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The Non-Canonical Roles of Histamine in Immunological Processes

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ABSTRACT

The non-canonical roles of histamine in immunological processes:

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Histamine is a well-known potent mediator during the elicitation of IgE-mediated allergic diseases. The most robust, and potentially fatal, response of histamine is observed in patients with food allergy that undergo IgE-mediated anaphylaxis. The key mechanisms of the biology of histamine in allergic disease are well-established, however there are still gaps in the knowledge of how histamine is regulating the allergic response. We investigated two different aspects of histamine biology: the first was focused on the kinetics of histamine during anaphylaxis and how histamine potentiates a prolonged response, and the second was to determine how newly identified histamine responding cells regulate antibody production in allergic disease. We designed our studies to specifically understand how histamine metabolites influence histamine-driven biology, and the role for histamine in regulating B cell antibody production.

Histamine is rapidly release during anaphylaxis and initiates responses primarily through histamine receptors 1 and 2. In the circulation histamine is quickly metabolized, with a half-life of about 1-2 minutes. Yet, antihistamines are effective at alleviating remaining symptoms hours after the initial response. This suggests that there is an unknown mechanism that promotes histamine-driven responses long after its degradation. We identified a histamine metabolite, imidazole acetic acid (IAA), that perpetuates histamine-driven inflammation by inducing histamine release from mast cells. We found that IAA does not bind to the histamine receptors itself, but instead IAA may be mediating its effects through an imidazole receptor, Nischarin. Interestingly however, in the absence of Nischarin from all cells resulted in heightened IAA-driven anaphylaxis, which suggests that IAA must mediate its effects on anaphylaxis through another, unidentified receptor. Together, these data demonstrated for the first time that IAA pays an important role during anaphylaxis by potentiating histamine release. Although Nischarin was not the receptor mediating these effects, our results suggest that Nischarin is a potential suppressor for anaphylaxis.

Histamine receptors themselves are known to be expressed on cells that are important for the development of allergic disease, such as T cells, dendritic cells (DC), and B cells. Yet, the effects of histamine on these cells, especially during sensitization, have not been well characterized. Previous

studies have shown that histamine receptor knock-out (KO) mice produce fewer antibodies in models of allergic air-way inflammation, however, it is not clear how histamine is regulating antibody production, nor whether histamine has any direct effects on B cells. In our studies, we found that both receptors were necessary for production of IgE and antigen-specific antibody responses in a model of allergic-airway hypersensitivity. In addition, we saw that histamine was necessary for the development of IgE^+ cells in the draining lymph nodes. Importantly, we also determined that histamine receptors 1 and 2 were necessary on both B and T cells for induction of IgE responses *in vivo*. We were able to characterize differential gene expression signatures in B cells and T cells lacking histamine receptors 1 and 2 compared to WT cells using RNA sequencing, and these signatures were characterized by defects in the expression of genes involved in pathways associated with cellular stress responses as well as other B cell regulatory pathways.

Collectively, this work has identified and characterized novel roles for histamine in allergic responses. We identified a mechanism whereby histamine can potentiate its own response through one of its metabolites and a role for histamine in regulating antibody responses. These findings highlight the non-canonical roles that histamine may play in the development of allergic disease, and may provide insights into the identification of targets for the development of improve therapeutics for the treatment of these diseases.

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LIST OF ABBREVIATIONS

α	alpha
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AD	atopic dermatitis
AID	activation-induced cytidine deaminase
ALDH	aldehyde dehydrogenase
Alum	aluminum hydroxide
AMPK	AMP-activated protein kinase
APC	allophycocyanin
APC	antigen presenting cell
AR	allergic rhinitis
ASC	antibody secreting cells
APC-Cy7	allophycocyanin-cyanine7
β	beta
BAL	bronchial lavage fluid
BCR	B cell-receptor
BMMC	bone marrow derived-mast cells
BSA	bovine serum albumin
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CCG	chicken gamma globulin
cDC1	type 1 conventional dendritic cell
cDC2	type 2 conventional dendritic cell
CFA	complete Freund's adjuvant
CFSE	carboxyfluorescein succinimidyl ester
CRSwNP	chronic rhinosinusitis with nasal polyps
CSR	class-switch recombination
DAG	1,2-diacylglycerol

DAO	diamine oxidase
ε	epsilon
ECL	enterochromaffin-like cell
EDTA	ethylenediaminetetraacetic acid
EoE	eosinophil esophagitis
ELISA	enzyme-linked immunosorbent assay
ELISpot	enzyme-linked immune absorbent spot
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorting
FBS	feta bovine serum
FDC	follicular dendritic cell
FITC	fluorescein isothiocyanate
FMO	fluorescence minus one
FSC	forward scatter
γ	gamma
GALT	gut-associated lymph nodes
GC	germinal center
GI	gastrointestinal
GPCR	g-protein coupled receptor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Het	heterozygous
HDC	histidine decarboxylase
HMT	histamine N-methyltransferase
HR	histamine receptor
HRP	horseradish peroxidase
i.d.	intradermal
i.p.	intraperitoneal
i.v.	intravenous

IAA	imidazole acetic acid
IFNα	interferon alpha
IFNγ	interferon gamma
Ig	immunoglobulin
IL	interleukin
IP3	inositol-1,4,5-triphosphate
IPEX	immune dysregulation, polyendocrinopathy, enteropathym X-linked
iPSC	induced pluripotent stem cells
K_d	dissociation constant
KO	knock out
LP	lamina propria
LN	lymph node
MAO-B	monoamine oxidase B
MAPK	mitogen-activated protein kinase
mDC	myeloid dendritic cells
MHCII	major histocompatibility complex II
moDC	monocyte-derived dendritic cells
mRNA	messenger ribonucleic acid
MZ	marginal zone
N.D.	not detectable
NP	4-hydroxy-3-nitrophenyl
NO	nitric oxide
OVA	ovalbumin
PAF	platelet activating factor
PBS	phosphate-buffered saline
PCA	principal components analysis
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell

