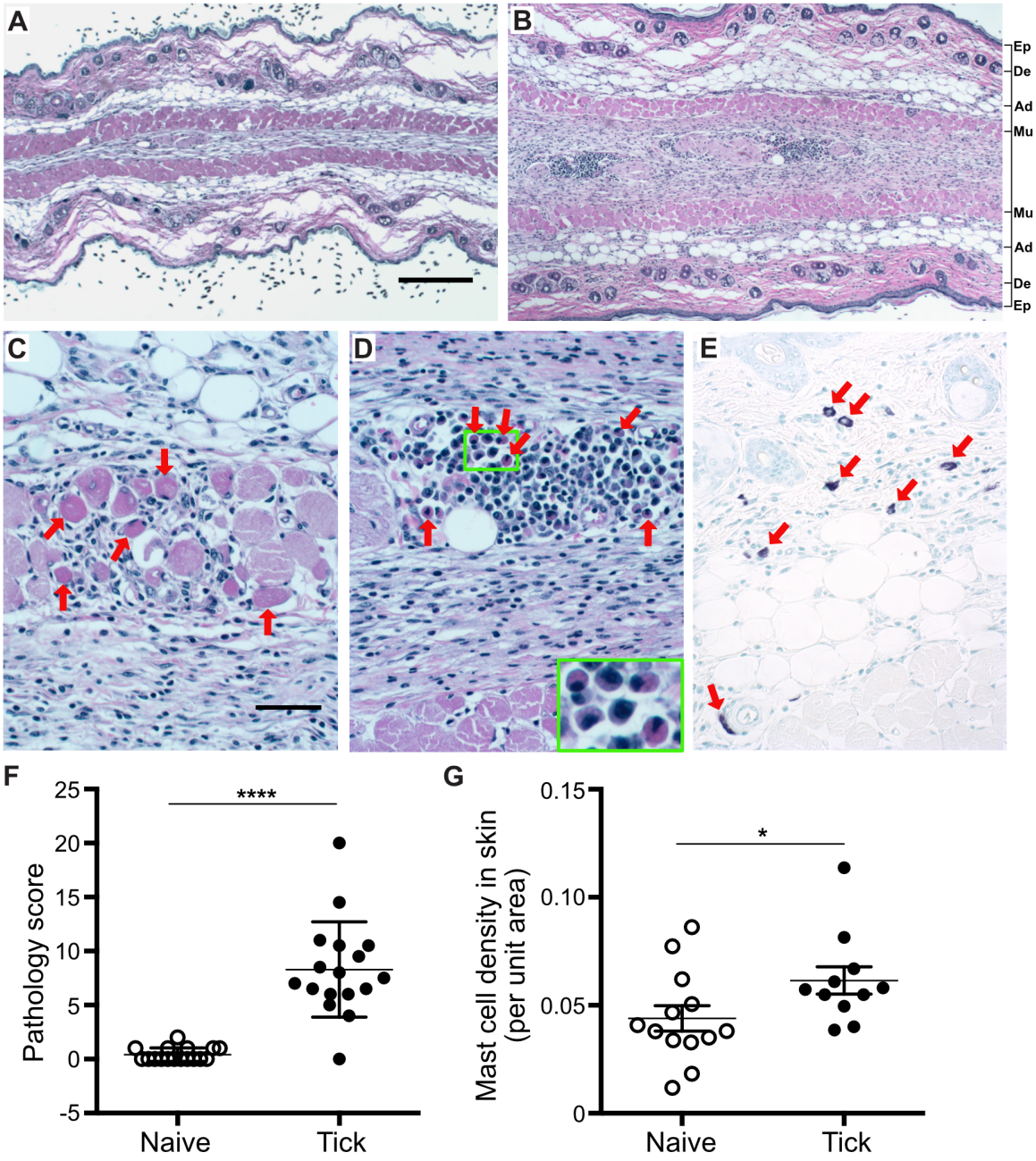


Figure 2.2 Subcutaneous immunization with tick extract induces a local inflammatory response.

(**A**-**E**), Skin samples were obtained from the site of tick extract injection on day 35 and analyzed by H&E and toluene staining. Representative skin sections from (**A**) naïve and (**B**) tick immunized mice showed increased cellular infiltration and enlarged dermis from immune mice. Two butterflied layers of skin per mouse are shown. Ep, epidermis; De, dermis; Ad, adipose; Mu, muscle. Original magnification x40. Bar scale = 100 µm. *C-E*, Representative skin sections from mice immunized with tick extract showed (**C**) atrophic muscle fibers (red arrows), (**D**) lymphocyte infiltration with clusters of plasma cells some

containing the Russell bodies (red arrows; inset), and (**E**) mast cells. Original magnification x200. Bar scale = 20 μm. *F*, Skin pathology was scored based on dermal thickening, muscular necrosis, leukocyte infiltration, and fibrosis. *G*, Mast cell infiltration in skin sections was assessed by toluidine blue staining and normalized to relative units. Each symbol represents an individual mouse and error bars show the mean ± SEM. *$p < 0.05$, ****$p < 0.0001$. Preparation of skin samples and quantification of mast cell infiltration was completed by Jessica Chandrasekhar. Pathology scores were determined by Dr. Kenneth Tung.

challenged with tick extract compared to those from naïve animals (Fig. 3.2, G). These findings establish the capacity of cutaneous tick exposure to induce local inflammation.

Skin inflammation is strongly linked with food allergy [157-159]. The heightened effect of environmental peanut allergen exposure in children with skin inflammation suggests that sensitization to allergens occurs through the skin that can lead to disease at other anatomic sites [160, 161]. Therefore, we assessed whether the development of an IgE response was dependent on cutaneous tick exposure or could also be induced through other routes of immunization. Mice sensitized and challenged intraperitoneally with tick extract failed to produce significantly increased levels of total and tick-specific IgE compared to those immunized subcutaneously (Fig. S3.2, A and B). These results indicate that the cutaneous route of exposure plays an important role in inducing an IgE response to tick extract.

Tick immunized mice exhibit a hypersensitivity response following oral exposure to beef

To determine if subcutaneous exposure to tick extract was capable of inducing a hypersensitivity response after consumption of red meat, we measured the activation of circulating basophils in peripheral blood of mice sensitized and challenged with tick extract following oral gavage with beef thyroglobulin (Fig. 3.3, A). Frequencies of basophils were substantially increased following oral gavage in immunized mice compared to naïve controls. (Fig. 3.3, B). Increased frequencies of basophils were detected at 30 and 60 minutes after gavage, and by 90 minutes were reduced to baseline levels observed in immunized mice that were not orally gavaged. Tick immunized mice also exhibited increased frequencies of basophils that expressed CD200R and CD41,