PE	phycoerythrin
PE-Cy7	phycoerythrin-cyanine7
PEI	polyethyleneimine
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PNA	peanut agglutinin
PSA	passive systemic anaphylaxis
RBC	red blood cell
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute-1640 media
ROS	reactive oxygen species
SEM	standard error of the mean
SSC	side scatter
SLE	systemic lupus erythematosus
T1	transitional type-1
T2	transitional type-2
TD	(T cell) thymus-dependent
Tfh	follicular T helper cells
Th	T helper cell
Th2	T helper type-2 cells
TI	(T cell) thymus-independent
TI-1	Type 1 T cell-independent
TI-2	Type 2 T cell-independent
TNF	tumor necrosis factor
Treg	$CD4^+$ regulatory ($FoxP3^+$) T cells

tSNE	t-stochastic neighbor embedding
UMI	unique molecular identifier
UPR	unfolded protein response
WT	wild type

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CHAPTER 1 – Introduction

GENERAL INTRODUCTION

Histamine was first described in 1907 when it was synthetically synthesized by Windaus and Vogt as an imidazole derivative of the amino acid histidine and was called imidazolethylamine [1]. Imidazole is a five-member aromatic heterocyclic ring, composed of three carbon and two nitrogen atoms that are at the first and third position. Imidazole derivatives contain an imidazole ring with additional substituents; in the case of histamine, the imidazole ring includes an ethyl amine group. The imidazole ring can act as weak acid or base and is susceptible to nucleophilic and electrophilic reactions [2], making it a biological skeleton for a variety of substituents. Consequently, imidazole derivatives have been described in a variety of processes including antibacterial, anti-inflammatory, antifungal, and antiparasitic, indicating their great biological potential[3]. Interestingly, upon the metabolism of histamine the remaining catabolites retain the imidazole ring, however it is not clear whether these molecules have any biological significance.

The biological roles of histamine itself have been well described in a variety homeostatic and pathological functions and are mediated through four histamine receptors (H1R, H2R, H3R, and H4R). Each histamine receptor utilizes a distinct signaling pathway, which allows for a plethora of responses depending on the localization and expression of the histamine receptors on a given cell type (see below). To date, studies on the role of histamine in allergic responses have focused mainly on its role during the elicitation phase. However, the continual identification of new histamine receptor-expressing cells and noncanonical sources of histamine allows us to investigate new histamine-dependent mechanisms that may be important for the fine-tuning of immune responses. The main goals of this thesis were to (1) characterize the role of histamine metabolites in allergic response and (2) further assess the non-canonical role of histamine in allergic disease, specifically in antibody production.

Histamine Synthesis and release

Histamine is synthesized through a decarboxylation reaction of the amino acid histidine, which is catalyzed by the enzyme histidine decarboxylase (HDC). HDC is mainly expressed by mast cells and basophils, although some other cells, including neurons, also express this enzyme at lower levels [4]. Upon its production, histamine will either be immediately released, known as nascent histamine, or stored

in granules for rapid released in great quantities upon cellular activation. The ability for mast cells and basophils to store histamine plays an important role in the levels of histamine that will be released upon their activation. Mast cells and basophils are the only known cells that can store histamine, which make them the major histamine secreting and producing cells [5]. Other significant sources of histamine come from histaminergic neurons and enterochromaffin-like cells (ECL) from gastric glands [6]. Minor cellular sources of histamine include DCs, T cells, macrophages, and neutrophils, in which HDC is induced in response to various stimuli [7]. However, a biological role for histamine from these cell types has only been shown *in vitro*, and the relevance of their ability to make histamine *in vivo* is not clear. For example, endogenous histamine expression from human DCs and HDC expression from human monocyte/macrophages has been shown to be important during their *in vitro* differentiation [8,9]. In addition, HDC KO murine T cells were shown to have increased levels of nitric oxide (NO) that resulted in decreased cytokine production, suggesting that histamine production in T cells may play an important role in their effector function [10]. Overall these studies demonstrate that non-canonical sources of histamine may be important for autocrine mechanisms of regulation. However, whether these cells are relevant sources of paracrine histamine is not known.

As mentioned previously, there are cells that induce HDC and are able to secrete histamine and there are cells that are able to store histamine in granules, such as mast cells and basophils. The process of secreting stored histamine occurs through cellular degranulation, and is most well-described through an antigen-specific IgE-dependent mechanism [11]. Studies have shown that there are also antigen-specific IgG-dependent mechanisms of mast cell degranulation, however the amount of antigen required for this mechanism is 100-1000 fold greater than for IgE, depending on the antigen [12]. IgE-dependent histamine secretion typically occurs in allergic responses and parasitic infections. In allergy, the mechanism of IgE-mediated histamine release starts with the production of antigen-specific IgE against a non-pathogenic antigen, such as food proteins, pet dander, or pollen. Once this antigen-specific IgE is produced, it can bind to the high-affinity IgE receptor, FCεRI, on the surface of mast cells and basophils. Re-exposure to the same antigen will lead to cross-linking of the IgE bound to the surface of mast cells and basophils via FcεRI, degranulation, and release of large quantities of histamine, among other inflammatory mediators.

IgE has two known receptors, FCεRI and CD23 (FCεRII; low affinity IgE receptor). While FCεRI is mainly expressed on mast cell and basophils and mediates degranulation [13], FCεRII is expressed on B cells and has been shown to regulate the synthesis of IgE by B cells [14]. FCεRI has the highest binding constant of all the immunoglobulin receptors, with a K_d ranging from 10^{-9} to 10^{-10} M, while FCεRII has a K_d ranging from 10^{-6} to 10^{-7} M [15]. FCεRI expressed by mast cells and basophils is composed of an alpha, beta, and two gamma chains. In mice, FCεRI is only expressed on mast cells and basophils, whereas in humans there is also reported expression on DCs [16], eosinophils [17], macrophages/ monocytes [16], and neutrophils [18]. However, the FCεRI structure on these cells is only composed of an alpha and two gamma chains, but lacks the beta chain found in mast cells and basophils. Expression of the beta chain has been shown to stabilize surface expression of the receptor and facilitate a more robust response that mediates degranulation in humans [19,20], and in mice the beta chain is necessary for surface expression of FCεRI [20]. Thus, it is not clear whether the FcεRI expressed by cells that lack the beta chain is functional *in vivo*.