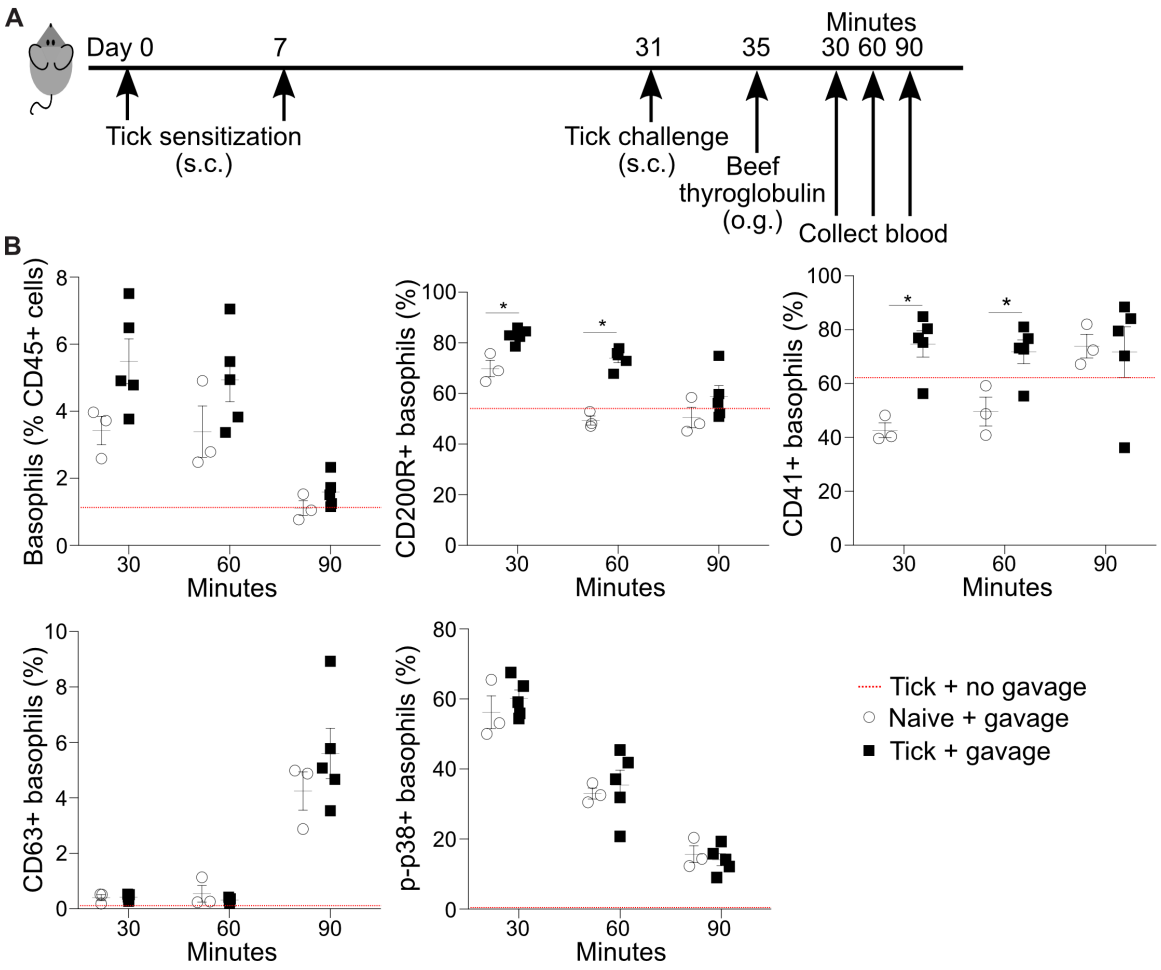


Figure 3.3 Mice cutaneously exposed to tick extract exhibit a hypersensitivity response following oral exposure to beef.
(**A**) Mice were injected subcutaneously with tick extract on days 0, 7 and 31, and oral gavaged with beef thyroglobulin on day 35. Circulating basophils in peripheral blood were analyzed 30, 60 and 90 minutes after oral gavage. (**B**) Frequencies and activation of basophils from gavaged naïve and immune mice were evaluated by flow cytometry. The red line indicates basophil frequencies from mice that were immunized with tick extract but were not orally gavaged. Each symbol represents an individual mouse and error bars show the mean ± SEM. *$p < 0.05$.

markers that are upregulated upon basophil activation [162-164], 30 and 60 minutes following oral gavage with beef thyroglobulin compared to naïve controls (Fig. 3.3, B). No differences in the frequencies of basophils expressing CD63 or phosphorylated p38, markers of human basophil activation indicative of anaphylactic degranulation [165-167], were observed in gavaged animals. Reduced body temperature, a sign of anaphylaxis, was not evident in mice (data not shown). Taken together, these data suggest that sensitization to tick antigens through the skin can lead to a hypersensitivity response in mice following exposure to beef thyroglobulin through the oral route.

T cell help is required for the IgE response to cutaneous tick exposure

Having established that cutaneous tick exposure induced Tfh and GC B cell frequencies in the skin draining lymph nodes (Fig. 3.1, E-H), we assessed the requirement of helper T cells for the IgE response. Sensitized mice were treated with the $CD4^+$ T cell depleting mAb GK1.5 before tick challenge and analyzed on day 35 (Fig. 3.4, A). This regimen efficiently depleted $CD4^+$ T cells (Fig. 3.4, B). Treatment with GK1.5 significantly reduced total and tick-specific IgE levels compared to isotype-treated controls (Fig. 3.4, C-E). We also measured tick-specific IgG1 levels since class switching to IgE is dependent on Th2 associated cytokines [168], and elevated levels of IgG1 to allergens often associate with IgE production including patients with red meat allergy [169, 170]. Results demonstrated that the tick-specific IgE response is characterized by elevated tick-specific IgG1, which is incompletely blunted in mice treated with GK1.5 (Fig. 3.4, F). These findings demonstrated that the IgE, and to a lesser extent the IgG1, recall response to cutaneous tick exposure is dependent on $CD4^+$ T cells.

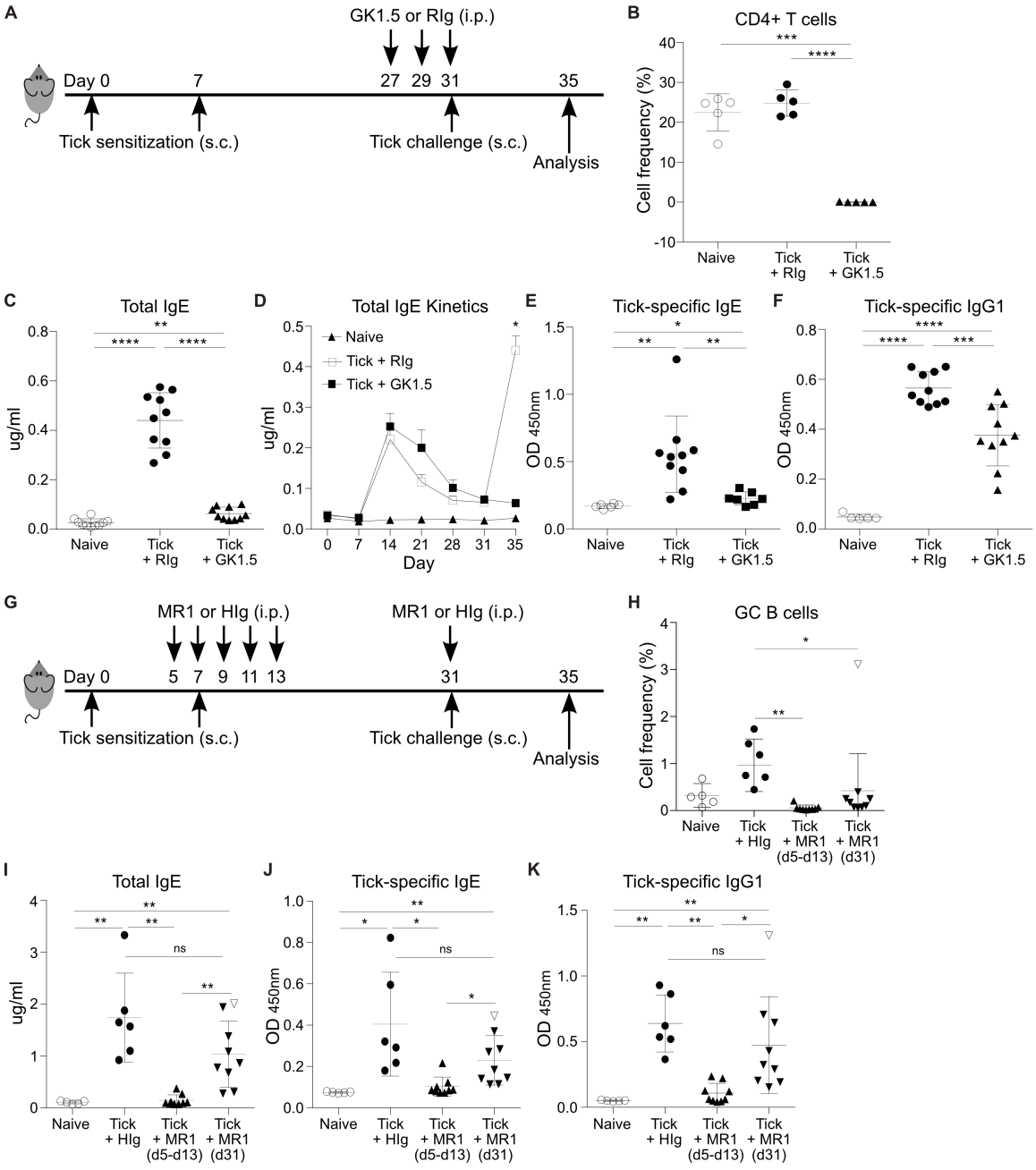


Figure 3.4 T cell help is required for the induction of IgE response to tick extract. (**A**) Mice were injected subcutaneously with tick extract on days 0, 7 and 31. GK1.5 or isotype control mAb was administered i.p. on days 27, 29 and 31, and mice were analyzed on day 35. (**B**) Depletion efficiency of $CD4^+$ T cells in the inguinal lymph nodes was assessed by flow cytometry. (**C** and **D**) Serum levels of total IgE and tick-specific (**E**) IgE and (**F**) IgG1 were assessed by ELISA. *G*, Mice were injected subcutaneously with tick extract on days 0, 7 and 31, and administered MR1 or isotype control mAb either on days 5, 7, 9, 11, and 13, or on day 31. *H*, Reduced GC B cells in the inguinal lymph nodes was determined by flow cytometry. *I*, Serum levels of total IgE, tick-

specific (**J**) IgE and (**K**) IgG1 were assessed by ELISA. Each symbol represents an individual mouse and error bars show the mean ± SEM. *$p < 0.05$, **$p < 0.01$. ▽ = a mouse that did not show reduced GC B cells with MR1 treatment. ns, not significant.

To evaluate further the contribution of T cell help in driving GC B cell responses and antibody production induced by tick immunization, we treated mice with the CD154 mAb (MR1) to block GC formation either when mice were sensitized or challenged with tick extract (Fig. 3.4, G and H). This approach allowed us to separately test the importance of GCs in allergen sensitization and recall responses. Results demonstrated that treatment with MR1 during tick sensitization prevented total IgE as well as tick-specific IgE and IgG1 production compared to isotype-treated controls (Fig. 3.4, I-K). In contrast, mice treated with MR1 during tick challenge showed no significant differences in the production of total and tick-specific antibodies despite reduced GC B cells. This experimental group of mice included a single mouse that did not show reduced GCs and was consistent with greater levels of total and tick-specific antibodies. Overall, these data suggest that GC responses to cutaneous tick exposure are required for allergen sensitization but are not essential for recall antibody production.

Tick extract has an adjuvant effect

In addition to allergen exposure, sensitization usually requires the presence of other factors that may function as adjuvants. These include experimental adjuvants such as bacterial toxins, bacteria colonization of lesional skin, or damage to the skin barrier [171-173]. The rapid increase in IgE titers in red meat allergy patients following tick bites suggests that, in addition to allergen, ticks contain inflammatory factors that drive IgE production to previously tolerated antigens [86, 91, 94]. To investigate whether tick extract has an adjuvant effect in our mouse model we sensitized and challenged mice with the exogenous T cell-dependent NP-KLH Ag in the presence of tick extract or in the

vaccine adjuvant alum and measured NP-specific antibody production (Fig. 3.5, A). Results demonstrated that total IgE levels were increased in mice that received NP Ag in tick extract compared to those immunized subcutaneously with NP Ag in alum and naïve controls (Fig. 3.5, B). Mice immunized with NP-KLH Ag in tick extract also exhibited significantly increased NP-specific IgE (Fig. 3.5, C), which included high affinity antibodies (Fig. 3.5, D). These data were consistent with increased frequencies of NP-specific B cells found in mice immunized with NP Ag in tick extract compared to other groups (Fig. 3.5, E), demonstrating that tick extract has an adjuvant effect.

Mice synthesize α-gal epitopes and therefore naturally do not produce α-gal antibodies because of immune tolerance [98, 174]. To determine whether the IgE response observed in wild type mice was influenced by α-gal expression, we sensitized and challenged wild type mice and mice deficient in α-gal [175] with tick extract and compared the serum levels of IgE. Levels of total IgE were induced both in wild type and α-gal deficient mice after tick immunization, with greater levels observed in α-gal deficient mice though not statistically significant compared to wild type mice (Fig. S3.1, C). No significant differences in the levels of tick-specific IgE were found between mouse strains (Fig. S3.1, D). These findings establish that the induction of IgE specific for tick proteins is not affected by α-gal expression, and suggest that α-gal expression may dampen the amount of total IgE produced.

MyD88 is required for the IgE response to tick extract, and this requirement is B cell intrinsic

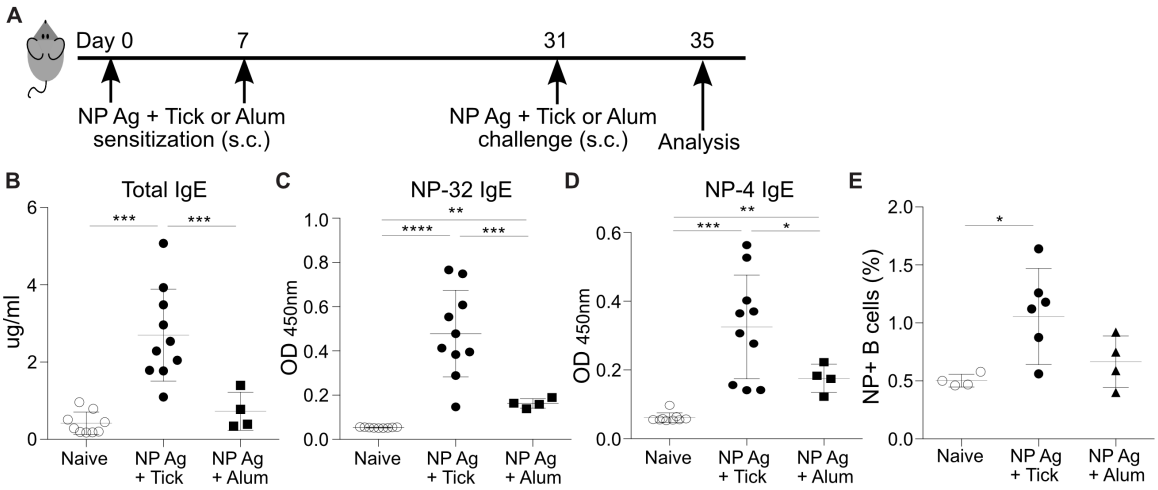


Figure 3.5 Tick extract functions as an adjuvant.
(**A**) Mice were injected subcutaneously with NP Ag in the presence of tick extract or in the presence of alum on days 0, 7 and 31, and analyzed on day 35. (**B**) Serum levels of total IgE, (**C**) total NP-specific and (**D**) high affinity NP-specific IgE were assessed by ELISA. (**E**) Frequencies of NP-specific B cells ($B220^+$ NP^+) in inguinal lymph nodes was quantified by flow cytometry. Each symbol represents an individual mouse and error bars show the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

To determine how tick extract might be providing an adjuvant effect in our mouse model, we assessed the ability of tick extract alone to activate toll-like receptors (TLR) through a commercial TLR screen (see materials and methods for more information). The screen indicated that tick extract had a robust stimulatory effect through TLR2, TLR4, TLR5, and to a lesser extent, TLR9 (data not shown). Because all of the TLRs identified by the screen signal through MyD88, we investigated whether signaling through MyD88 was important for the IgE response to cutaneous tick exposure. $MyD88^{-/-}$ and wild type mice were sensitized and challenged with tick extract using the same immunization strategy shown in Fig. 3.1A. Results demonstrated that total IgE as well as tick-specific IgE and IgG1 production was attenuated in the absence of MyD88 (Fig. 3.6, A-C).

Several allergens have been reported to contain TLR ligands and activate MyD88 signaling in innate and adaptive immune cells, including B cells [176-180].
Using mice expressing Cre recombinase from the endogenous immunoglobulin heavy constant gamma 1 (Ighγ1) locus [181], crossed with $MyD88^{fl/fl}$ mice [182], we generated conditional knockout mice that delete MyD88 in $IgG1^{+}$ B cells to study the B cell intrinsic function of MyD88 to control IgE responses. Attenuated levels of total IgE and tick-specific IgE and IgG1 antibodies were found when we performed the same experiment using the $Igh\gamma1^{Cre}MyD88^{fl/fl}$ mouse strain as with germline $MyD88^{-/-}$ mice (Fig. 3.6, D-F). These data demonstrate that the requirement for MyD88 signaling in IgE responses to cutaneous tick exposure is B cell intrinsic and, moreover, suggests that IgE class switch recombination occurs through an IgG1 intermediate B cell.

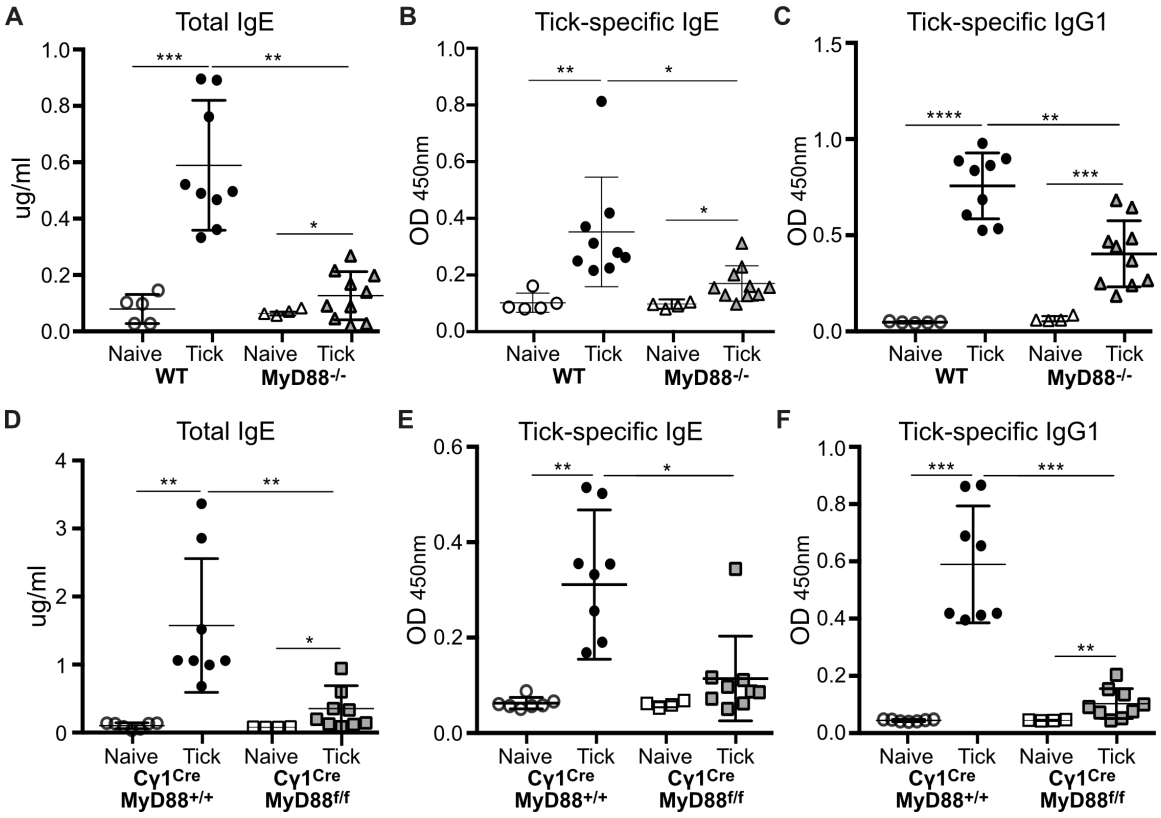


Figure 3.6 B cell intrinsic expression of MyD88 is required for the induction of a robust IgE response to tick extract.