HISTAMINE FUNCTION

Once histamine is released it can bind to its known receptors, H1R-H4R, which are g-protein couple receptors (GPCR). Structurally, GPCRs have seven transmembrane-spanning domains, where the N-terminus is outside the cell and the C-terminus is inside the cell. GPCRs signal through the activation of heterotrimeric G-proteins Gα, Gβ or Gγ, that can be further classified into subsets [21]. Histamine receptors signal through three different Gα subsets, $G\alpha_q$, $G\alpha_s$, $G\alpha_{i/o}$ [22]. Aside from sharing structural motifs, histamine receptors have low sequence homology that ultimately results in varying binding affinities to histamine. H3R has the highest binding affinity for histamine, with a K_d of approximately 10 nmol/L, followed by H4R, H1R, and H2R, with K_d of approximately 20-40 nmol/L, 10 μmol/L, and 30 μmol/L, respectfully [23]. H1R and H2R are widely expressed on a variety of cells in the body, while H3R is localized mainly to the brain, and H4R is expressed in hematopoietic and intestinal cells [24]. The physiology of histamine and functions of each of the histamine receptors have been characterized

through the use of agonists, antagonists, and histamine receptor deficient mice, which will be discussed in more detail below.

Histamine-driven biology

Before the extensive characterization of histamine receptor signaling, histamine was first characterized based on physiological observations. A pivotal early study demonstrated that histamine could induced vasodilation, contraction of smooth muscles in the airways, uterus, and intestine, stimulated heart rate contractility, and included shock in animals [25]. The first histamine receptor antagonist was discovered in the 1940s and it targeted H1R; mepyramine was the first widely used H1R antagonist to alleviate symptoms induced by histamine [26]. Along with mepyramine, there were other antihistamines developed that are now deemed the first generation of antihistamines including diphenhydramine (Benadryl), carbinoxamine (Clistin), clemastine (Tavist), chlorpheniramine (chlorTrimeton), and brophern iramine (Dimetane). It was soon discovered that mepyramine was not able to block all histamine-driven physiology, specifically increased heart rate and gastric hydrochloric acid secretion, which led to the discovery of H2R. Black and colleagues discovered that Burimamide blocked gastric acid secretion [27], which sparked the discovery of other H2R antihistamines such as cimetidine, ranitidine, and famotidine. Cimetidine was the first effective and non-toxic antagonist and was found to inhibit gastric acid secretion upon oral and intravenous administration [28]. Concurrently there were studies on the identification of where histamine was being expressed that highlighted the significance of histamine biology in the brain [29], and helped to explain the off-target effects of first generation antihistamines that induced sedation [30,31]. As mentioned before, H3R has a higher affinity to histamine than H1R and H2R, and this characteristic led to the discovery of H3R, since specific histamine agonists were found to be more potent than the known histamine antagonist that were targeting H1R and H2R [32]. Early studies focused on the roles of H3R in brain biology, and to date, it has been established that the primary expression and function of H3R is exclusive to the brain [33]. Finally, the most recently discovered receptor, H4R, occurred with advances that allowed for sequencing of the human genome [34,35]. Although there are no strong physiological manifestations that have been characterized to be

driven specifically by H4R, it has many roles in the regulation of a variety of immune cells, which will be discussed below in the context of allergy.

Histamine agonists and antagonists served as useful tools to define the physiological roles of each histamine receptor. H1R activation specifically induces airway and vascular smooth muscle cell contraction, increased vascular endothelial cell permeability, and pruritus. H2R activation induces gastric acid secretion, increased heart rate, and relaxation of smooth muscle cells, uterus, and airways [22,24]. H3R is involved in the sleep-wake cycle, cognition, and homeostatic regulation of energy levels [36]. H4R has less obvious physiological roles, but it is important for immunomodulation and inflammation [37]. The characterization of the specific signaling pathways is primarily attributed to advances in cloning of the histamine receptors, which will be described below.

H1R

H1R is expressed in a variety of cells, including immune cells such as monocytes, neutrophils, DCs, and T cells, as well as neurons, endothelial cells, and epithelial cells [22,38]. Classically, H1R signals mainly through the g-protein $G\alpha_{q/11}$, which activates phospholipase C (PLC) to cleave phosphatidylinositol 4,5-bisphosphate to form inositol-1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG). The increase in IP3 leads to increased binding to its receptors, such as calcium (Ca^{2+}) channels in the endoplasmic reticulum, increasing the levels of cytosolic Ca^{2+}. The increased levels of Ca^{2+} and DAG activate protein kinase C (PKC), which can further phosphorylate and activate downstream mediators [22]. There are also several other less common signaling pathways of H1R, including nitric oxide (NO) [39], liberation of arachidonic acid metabolites [40], and increased levels of cyclic adenosine monophosphate (cAMP) [41]. Histamine induced cAMP signaling is primarily driven through H2R which will be discussed below.

H2R

H2R is also widely expressed and found on a variety of cells including immune cells, such as DCs, and T cells, as well as parietal cells, smooth muscle cells and in brain and cardiac tissues [22]. H2R mainly signals through the $G\alpha_s$ G-protein, and enhances adenylate cyclase activity, which significantly

increases cAMP formation and the activation of protein kinase A (PKA). However, H2R has also been shown to couple with $G\alpha_q$ to mobilize intracellular Ca^{2+} through the PLC/IP3 pathway, as discussed above for H1R [24].