(**A**-**C**) WT and MyD88$^{-/-}$ mice were injected subcutaneously with tick extract or PBS (naïve) on days 0, 7 and 31, and analyzed on day 35. (**A**) Serum levels of total IgE, (**B**) tick-specific IgE and (**C**) IgG1 were assessed by ELISA. *D-F*, Ighγ1^{Cre}MyD88$^{fl/fl}$ conditional knockout mice and Ighγ1^{Cre}MyD88$^{+/+}$ littermate controls were injected subcutaneously with tick extract as described above. (**D**) Serum levels of total IgE, (**E**) tick-specific IgE and (**F**) IgG1 were quantified by ELISA. Each symbol represents an individual mouse and error bars show the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Discussion

The mechanisms that lead to sensitization to food allergens and the production of IgE are poorly understood. The objective of this study was to develop and characterize a mouse model of allergic sensitization to cutaneous tick exposure that potentially could be used to identify mechanisms controlling the induction of IgE antibodies associated with tick-borne allergic diseases. Subcutaneous immunization with proteins isolated from lone star ticks induced IgE antibody production, including tick-specific IgE, as well as tick-specific IgG1 antibodies. These results also supported a subcutaneous route was needed for an IgE antibody response to tick immunization compared to a intraperitoneal route, and suggested a critical role for T cell help within the skin draining lymph nodes in this process. Inactivation of cognate T cell help to B cells by blockade of CD40 ligand-CD40 interactions during the sensitization phase but not the challenge phase prevented both IgE and tick-specific IgG1 production in immunized mice. Depletion of $CD4^+$ T cells during the challenge phase prevented IgE production whereas tick-specific IgG1 was induced but at lower levels. Together, these results suggest an important role for cognate T cell help in sensitization to tick proteins and non-cognate T cell help in driving recall antibody responses. Moreover, MyD88 signaling was found to be required for the generation of IgE in response to immunization with tick extract, and this requirement was B cell intrinsic. Our findings also demonstrated that cutaneous tick exposure induced systemic basophil activation following oral consumption of beef thyroglobulin, consistent with hypersensitivity responses found in patients with red meat allergy after the ingestion of mammalian meat [183].

These studies support a useful model to further investigate the signals required to induce IgE in tick-borne allergic responses. Several questions remain regarding the mechanisms of IgE production in response to cutaneous tick exposure. First, further work is needed to explore the composition of B cells that have a propensity for allergic sensitization and the molecular pathways by which B cells differentiate from naïve to IgE-producing cells. Recent studies using IgE-reporter mice support two differentiation pathways for the generation of IgE-producing B cells. The first is that IgE-secreting plasma cells can develop from the responding progeny of germinal center (GC)-derived IgE^+ B cells and IgE^+ memory B cells that are directly derived from IgM^+ naïve B cells [59]. The second is that IgE plasma cells develop from sequential class switching of the responding progeny of GC-derived $IgG1^+$ B cell intermediates and from $IgG1^+$ memory B cells after antigen exposure [48-50, 53-55, 57, 61-63]. Discrete subsets of $IgG1^+$ memory B cells based on CD80 and CD73 expression levels have been recently shown to contribute distinctively to the affinity and pathogenicity of IgE [65]. However, the immunophenotypes of these putative $IgG1^+$ B cell intermediates and memory B cells and their function in allergy are unknown. Characterization of these cell types in our model might help elucidate the contribution of sequential class switching to IgE during recall responses to tick proteins and the roles of cognate and non-cognate T cell help in this process.

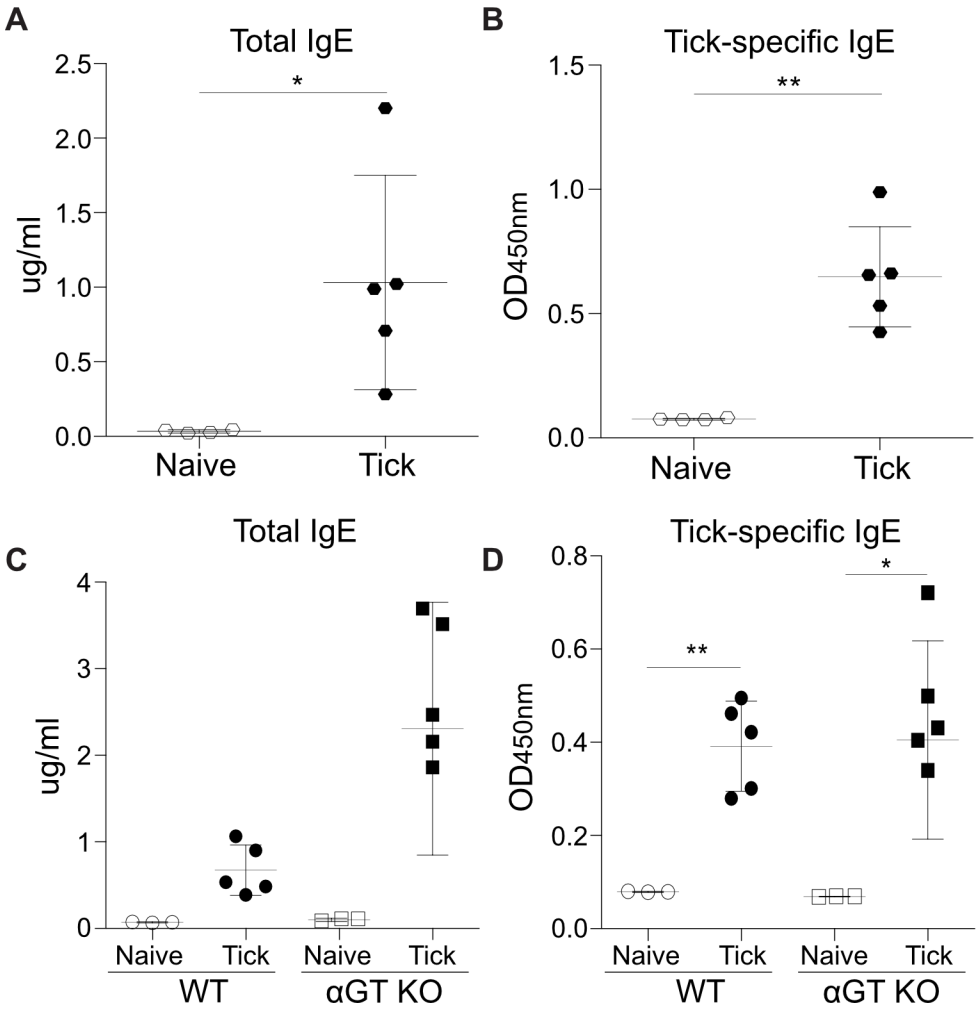
Second, our findings implicate the production of tick-specific IgE is mediated through TLRs expressed in B cells and in particular that MyD88 signaling in $IgG1^+$ B cells is important for driving sequential class switch recombination to IgE. Future studies are focused on identifying the TLRs in B cells that mediate downstream MyD88 signals

for IgE production. Interestingly, work using an airway allergy model demonstrated that B cell specific loss of MyD88 resulted in significantly reduced production of both IgE and IgG1 [177], supporting a role for MyD88 expression in B cells is important for sensitization to respiratory allergens. Several allergens have been reported to contain TLR ligands and activate MyD88 signaling in innate and adaptive immune cells [176] [176-180]. For example, TLR2, TLR4 and TLR9 have modulatory effects on the mucosal immune system and have been shown to help shape IgE production [184-186]. Although initially shown to be involved in Th1 responses [187], more recent work has demonstrated that MyD88 signaling plays a role in Th2 cell-mediated responses as well [177, 188, 189]. Previous work from animal models has demonstrated that allergic sensitization can be induced by topical allergen exposure. However, these models show that additional factors beyond exposure are necessary to induce sensitization, including adjuvant or damage to the skin [190-192]. Our finding that tick extract had an adjuvant effect and induced IgE antibodies specific for NP hapten points to a possible role of TLR ligands within the tick extract to mediate IgE responses. Little is known about the role of TLRs in skin sensitization and food allergy, and it might be expected that multiple TLRs are involved. Our observation that a commercial screen identified several TLR ligands are present in lone star ticks suggests that microbes contained within ticks, or the ectoparasitic tick itself, are sources of TLR ligands and might be important for sensitization to tick proteins.

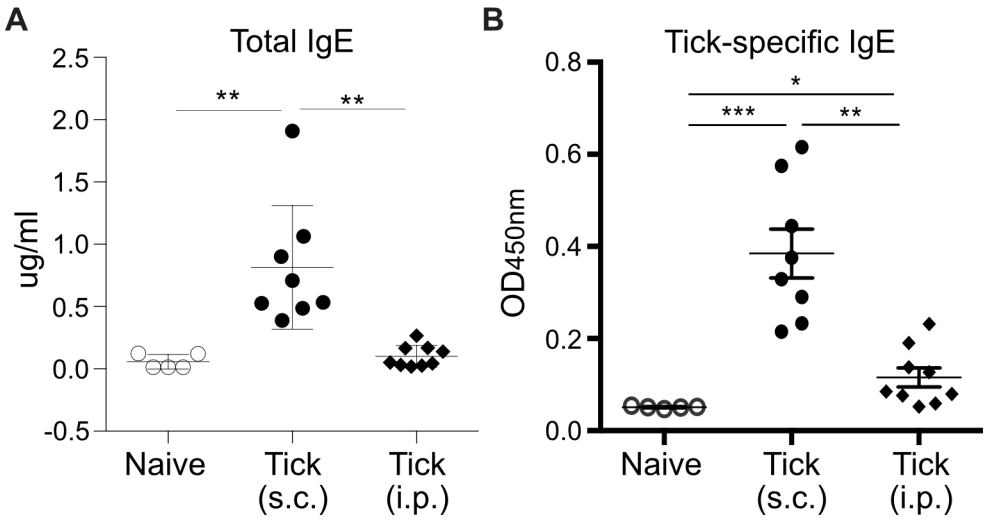
Finally, an important question in the study of food allergy is how an immune response to antigen exposure in the skin leads to a loss of tolerance to food antigens in the gut. Further work will be required to assess how gut-associated lymphoid tissue

responds to food antigens and its relationship with cutaneous immune responses such as the potential trafficking of lymphocytes from the skin draining lymphoid tissue to the gut. Along these lines, it will be of interest to explore whether the induction of tick-specific IgE is associated with α-gal specificities in mice immunized with tick extract. Given that wild type mice express α-gal and thus do not naturally produce α-gal antibodies, were alpha-gal IgE to be induced following cutaneous tick exposure it would suggest a loss of tolerance. Sensitization of α-gal deficient mice by subcutaneous injections of saliva obtained from the Amblyomma sculptum tick species resulted in the production of IgE antibodies against α-gal [96]. In contrast, no detectable levels of IgE against α-gal were observed in α-gal deficient mice when sensitized to virus-like particles containing alpha-gal epitopes. This suggests that A. sculptum saliva might have an adjuvant effect that is needed to induce IgE against α-gal despite α-gal not present in these mice, similar to humans; however, this study did not include wild type mice for comparison. Increasing our understanding of the mechanisms underlying tick-borne allergic diseases is critical for identifying new targets to treat and prevent allergies.

Supporting Information



Supplemental Figure S3.1 The IgE response to tick extract is not strain specific. (**A** and **B**) Balb/C mice were immunized subcutaneously with tick extract on days 0, 7 and 31, and analyzed on day 35 for serum levels of total IgE and tick-specific IgE by ELISA. (**C** and **D**) WT and $\alpha Gal^{-/-}$ mice were immunized with text extract as described above and analyzed on day 35 for serum levels of total and tick-specific IgE by ELISA. Each symbol represents an individual mouse and error bars show the mean ± SEM. *$p < 0.05$, **$p < 0.01$.



Supplemental Figure S3.2 Subcutaneous but not intraperitoneal immunization with tick extract induces an IgE response.
(**A** and **B**) Mice were sensitized and challenged s.c. or i.p. with tick extract on days 0, 7 and 31, and analyzed on day 35. Serum levels of total IgE and tick-specific IgE were measured by ELISA. Each symbol represents an individual mouse and error bars show the mean ± SEM. *$p < 0.05$, **$p < 0.01$., ***$p < 0.001$.

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

Individuals with red meat allergy have an altered B cell profile

B cells play a vital role in the pathogenesis of allergic disease through the production of IgE antibodies. However, the B cell phenotypes responsible for long-lived, allergen-specific IgE antibodies have not been identified. Numerous murine allergy models have been used in attempts to identify long-lived, IgE+ PCs, however while some models support the notion of long lived IgE+ PCs, other models do not, and long-lived IgE+ PCs have not been identified in humans. Additionally, a direct comparison of the B cell compartment between allergic and non-allergic individuals, which could help identify target populations that might contribute to IgE production, has not been completed. Through CyTOF analysis, we have identified a unique B cell signature in the blood of patients with red meat allergy (Fig 2.3B). Furthermore, we have identified several novel B cell subsets that are enriched in individuals with red meat allergy, and are capable of secreting α-gal specific IgE following in vitro stimulation (Fig 2.5C).

Previously, the surface markers CD27 and IgD were largely used to divide B cells into multiple subsets based on antigen experience [66, 70]. However, our data clearly shows that there is considerable heterogeneity within these traditional B cell subsets, suggesting that the use of additional markers are vital for identifying disease related differences in human B cells. Previously, the number of surface markers that could be assessed simultaneously was limited by spectral overlap and the corresponding compensation issues between different fluorophores [114]. While CyTOF has overcome these limitations through the use of metal tags, which allow for single-cell analysis of more than 40 parameters simultaneously [113], cells analyzed by CyTOF are vaporized, thus preventing the purification of live cell subsets to further interrogate functional

properties. Through the use of the flowType algorithm, we have identified the minimum number of markers that are required to identify the novel B cell subsets that correlated with allergy in our analysis (Fig 2.4B), facilitating assessment of the functional properties of these cells. This method provides a framework not only for the identification and analysis of cellular subsets that may play a role in red meat allergy, but could be applied to any disease state to identify cellular populations that could play a role in the pathogenesis, or even prevention, of disease.

Non-canonical B cells subsets are associated with the production of α-gal specific IgE

My data indicates that a population of CD43+ IgD+ cells are enriched in patients with red meat allergy compared to non-allergic controls (Fig 2.4A), and, in a subset of allergic patients, these cells are capable of producing α-gal specific IgE upon in vitro stimulation (Fig 2.5C). The function of CD43 expressing B cells is an area of interest for a number of groups. Recent studies have suggested that CD20+CD27+CD43+ B cells located in peripheral blood are the human counterpart to mouse B1 B cells [193]. However, other groups have suggested that these cells exhibit a pre-plasmablast-like phenotype based on spontaneous Ag secretion [194] and later work supported this conclusion [195]. Additionally, analysis of gene expression data has suggested that these cells developmentally lie between memory B cells and plasmablasts [194]. Unlike the pre-plasmablast-like cells identified in these recent studies, our population of interest exhibits very low or negative expression of the canonical memory B cell marker, CD27 (Fig 2.S2B). It is possible that the B cell clusters we have identified represent several precursor populations to the CD20+CD27+CD43+ cells identified by other groups, or

these subsets may be unrelated (Fig 4.1). Further work is needed to understand if and how these B cell subsets are related. Additionally, a lack of CD27 expression, the classical marker used to distinguish memory B cells, does not preclude a memory-like phenotype. Indeed, more evidence is being discovered for memory B cells that do not fit the classical definition of class switched, CD27+ cells [135, 196], and CD27-IgE+ cells exhibiting a memory phenotype have been found to be enriched in children with asthma, allergy and other atopic disease [130]. Taken together, emerging data clearly indicates that significant heterogeneity exists within traditional B cell subsets, in agreement with the findings presented in chapter 2. Future studies will be aimed at more fully characterizing the phenotype of the cellular subsets we have identified in red meat allergic patients.

The analysis of up to 40 markers that can be assessed simultaneously through CyTOF allows for a more comprehensive analysis of cell surface markers than previously possible with traditional flow cytometry. However, analysis of surface marker expression alone is not sufficient to fully characterize the phenotype of novel cellular subsets. We propose that the B cell clusters identified through our CyTOF analysis constitute populations of precursors for α-gal specific IgE secreting cells. Deep sequencing of the immunoglobulin gene expressed by these cells could help to identify if these cells constitute a memory-like population. Detection of somatic mutations in the DNA of the V(D)J rearrangement, which usually result from antigen experience in the GC, provides evidence of prior activation of these cells [197]. However, the relative scarcity of these cells within the circulation in even those patients with very high allergen specific antibody titers will make these studies challenging. Analysis of transcription factor

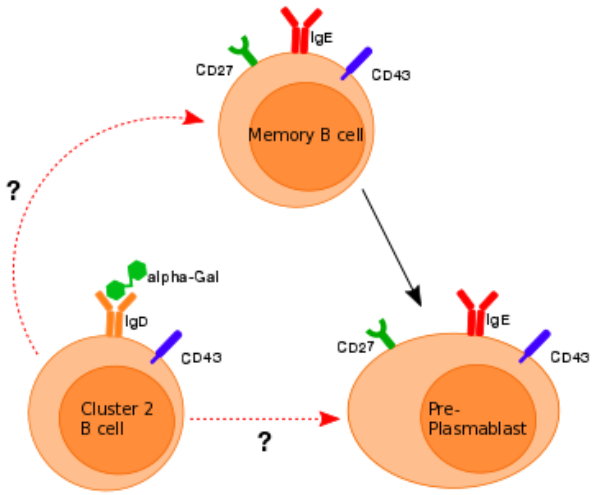


Figure 4.1 The relationship of novel B cell clusters to previously identified B cell subsets is not clear.
CD27+CD43+ B cells have been identified as pre-plasmablast like cells, and developmentally appear to arise from memory B cells. Whether the CD27-CD43+ B cells enriched in patients with red meat allergy are developmentally related to these pre-plasmablasts, potentially as precursors, remains to be determined.

expression in novel B cell subsets can also be used to inform predictions on the function of novel cellular subsets, as expression of Paired box protein 5 (Pax5) and Bcl6 would indicate that these cells have not become terminally differentiated PCs. In contrast, expression of BLIMP1 and X-box binding protein 1 (XBP1) would suggest that these B cells exhibit a plasmablast or PC phenotype. Although no deterministic transcription factor for memory B cells has been identified at this point[198], analysis of the transcription factors that have been associated with other B cell subsets, such as those listed above, could still be informative in identifying the function of the novel subsets we have identified.