H3R

H3R is mainly expressed in brain by histaminergic neurons, and at low levels by glial cells. H3R primarily signals though $G\alpha_{i/o}$, to reduce cAMP formation and inhibit voltage-activate Ca^{2+} channels, which leads to decreased levels of intracellular Ca^{2+} [24,36]. However, there is also evidence that signaling of H3R through $G\alpha_{i/o}$ activates phosphoinositide 3-kinase (PI3K), leading to phosphorylation of Akt, and activation of mitogen-activated protein kinase (MAPK) pathways [24,42]. Unlike H1R and H2R, H3R has many identified isoforms that can influence g-protein coupling and signaling events [36].

H4R

H4R is the most recently discovered histamine receptor, and is primarily expressed in immune cells, including eosinophils, DCs, Langerhans cells, neutrophils, T cells, basophils, and mast cells. H4R also signals through $G\alpha_{i/o}$, and has been shown to primarily increase intracellular Ca^{2+} [24]. Although H3R has the highest homology with H4R and signals through the same g-protein, the major differences in signaling can be attributed to the formation of different isomeric forms of H3R.

Taken together, histamine receptor signaling is very complex, and, although there are classical signaling pathways defined, histamine can facilitate a completely different response through the same receptor in a different cell type depending on which g-protein subunit is utilized. For example, the release of arachidonic acid through H1R results from its coupling to $G\alpha_{i/o}$ instead of $G\alpha_q$. This phenomenon is also observed for H2R where coupling occurs with $G\alpha_q$ instead with $G\alpha_s$, however, it was found that this property was restricted to certain cell types [24]. Additionally, studies have shown that the same receptor can have differential signaling pathways dependent on the stereochemistry of the same agonists [43], which demonstrates the sensitivity of signaling regulation through the histamine receptors. Overall, It is

important to consider these properties of histamine receptors in order to understand how histamine regulates specific mechanisms in disease.

HISTAMINE IN ALLERGY

Histamine is a key mediator of inflammation, and has been detected at elevated levels in multiple sclerosis [44] and rheumatoid arthritis [45], but it is predominantly associated with allergic diseases. Specifically, histamine levels are increased in the BAL fluid from patients with allergic asthma [46], and in the skin of patients with atopic dermatitis (AD) [47] and chronic urticaria [48]. More importantly, levels of histamine also correlates with severity of disease in allergic asthmatic patients [49,50].

Allergic diseases are a heterogenous group of diseases, and can be categorized based on specific biological markers, such as the presence or absence of IgE, the type of antigen-specific IgE, and the presence of eosinophils or neutrophils in the lung, to name a few. However, the classical model of allergy is driven by a T helper cell type 2 (Th2) response through antigen-specific IgE. In this model, allergic responses are driven through antigen binding to antigen-specific IgE to activate cells such as mast cells, as described above. This type of activation leads to the release of histamine, and the physiological responses observed are similar to those in studies with administration of histamine. In allergy, these responses are mediated primarily through binding to histamine receptors 1,2, and 4 [51].

Murine models of allergic diseases such as asthma and atopic dermatitis have help to elucidate important mediators of disease. In addition, these diseases have been studied in histamine receptor KO mice, which have been a useful tool to understand the cellular mechanisms driven through specific histamine receptors. Recently, it has been shown in a model of atopic dermatitis that H4R KO mice have decreased skin lesions, reduced infiltration of inflammatory cells and reduced epidermal hyperproliferation at lesional sites in the skin. Systemically, these mice had fewer cells in the spleen and lymph nodes and reduced levels of antigen-specific-IgE. Studies using a model of allergic airway hypersensitivity have demonstrate that H1R is necessary for the recruitment of $CD4^+$ T cells to the lung [52], and H2R is necessary for the recruitment of eosinophils into the lung upon allergen exposure. Interestingly in the same model, multiple studies have shown that H2R is necessary for total and antigen-specific IgE production [53,54].

Although multiple studies have shown that the absence of histamine receptors results in reduced antibody production, the mechanisms that mediate this phenotype have not been characterized. A major contributing reason to the lack of studies on histamine-driven regulation of antibody production is that histamine is canonically recognized as a strong effector during allergic elicitation, rather than a regulator of antibody production. However, there are multiple studies that report histamine receptor expression on the cells necessary for antibody production including T cells, DCs, and B cells. In addition, the production of IgE does not only occur upon the development of allergies, but also throughout disease including allergic elicitation. Therefore, it is possible that histamine may be regulating these processes, but as of now has been overlooked. Currently, there are defined roles of histamine receptors on T cells and DCs. There are few studies on histamine receptors on B cells, and none have investigated the potential role of histamine receptors on B cells *in vivo*. Given the presence of histamine throughout the allergic response, it is important to understand how it may be regulating antibody responses.

HISTAMINE METABOLISM AND METABOLITES

There are two major pathways of histamine metabolism, an intracellular mechanism driven by histamine N-methyltransferase (HMT), which catalyzes ring methylation, and an extracellular mechanism driven by diamine oxidase (DAO), which catalyzes oxidative deamination [55] (**Figure 1.**). The resulting products from HMT breakdown are *N*-methylhistamine, which is further metabolized to *N*-methylimidazole acetaldehyde by monoamine oxidase B (MAO-B), followed by further break down to *N*-methylimidazole acetic acid by aldehyde dehydrogenase (ALDH). DAO breaks down histamine to imidazole acetaldehyde, followed by modification to IAA by ALDH, and finally conjugation with ribose to derive imidazole acetic acid riboside [55-57]. The major products that have been quantified in human studies are *N*-methylhistamine, *N*-methylimidazole acetic acid, imidazole acetic acid, and imidazole acetic acid riboside (**Figure 1.**). Few studies have investigated which of these two pathway dominates, however it is known that the affinity for histamine is relatively similar between HMT and DAO and metabolites from both pathways are detected upon injection of histamine [55,57]. Studies on the kinetics of histamine have determined that histamine in the periphery has a half-life of 1-2 minutes [58], however it has also been shown that disease states, such as allergies, may affect histamine clearance [59].