Biomarkers of Food Allergy

The evidence suggesting that tick bites are the initiating event that leads to the development of red meat allergy continues to build [86, 91, 92, 94, 96]. However, not all individuals who experience tick bites will develop the allergy, and what differentiates individuals who will develop the allergy from those who will not is not clear. Identifying the factors that predispose some individuals to develop the allergy would be valuable for quickly identifying and preventing life-threatening allergic responses, and as a source of information to more fully understand the signals that drive the development of the allergy. We propose that the altered B cell signature identified by the CyTOF analysis presented here, specifically the frequency of the 4 clusters of interest identified through our analysis (Fig 2.4B) may serve as a biomarker for red meat allergy, in that an increased frequency of these populations in circulation may indicate that an individual has developed, or will develop the allergy before an allergic response to consumption of

red meat occurs. Whether or not these subsets could also serve as biomarkers for other food allergies will require further investigation. While symptoms of red meat allergy are reminiscent of other common food allergies, often manifesting as urticaria, angioedema, and potentially systemic anaphylaxis [84, 95], other aspects of red meat allergy are rather unique including the delay between consumption of mammalian meat and the onset of symptoms and a clear causative agent. Additionally, unlike with other food allergies, many individuals who develop red meat allergy have a history, often decades long, of red meat consumption without incident prior to the development of the allergy. Further study will be required to assess the presence and function of these subsets in the context of other food allergies.

Quantification of the frequency of these B cell clusters in patients with peanut or other traditional food allergy could be used as an initial step in determining if these cells are enriched in patients with any food allergy compared to non-allergic controls. Even if no relationship between the B cell clusters we have identified and other food allergies is identified, this analysis could be useful as the analysis pipeline described in Chapter 2 could be applied to identify new cellular subsets that could contribute to other food allergies. Regardless of the whether the same or different subsets are implicated in pathogenesis of other food allergies, functional analysis, similar to that carried out in Figure 2.5, would be required to provide evidence of a functional role for these populations in other food allergies.

Although in vivo experiments are not possible with human subjects, ex vivo analysis of these B cell subsets to assess the frequency of these cells over time in allergic patients, as well as to more fully characterize the function of these cells could still be

useful for evaluating the utility of these clusters as biomarkers. Assessing changes in the frequency of these populations over time in allergic individuals would provide a model of how these populations change in response to exposure to α-gal in allergic individuals, providing more evidence for the role of these populations in driving pathogenesis of red meat allergy. This model could then be applied to assess the risk of developing the allergy in someone who has received bites from the lone star tick. Assessing changes in the frequency of these clusters following oral challenge with mammalian meat, as used in previous studies by Commins and colleagues [183] would also be useful in developing this model. Finally, tracking the presence or frequency of these clusters over time in a population of individuals at high risk of developing the allergy, could be used to test the functional utility of using these populations to predict the development of red meat allergy. Given the evidence linking bites from the lone star tick to the development of red meat allergy, individuals who are frequently exposed to lone star ticks, such as park rangers, military personnel, and hikers, would likely exhibit a higher chance of developing the allergy and therefore comprise an ideal cohort for such a study. While the analysis of these populations is relatively straight forward, recruiting a suitable number of participants with the allergy and those at risk for developing the allergy, and completing frequent analysis of circulating B cell subsets is not trivial, making these studies challenging. However, the functional analysis need not be run in tandem and the cytof analysis has been optimized for frozen cells, which would allow for the accumulation of samples over time. Although the time required to recruit a sufficient cohort of patients may be extended, the large amount of information that can be obtained through unbiased,

high-dimensional CyTOF analysis of PBMCs from allergic patients would far outweigh this cost.

A murine model of tick-induced IgE

Although human studies can be highly informative, uncontrollable variables complicate the conclusions that can be drawn from the data, and mechanistic studies are often not feasible. Therefore, the development of relevant animal models is necessary to further dissect the components of an immune response that cannot be fully interrogated in human studies. Although previous models have been used to assess the presence of long-lived IgE secreting plasma cells and to assess the contribution of direct vs indirect class switching to IgE in the production of pathogenic allergic antibodies, the results have been inconclusive. Here we have detailed a novel mouse model of tick-induced IgE (Fig 3.1A). This model allows for investigation into the mechanisms which drive production of IgE in response to tick extract immunization.

We have established that subcutaneous immunization with extract prepared from the lone star tick induced a robust, tick specific IgE response in B6 mice (Fig 3.1B), providing a physiologically relevant system in which to study the signals that lead to the development of an IgE response. A robust IgE response to subcutaneous tick extract immunization was observed not only in C57B6 mice, but also in Balb/C mice (Fig 3.S1A), and alpha-gal deficient mice (Fig 3.S1B), which were generated on a mixed strain background, suggesting that the IgE response to tick extract is induced similarly across different strains of mice, providing further strength to the utility of this model. Interestingly, the IgE response was significantly diminished when tick extract was

administered by intraperitoneal injection (Fig 3.S2A), suggesting that the route of exposure to tick extract has a significant impact on the development and type of inflammatory response that occurs in response to tick extract. These data further support the growing evidence that epithelial barrier disruption plays a strong role in atopic disease [11, 12] [3, 13, 17]. These data also suggest that the findings from other models of IgE induction, which make use of non-physiological routes of sensitization, may not accurately describe what occurs during allergic sensitization in humans. Additionally, an extensive, unbiased analysis B cell subset in the context of these allergy models has not been completed, which, as our human studies have shown, is important for identifying B cells which may contribute to the allergic response. These shortcomings highlight the need for models which more accurately recapitulate what occurs in human allergic disease.

Currently, it is not clear how the immune response to subcutaneous tick extract exposure differs from that following I.P. immunization. However, further understanding of these differences could improve our understanding of the signals that drive the induction of an IgE response. Ideally, a comparison of the cytokine profile and immune cell milieu found at each injection site following immunization with tick extract could aid in identifying the differences in the immune response generated following subcutaneous versus I.P. immunization. However, these studies would pose significant challenges. One major challenge would be the isolation of immune cells from the skin following subcutaneous immunization. While there are protocols for isolating immune cells from the skin, low cell numbers often result, further compounding the low frequency of immune cells present in the skin to begin with. To avoid issues of cell loss during

isolation, we could employ Immunohistochemistry (IHC) to assess the presence of different immune cell populations in the skin at the site of tick extract immunization. A shortcoming of IHC is the limited number of parameters that can be assessed simultaneously. New techniques such as Imaging Mass Cytometry (IMC) which combine the advantages of traditional IHC with the ability to assess a high number of parameters at once could be employed to overcome parameter limitations. Although this would not allow a direct comparison between the immune cells present in the peritoneal cavity following I.P. immunization, this method could provide an initial indication of differences in the cellular response to tick extract immunization resulting from different immunization routes.

Previously, we have attempted to assess the production of Th2 cytokines, such as IL-4, IL-5, and IL-13, following subcutaneous immunization with tick extract by ELISA, however the level of these cytokines was below the limit of detection. The concentration of these cytokines in circulation following I.P. immunization would likely be even lower due to the significantly reduced IgE response in I.P. immunized mice, and therefore not detectible by ELISA. To overcome the detection issues we encountered using ELISAs we could use ELISpot assays to assess cytokine production, as this assay is much more sensitive than an ELISA. To determine if I.P. immunization with tick extract induces a Th2 response we could isolate T cells from the peritoneal cavity of mice immunized I.P. and the skin of mice immunized subcutaneously, and assess production of IL-4, IL-5, and IL-13 by ELISpot. Additionally, to determine if I.P. immunization skews the immune response to tick extract towards a Th1 or Th17 phenotype the production of non-Th2 cytokines, such as interferon (IFN)-gamma, IL-2, and IL-17 could be assessed by

ELISpot. A Th1 or Th17 skewed immune response could explain the reduction in IgE we observed in I.P. immunized mice compared to mice immunized subcutaneously.

Tick-specific IgE secreting cells are generated through a germinal center response in subcutaneously immunized mice

The mechanisms by which IgE secreting cells are generated has long been a question in the field of allergy. B-cell lymphoma 6 (Bcl6), which antagonizes binding of a key transcription factor for IgE expression, is highly expressed by GC B cells, suggesting to some investigators that CSR to IgE is unlikely to occur in the germinal center [60, 61]. Studies in IgE reporter mice, which have been completed by various groups, have provided conflicting data on whether or not IgE+ B cells are found within the GC [59, 63]. Our data clearly indicates that the development of GCs are required for the production of IgE in response to tick extract (Fig 4.2), as mice that do not develop GCs due to treatment with the monoclonal antibody MR1, exhibit a reduction in total and tick specific IgE in mice treated with MR1 (Fig 3.4H, I). Interestingly, tick-specific IgG1 is not impaired in mice that receive MR1 treatment during the recall response (Fig 3.4J), suggesting different requirements for T cell help between the IgE and IgG1 responses.

In addition to questions about the anatomical location of CSR to IgE, the route by which IgE is generated has also long been debated. IgE reporter mice have also been used in an effort to determine whether IgE is generated through direct switching from an IgM+ B cells, or whether indirect switching, through an IgG1 intermediate, is required for the production of pathogenic IgE producing B cells. Again, the results have varied, potentially due to changes in signaling following genetic alteration to the IgE locus used

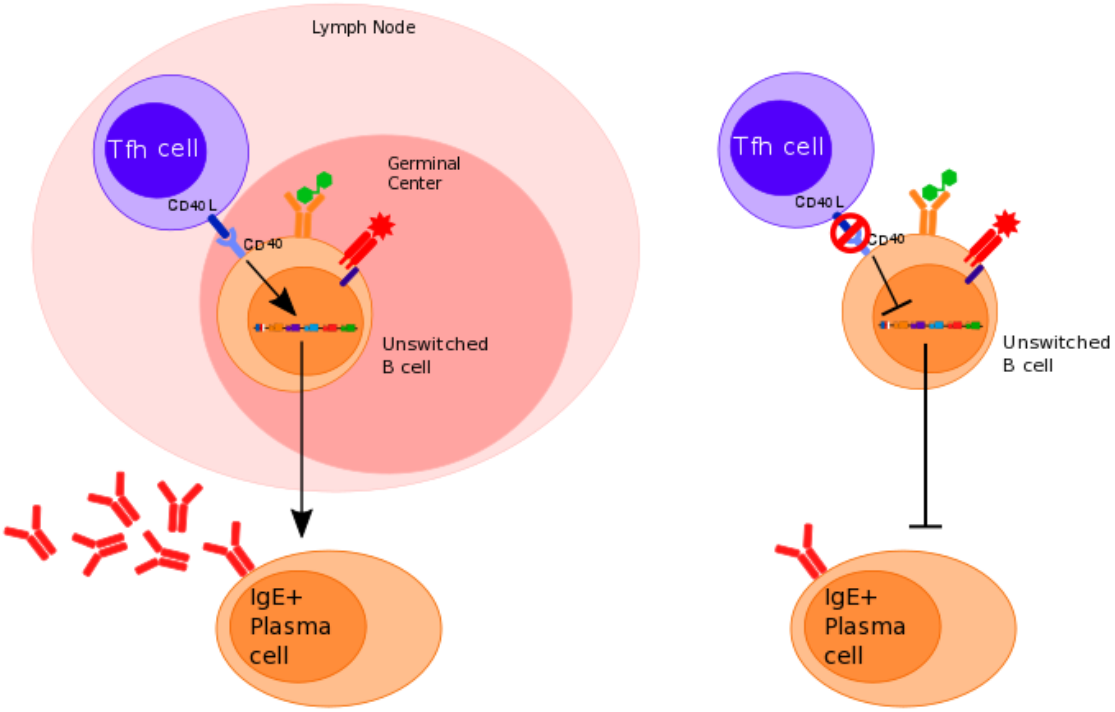


Figure 4.2 IgE+ B cells are generated through a germinal center response.
T cell help, in the context of a germinal center response, is required for the development of IgE+ B cells following immunization with tick extract (left). In the absence of a GC response, due to impaired CD40-CD40L signaling, the development of IgE+ B cells is blocked (right).

to generate the reporter mice, or due to differences in the antigen used in each case. Our data indicates that total and tick-specific IgE is completely ablated when the IgG1 response is suppressed (Fig 3.4H-J), suggesting that IgE is produced through sequential switching in the context of subcutaneous immunization with tick extract. However, further studies are required to confirm that IgE is produced through indirect switching.

Although we have attempted to confirm that IgE is produced through sequential switching in the context of tick extract, our results have been inconclusive. We first attempted to directly assess if IgE producing cells arise from IgM+ or IgG1+ cells. IgM+ or IgG1+ cells were sorted from tick immunized mice and transferred to genetically mismatched recipients before challenge with tick extract. Analysis of the serum of these mice indicated that there were no significant differences in serum IgE titers between control mice that did not receive transferred cells and mice that received transferred IgM+ or IgG1+ cells (data not shown). The lack of a significant increase in IgE production in either group of transfer recipients may indicate these mice did not receive a sufficient number of cells to drive a robust IgE response. Unfortunately, because tick immunized mice do not mount a GC response in the spleen, and other routes of immunization, which would drive development of a GC response in the spleen, may not result in a similar IgE response, donor cells must be isolated from the inguinal lymph nodes, limiting the number of cells that can be transferred to recipient mice. To indirectly assess the route of IgE CSR, we attempted to assess the production of Sμ-Sε and Sγ-Sε by quantitative polymerase chain reaction (q-PCR). To overcome the low number of cells producing IgE in vivo, we developed a culture system to generate IgE-secreting cells. Although we were able to detect IgE germline transcript by q-PCR, we could not

successfully detect switch circle transcripts. Despite readily detectible IgE production and improved cell numbers in the culture system, the production of switch circles is likely below the limit of detection. To overcome this limitation, it may be necessary to enrich for IgE producing cells from the culture prior to DNA isolation. As not all cells from the culture system will undergo class switching to IgE, non-IgE+ cells will be present in the population from which we are isolating DNA and may dilute out those cells which do contain Sμ-Sε and Sγ-Sε switch circles.

The adjuvant effect of tick extract

Despite the mounting evidence suggesting that tick bites are responsible for the induction of red meat allergy, how tick bites actually drive the development of IgE against α-gal has not been identified [95]. Our data clearly indicates that tick extract is capable of serving as an adjuvant, likely through activation of TLR(s) signaling (Fig 4.3), resulting in a significantly stronger IgE response when given in combination with NP compared to NP administered in combination with alum (Fig 3.5B). The adjuvant effect we observed in our mouse model provides some explanation for how tick bites might induce an inflammatory response to a previously tolerated antigen. Identification of the component or components of the tick extract that drive the response will require further investigation.

To identify the component(s) of tick extract that are capable of driving the adjuvant effect of our mouse models, we could isolate tick specific IgE antibodies from the serum of mice immunized with tick extract and use these antibodies to label antigenic molecules in the extract. The labeled tick extract antigens could then be isolated via

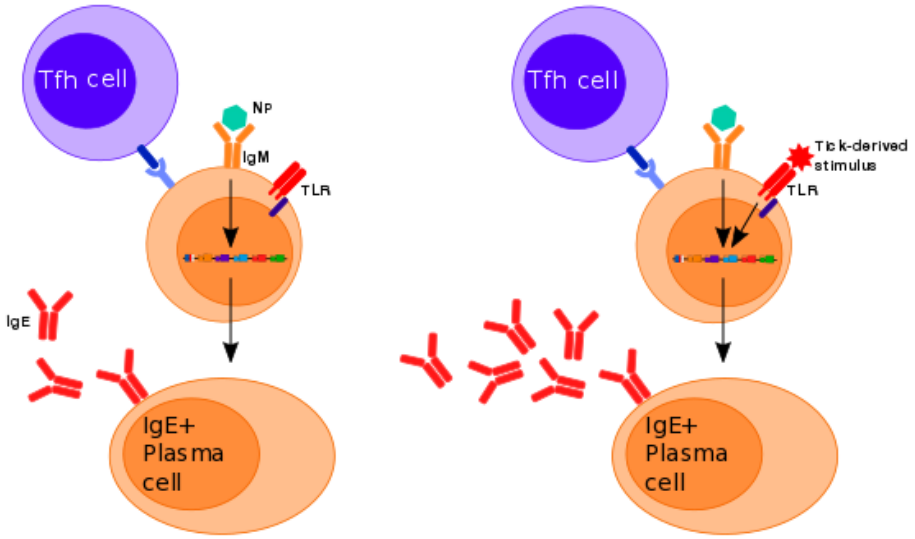


Figure 4.3 Tick extract provides an adjuvant effect.
Immunization with NP-OVA is capable of inducing an IgE response (left), however, the addition of tick extract amplifies this response, resulting in significantly increased IgE production (right).

immunoprecipitation with an anti-IgE antibody. Alternatively, tick-specific IgE+ B cells from tick immunized mice could be used to generate hybridomas, and the antibodies produced by these hybridomas could be used to immunoprecipitate tick extract antigens. Once isolated, mass spectrometry could be employed to identify the proteins found in the tick extract that lead to the production of IgE. This method would be complicated by the fact that no amino acid or nucleotide sequences from the lone star tick have been cataloged, inhibiting protein identification. Although some protein may be identified based on homology to proteins from other previously cataloged species, the development of an expressed sequence tag (EST) library for the lone star tick could help to facilitate protein identification. Once candidate molecules have been identified, the ability of each protein to act as an adjuvant can be assessed through analysis of serum antibody responses following subcutaneously immunization of the protein in combination with NP-KLH.

TLR signaling involvement in the induction of the IgE response to tick extract

Despite the challenges involved in identifying the molecules within the tick extract that drive the IgE response, the availability of reporter cell lines enabled the identification of several pattern recognition receptors that may be involved in the signaling cascade that leads to IgE production in this context. A commercially available TLR screen revealed that tick extract generated from *A. americanum* is capable of stimulating multiple TLRs, namely TLR2, TLR4, TLR5, and TLR9, all of which signal through the adaptor protein MyD88. Although MyD88 was originally thought only to be involved in Th1 response [187], previous work in a lung ragweed allergy model did

identify MyD88 signaling as a key component of both the IgE and IgG1 response to ragweed [177]. Interestingly, in our murine model of tick induced IgE, the IgE response is ameliorated in the absence of MyD88, while the IgG1 response to tick extract is only slightly reduced (Fig 3.3.A-C). These data suggest a divergence in the signals that drive the development of the IgE vs IgG1 response to tick extract, providing a useful model to dissect the signals that promote CSR to each of these isotypes (Fig 4.4). Identifying the signaling pathways that drive the development of IgE will be vital for identifying new targets for improving treatment of allergies. Although our data clearly implicate MyD88 as playing a key role in the development of an IgE response, it serves as a signaling adaptor for all TLRs except for TLR3, and defects in MyD88 result in a predisposition to severe bacterial infection [199]. Therefore, targeting MyD88 as a treatment for red meat allergy would likely result in a number of detrimental off target effects, suggesting that it may not be a viable treatment strategy.