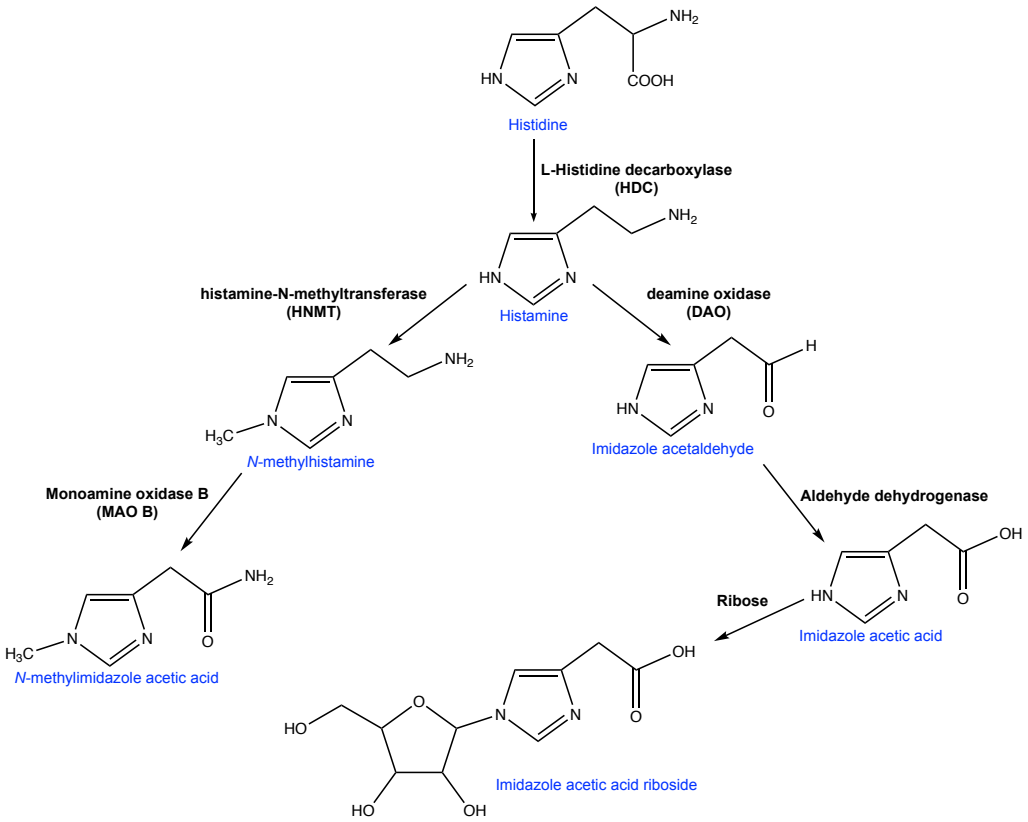


Figure 1: Histamine metabolism and major metabolites

Histamine is metabolized by two major pathways: an intracellular pathway catalyzed by HNMT and an extracellular pathway catalyzed by DAO. The major metabolites that are characterized are *N*-methylhistamine, *N*-methylimidazole acetic acid, imidazole acetic acid, and imidazole acetic acid riboside.

Regardless of the mechanism of their generation, histamine metabolites retain the imidazole ring, and thus, are imidazole derivatives. Importantly, early studies demonstrated that these metabolites have a much longer half-life than histamine itself and are relatively stable [60]. This is noteworthy because imidazole derivatives have been described in a variety of processes including antibacterial, anti-inflammatory, antifungal, and antiparasitic, indicating their great biological potential [3]. Thus far, histamine metabolites have not been considered as biologically active, even in responses where vast amounts of histamine are released, such as during anaphylaxis. Histamine metabolites are instead used as markers to confirm the diagnosis of anaphylaxis [61]. There are only few studies on a histamine metabolite, IAA, that describe a biological activity [62,63]. In the first study, the authors were interested in the potential of histamine metabolites to act as chemotactic agents for human eosinophils, since histamine had been described to have this activity. Of the metabolites tested, only IAA was shown to act as a chemotactic agent for human eosinophils *in vitro*, and it was described that the chemotactic mechanism was similar to that of histamine. Results from this study suggested that a histamine metabolite could perpetuate a histamine-driven response long after histamine is metabolized. The second published study on the biological activity of IAA examined the potential receptors for this histamine metabolite in the central nervous system. Altogether, the roles for histamine metabolites are not fully understood, and in cases where histamine is robustly secreted, it is essential to characterize how histamine metabolites may be mediating disease.

HYPOTHESIS AND SIGNIFICANCE

While histamine has well-defined roles in immune regulation, the effects of histamine are likely very complex, due to differential expression of multiple histamine receptors on distinct cells. Thus, it is important to study the diverse effects that histamine may mediate at different points in an inflammatory response. We derived two different hypotheses of based on histamine biology (1) Histamine metabolite IAA drives histamine-driven responses and (2) Histamine regulated antibody responses through histamine receptors on B cells. The studies in this thesis were designed to directly test this hypothesis by investigating the role of the histamine metabolite IAA in a variety of allergic responses, and the role of histamine receptors on B cells in models of allergic immunization. The work presented in this thesis

shows for the first time that IAA is capable of perpetuating histamine-driven allergic responses through the induction and release of additional histamine, and that histamine itself plays a key role in the development of allergen-specific IgE. Altogether, these studies demonstrate that histamine plays a variety of roles during the allergic response, and they provide novel insights into the mechanisms that may regulate allergic disease.

CHAPTER 2: Materials and Methods

MICE

C57BL/6 (B6) and (B6.SJL) mice were obtained from Taconic Farms, Hudson, NY. Mast cell deficient mice (SASH, $Kit^{W\text{-}sh}$) were obtained from the Jackson Laboratory. Nischarin LacZ-reporter/conditional KO mice were derived through the Northwestern mouse core. Histidine carboxylase deficient mice (HDC KO) were obtained from H. Ohtsu [64]. Histamine receptor 1 KO mice (H1R KO) [65] and H2R KO [66] mice were obtained from Dr. Takeshi Watanabe, Kyoto University, Japan, and they were crossed to derive H1R/H2R double KO (DKO) mice in our lab. Rag1 KO mice were a gift from Dr. Deyu Fang. B cell-deficient (μMT) mice were a gift from Dr. Xunrong Luo. Mice were housed under specific pathogen-free conditions. All experiments were approved by the Northwestern University Animal Care and Use Committee.

IN VIVO HISTAMINE AND IAA ASSAYS

Eosinophil recruitment to peritoneum

Mice were administered IAA or histamine by intraperitoneal injection (i.p.) at increasing doses (500 μL; 6.0 mM, 0.6 mM, or 0.06 mM). 24 hours following injection, peritoneal lavages were collected, and eosinophils were quantified from cytospins using Diff Quick (Dade Behring, Newark, DE).

Induced pruritus

IAA or histamine were administered by intradermal (i.d.) injection in the back of the neck (shaved) via a 26 G needle at (100 μL; 0.1 M). This injection site was chosen as it is only accessible by the animal's hind paws, and therefore scratching behavior can be separately identified from grooming, which is performed by the forelimbs. Mice were placed in buxco chambers and filmed using a MacBook Pro and imovie software. Itch was measured by counting the number of 'bouts' of scratching in the 30-minute period immediately following injection. A bout of scratching was defined as three or more individual rapid scratch movements with the hind paws to the area around the injection site (i.e. the back of the neck).

Plasma histamine quantification

IAA (100 μL; 0.1 M) or PBS was administered by intravenous (i.v.) injection as above, blood was collected prior to injection and at 1 minute, 3 minutes, 10 minutes, and 30 minutes after injection. Serum histamine levels were quantified by Histamine ELISA kit (Abcam).

ANAPHYLAXIS MODELS

IAA and Histamine challenge

IAA and Histamine were administered by i.v. injection (100 μL; 0.1 M) and rectal temperatures were taken every 10 minutes for 60 minutes.

Passive systemic anaphylaxis (PSA)

Mice were passively sensitized overnight by intravenous injection (i.v) of 50 μg of monoclonal OVA-specific murine IgE (TOε clone) [67], generated by Northwestern University Recombinant Protein Production Core Facility. The next day mice were challenged i.v. with 50 μg of OVA (Sigma). Rectal temperatures were taken every 10 minutes for 60 minutes.

ALUM

To prepare alum (aluminum hydroxide, $Al(OH)_3$), 22.8 mL of sodium hydroxide (0.25 N NaOH; Ricca) was added dropwise to 10 mL of 10% of potassium alum (aluminum potassium sulfate, $AlK(SO)_4$, • 12 H_2O; Sigma) while vortexing (at low speed). This solution was incubated at room temperature for 10 minutes, followed by centrifugation at 1000 x g for 10 minutes. The supernatant was discarded and the pellet ($Al(OH)_3$), was washed with 30 mL of molecular biology grade water (Corning). $Al(OH_3)$ was centrifuged at 1000 x g for 10 minutes, the supernatant was discarded, and $Al(OH)_3$ was resuspended with molecular biology grade water to a total final volume of 50 mL. Alum was store at 4 °C for up to 2 months.

MOUSE MODELS OF IMMUNIZATION

For OVA-alum immunization, mice were immunized by i.p. injection of 10 μg of OVA (Sigma) conjugated to 30 μg of alum on day 0 and 14, followed by aerosolized 1 % OVA inhalation (10 mg/mL) for 20 minutes on days 21, 22, and 23. Mice were sacrificed 24 hours after the last challenge. For T cell-dependent and T cell-independent assays, mice were injected i.p. with 100 μL of either 50 μg 4-hydroxy-3-nitrophenyl$_{30}$-ficoll, (NP_{30} ficoll; Biosearch technologies) or 10 μg of $NP_{(20\text{-}26)}$ acetyl-chicken gamma globulin (NP-CGG; Biosearch technologies) conjugated to 3 μg of alum. After 14 days mice were sacrificed. For germinal center analysis of lymph nodes, 150 μg total of $NP_{(20\text{-}26)}$ CGG emulsified in Freund's incomplete adjuvant (Sigma) was injected into mice by subcutaneously in the flanks behind legs and scruff of neck [68], after 14 days mice were sacrificed.

BRONCHIAL ALVEOLAR LAVAGE (BAL) FLUID OF TOTAL AND DIFFERENTIAL CELL COUNT

BAL was collected by flushing the lung with 0.7 mL of sterile BAL fluid (10% FBS, 1mM EDTA, 1 X PBS) via the trachea, and cells were counted. For differential cell counts, 100 μL of collected BAL was used for cytospins onto slides and stained with Diff Quick (Dade Behring, Newark, DE). Differential cell numbers for neutrophils, lymphocytes, and eosinophils were calculated based on the percent by counting 100 cells per slide.

CELLULAR RECONSTITUTION IN MICE AND EXPERIMENTAL MODELS

Kit$^{W\text{-}sh}$ bone marrow-mast cell reconstitutions

Mast cell deficient mice (Kit$^{W\text{-}sh}$) were reconstituted with bone marrow-derived mast cells (BMMC). BMMC were derived from cells isolated from femurs and tibias of WT or HDC KO mice and cultured at 5.0 x 10^5 cells/mL in supplemented RPMI (2 mM L-glutamine, 1 mM sodium pyruvate [Sigma], 0.1 mM nonessential amino acids [Sigma], 10% FBS [Atlanta Biologicals], 25 mM HEPES [Sigma], 100 U/ml penicillin [Corning], 100 mg/ml streptomycin [Corning] and 0.05 mM 2-Mercaptoethanol [Sigma]) for 4-6 weeks with 30 ng/ml recombinant mouse (m)IL-3 (Miltenyi Biotec). 10^7 BMMC were i.v. injected into 4-6 week old W^{sh} mice via retro-orbital injection on day 0, and then again at week 8 with 10^6 BMMCs. 12

weeks after the first injection, mice were challenged with 130 mg/kg of IAA to induce anaphylaxis, and rectal temperatures were taken every 10 minutes for 60 minutes.