In an effort to identify more promising treatment targets, and to begin to elucidate how tick extract serves as an adjuvant, we have attempted to identify the TLRs that are required for the development of the IgE response to tick extract. Based on the results of the TLR screen, we assessed the ability of mice deficient in TLR2 or TLR4 to mount an IgE response to tick extract. Although an initial experiment suggested that TLR2 may be required for the IgE response to tick extract, the data was not consistent when the experiment was repeated. No defect in the IgE response to tick extract was observed in $TLR4^{-/-}$ mice. More investigation will be required to determine if TLR2 or TLR4 signaling plays a vital role in the IgE response to tick extract, however it is possible that, despite playing a role in initiating the IgE response, the loss of signaling from a single

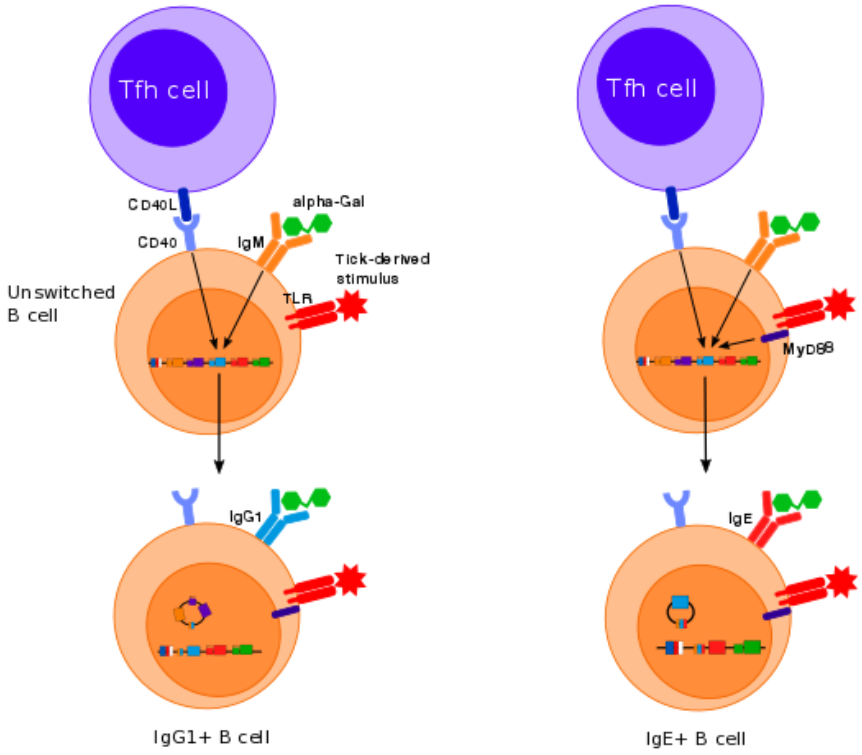


Figure 4.4 MyD88 signaling is required for the generation of an IgE response.
In the absence of MyD88 signaling tick extract immunization induces a tick-specific IgG1 response (left), however IgE production is impaired. When B cell intrinsic MyD88 signaling is intact, a robust IgE response develops (right).

TLR may not be sufficient to ablate the IgE response. Because tick extract is capable of activating multiple TLRs, it is possible that there is some redundancy in TLR signaling, and signaling through multiple TLRs must be blocked in order to observe a significant defect in IgE production. Mice deficient in multiple TLRs have been generated previously, however generating mice deficient in the specific TLRs that are activated by tick extract would require extensive breeding. An alternative would be to use a combination of commercially available TLR inhibitors, such as the TLR2 and TLR4 inhibitor, OxPAPC (Invivogen), in other TLR deficient mice, but further work will be required to optimize the use of these inhibitors.

In addition to investigating the specific TLRs that are required for the IgE response to tick extract, we have also worked to identify the downstream signaling molecules that promote CSR to IgE. Nuclear factor, IL-3 regulated (NFIL3) is a transcription factor found to be induced by LPS and IL-4 in the presences of STAT6 [200, 201] and suppressed by TGF-β stimulation [202], suggesting that it could play a role in Th2 responses. Characterization of mice deficient in NFIL3 showed that loss of NFIL3 resulted in a defect in CSR to IgE, but not IgG1 [203]. Because NFIL3 deficient mice exhibited impaired IgE but not IgG1 response, similar to our results in tick immunized MyD88 KO mice, we hypothesized that the loss of MyD88 could result in decreased expression of NFIL3. To investigate the potential role for NFIL3 in the development of a MyD88-dependent IgE response we assessed whether NFIL3 expression was reduced in MyD88$\gamma 1^{-/-}$ mice following in vitro stimulation. Stimulation with LPS and IL-4 in vitro was sufficient to induce the production of IgE in cells from B6 mice, whereas the defect in CSR to IgE observed in MyD88$^{-/-}$ and MyD88$\gamma 1^{-/-}$ was

maintained, and we were able to employ this system for our NFIL3 studies. Our data revealed no significant difference in the level of NFIL3 expression following in vitro stimulation, both at the mRNA level, as assessed by qRT-PCR, and at the protein level, as assessed by western blot, suggesting that NFIL3 expression is not dependent on MyD88 signaling in this context (data not shown). Because MyD88 signaling is involved in a large number of signaling pathways, a microarray comparing B cells from B6 and MyD88$^{-/-}$ or MyD88γ1$^{-/-}$ following stimulation would be useful for identifying candidate genes that may play a role in CSR to IgE.

Concluding Remarks

The work described in this dissertation has established a unique B cell signature present in the blood of patients with red meat allergy, and identified novel B cell subsets that are associated with α-gal specific IgE in allergy patients. Furthermore, we have shown that these B cells are capable of producing allergen specific IgE following *in vitro* stimulation, suggesting that these cells play a functional role in the pathogenesis of red meat allergy. These results provide new insight into the cellular mechanisms of the allergic response, and identify new potential targets for the treatment of food allergy. Additionally, the innovative bioinformatics pipeline for the unbiased identification of cellular subsets associated with markers of clinical disease we describe here has broad utility for identifying cellular mediators of disease in other conditions. In addition, we have generated and characterized a novel murine model of the tick induced IgE response, providing a physiologically relevant system in which to interrogate the mechanisms that drive the development and maintenance of the IgE response. Our data identifying B cell

intrinsic MyD88 signaling as a vital component of CSR to IgE (Fig 4.5) provides greater understanding of the mechanisms that drive the development of allergic IgE response. By further elucidating the signaling pathway that drives CSR to IgE we have also identified new potential targets for the prevention of allergic disease. Although the data presented here focuses specifically on tick-induced red meat allergy, these findings may apply to other food allergies as well.

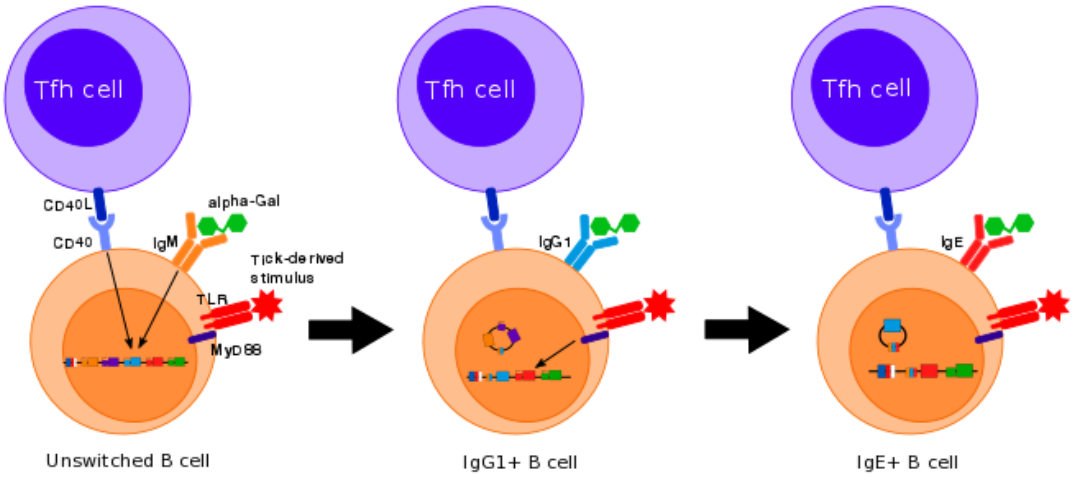


Figure 4.5 IgE is generated through GC and MyD88 dependent mechanisms following subcutaneous exposure to tick extract.
In the context of subcutaneous immunization with tick extract a GC response develops, and CSR to IgG1 occurs in a T cell dependent manor. Additional signaling through MyD88, likely due to activation of TLRs by tick extract, results in CSR to IgE and the generation of a robust IgE response.

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