Rag1 KO T and B cell reconstitutions

Rag1 KO mice were reconstituted with B and T cells from the spleen and lymph nodes of WT mice (superficial cervical, axillary, brachial, mesenteric, inguinal, and lumbar). B cells were purified by positive selection using CD45R (B220) conjugated MicroBeads, (Miltenyi Biotec) according to the manufacturer's instructions. T cells were purified from the negative fraction of the B220 isolation, followed by a secondary negative selection using EasySep^{TM} Mouse T cell Isolation kit (Stemcell). Each mouse received 10^7 T cells and 10^7 B cells. After 8 weeks of reconstitution mice were immunized with OVA-alum as above.

Bone marrow chimeras

Mixed bone marrow chimeric mice were generated in B6.SJL (CD45.1) mice. B6.SJL mice were lethally irradiated with 11 Gy gamma-radiation followed by reconstitution with 2.5 x 10^6 mixed-inoculum bone marrow cells (80% μMT or Rag1KO marrow and 20% H1R/H2R DKO or WT marrow; CD45.2). After 12 weeks of reconstitution mice were immunized with OVA-alum as above.

IN VITRO T CELL CYTOKINE PRODUCTION

Spleens and mediastinal lymph nodes were removed from OVA-immunized mice, homogenized, and red blood cells were lysed using eBioscience 1 X RBC lysis buffer (Invitrogen). Splenocytes were plated at 2.5 x 10^6 cells/mL in 24-well plates at 1 mL/well, while mediastinal lymph nodes cells were plated at 1×10^6 cells/mL. Cells were cultured in supplemented RPMI in the presence of OVA (50 μg spleen, 10 μg lymph node), 50 μL anti-CD3/CD28 (Dynabeads Mouse T-Activator CD3/CD28 for T cell expansion and activation, Thermo Fischer Scientific), or supplemented RPMI alone. Supernatants were collected 72 hours after culture and production of IL-5 and IL-13 was quantified by sandwich ELISA described below (Antibodies used for ELISAs are in **Table 2**.). IL-4 was quantified intracellularly by flow

cytometry using a Fixation/Permeabilization Kit with BD GolgiStop™ (BD Bioscience) as described in detail below.

B CELL CULTURES

Naïve B cells ($B220^+$) were purified from the spleen by positive selection using CD45R (B220) conjugated MicroBeads, (Miltenyi Biotec) according to the manufacturer's instructions. Cells were plated at 250,000 cells/mL in supplemented RPMI 1640 the presence of IL-4 (10-50 ng/mL; Peprotech) and anti-CD40 (1 μg/mL; Invitrogen) for 4-6 days.

Antibody secretion

For analysis of IgE and IgG1 antibody secretion, B cell culture supernatants were collected at day 4 of culture and spun down at 300 x g for 5 minutes. The supernatant was then isolated from the cell pellet. IgE and IgG1 were quantified in the supernatant by sandwich ELISA (described below).

Intracellular antibodies

For analysis of intracellular IgE and IgG1, cells were collected at day 4 of culture and spun down at 300 x g for 5 minutes, the supernatant was discarded and the pellet was collected. Cells were washed with 1 x PBS, spun down and 300 x g for 5 minutes, and the supernatants were decanted. Surface IgE and IgG1 were removed by incubating cells with 0.1 % (2X) trypsin-EDTA at room temperature. Cells were stained with a live/dead dye and Fc receptors were blocked as described below. Cells were stained for surface B220, CD138, and IgM at 4 °C for 30 minutes, fixed with 10 % neutral buffered formalin solution at 37 °C for 10 minutes, and permeabilized with cold (-20 °C) methanol for 30 minutes on ice. Cells were spun down at 300 x g and washed with 1 X PBS. Intracellular staining for IgE and IgG1 was performed at room temperature for 5 minutes. Cells were washed with FACs buffer (1% FBS in 1 X PBS) and analyzed by flow cytometry.

CFSE staining

$B220^+$ B cells were isolated as described above and brought to a concentration of 8×10^6 cells/mL in 5% FBS in 1 X PBS. CFSE (Thermo Fischer Scientific) was added at a concentration of 5 μmol/L, and cells were stained for 7 minutes at room temperature in the dark. Cells were immediately washed three times with 5% FBS in 1 X PBS. Cells were resuspended and plated at a concentration of 250,000 cells/mL in supplemented RPMI in the presence of IL-4 (10 ng/mL; Peprotech) and anti-CD40 (1 μg/mL; Invitrogen) for 6 days in 6 well plates at a volume of 4 mL/well (Final culture volume = 24 mLs) Cell proliferation was analyzed by quantifying CFSE staining by flow cytometry, in conjunction with surface markers against B220, CD138, IgE, and IgG1.

Apoptosis

$B220^+$ cells were isolated as above and cultured for 4 days in IL-4 (20 ng/mL; Peprotech) and anti-CD40 (1 μg/mL; Invitrogen) in 6-well plates at 4 mL per well. Cells were collected and washed with 1 X PBS and counted. Staining for apoptosis was conducted using Annexin V – FITC + PI Apoptosis Detection Kit (Leinco Technologies, Inc.).

QUANTIFICATION OF ANTIBODIES AND PROTEINS BY ELISA

Serum and culture immunoglobulin levels

Blood was collected from euthanized animals by cardiac puncture, placed into serum separation tubes (BD), and spun at 8,000 rpm for 8 minutes. Cell supernatants and serum were used to quantify antibody levels. Total antibodies (IgE, IgG1, IgM, and IgG2c) were determined sandwich ELISA by coating 96-well plates with primary antibody (all antibodies used for ELISA are in **Table 2.**) against the specific isotype of interest at a concentration of 2 μg/mL (100 μL) in carbonate buffer (pH 9.6) and incubated overnight at 4 °C. The next day plates were washed 3 times with ELISA was buffer (0.05 % Tween-20 [sigma] in 1 X PBS), and blocked with 100 μL of 3 % BSA (sigma) for 2 hours at room temperature. Plates were washed 3 times with ELISA wash buffer and samples and standards (antibody standards are in **Table. 2**) were added to the appropriate wells at 100 μL and incubated overnight at 4 °C.

Plates were washed 3 times with ELISA wash buffer and incubated with 1-2 μg/mL of biotinylated secondary antibody against the specific isotype of interest for 2 hours at room temperature. Plates were washed 3 times with ELISA wash, incubated with streptavidin-HRP (R&D Systems) in 1 X PBS for 30 minutes at room temperature in the dark, and washed 3 more times with ELISA wash buffer. ELISA plates were developed using ABTS (Invitrogen) and read at 415 nm.

OVA-specific IgG1, IgG2c, and IgM antibodies were determined by sandwich ELISA as described above, only the primary coat was with OVA (10 μg/mL) to capture antibodies that will specifically bind OVA, specific binding of isotypes were detected through secondary biotinylated antibodies to either IgG1, IgG2c, or IgM. Additionally, combined serum from a previous experiment was used to generate a standard curve of relative units (U). OVA-specific IgE was determine by sandwich ELISA as described above with the following specifics; primary anti-mouse IgE antibody (3 μg/mL), biotinylated OVA as secondary conjugate, and the TOε clone was used to derive the standard curve. NP-specific antibodies were determined by sandwich ELISA as described above. NP-BSA was used to coat the wells (5 μg/mL), followed by a biotinylated secondary antibody against the specific antibody isotype, and combined serum from a previous experiment was used to generate a standard curve of relative units.

Cytokine protein measurements

Cytokines from culture supernatants were determined by sandwich ELISA as described above. Mouse antibodies against IL-4, IL-5, IL-13, and IFNγ were used as the primary antibodies, biotinylated antibody against specific protein was used as the secondary antibody, and the recombinant protein of interest was used to generate the standard curves.

ELISpot assays

To determine the frequency of IgE-secreting B cells from OVA-alum immunized mice, ELISpot plates were prepared a day prior to adding cells plating. Sterile high protein binding immobilon-P membrane 96-well plates (Millipore) were prepared according to manufacturer's instructions. Plates were pre-coated with murine anti-IgE (Mabtech; Mouse IgE ELISpot Basic) in 1 x PBS overnight at 4°C. The

next day, the plate was washed with 200 μL of 1 x PBS 5 times and blocked with supplemented RPMI at room temperature for 30 minutes. The spleens and draining lymph nodes (mediastinal) of OVA-alum immunized mice were isolated and brought to a single-cell suspension as described above. Cells were plated at 1×10^6 cells/mL in supplemented RPMI in the presence of rIL-4 (Peprotech; 10 ng/mL) and anti-CD40 (1 μg/mL; Invitrogen) overnight (~16 hours) at 37 °C. ELISpots were developed according to the manufacturer's instructions, and quantified using an ImmunoSpot analyzer.

FLOW CYTOMETRY

To prepare tissues for flow cytometry, splenocytes, bone marrow, or lymph node (mediastinal) cells were isolated and homogenized mechanically between two glass slides on the frosted sides and run through a 100 μM nylon cell strainer (Falcon). Cells were then RBC lysed (eBioscience) following the manufacture's protocol. Cells were brought to single cell suspension, run through a 70 μM nylon cell strainer (Falcon), and counted. To isolated peritoneal cavity cells, the peritoneal cavity was flushed with 5 mL of lavage fluid (10% fetal bovine serum (FBS) in 1 X PBS and 1 mM EDTA). Cells were then brought to a single-cell suspension and counted. 1×10^6 - 5×10^6 cells were used for staining. Cells were washed with 1 X PBS and stained with 0.25 μL of LIVE/DEAD Fixable Aqua Dead Cell Stain kit (Thermo Fischer Scientific) in 500 μL of PBS for 20 minutes at room temperature in the dark. Cells were washed with 1 X PBS and resuspended in 100 μL of FACs buffer with Fc blocker; Murine cells were blocked with anti-mouse CD16/CD32 (BD bioscience) and human cells were blocked with FcR blocking reagent (Miltenyi Biotec). Cells were stained with designated markers by adding 50 μL of antibody cocktail and incubated at either 4 °C for 30 minutes in the dark or 15 minutes at room temperature in the dark; for antibodies used please see (**Table. 1**). Intracellular flow staining was conducted as below. Stained samples were run on an LSRII flow cytometer (BD Biosciences) or sorted on a FACSAria SORP system in the flow cytometry core facility at Northwestern University. Data were analyzed using FlowJo 10.7 (Tree Star, Ashland, OR).

Table 1: Antibodies used for flow cytometry

Panel 1:Bone marrow B cells

Target	Clone	Conjugate	Vendor
B220	RA3-6B2	AF700	Biolegend
BP-1	6c3	PE-Cy7	Biolegend
CD24	M1/69	PerCP-Cy5.5	Biolegend
CD43	122/60	FITC	Thermo Fisher
IgM	11/41	APC	BD Bioscience
IgD	1126c.2a	PE	BD Bioscience

Panel 2: Splenic B cells

Target	Clone	Conjugate	Vendor
CD19	1D3	APC-Cy7	BD Bioscience
CD21	7E9	BV421	Biolegend
CD24	M1/69	PerCP-Cy5.5	Biolegend
IgM	11/41	APC	BD Bioscience
IgD	1126c.2a	PE	BD Bioscience

Panel 3: Peritoneal B cells

Target	Clone	Specificity	Vendor
B220	RA3-6B2	AF700	Biolegend
CD19	1D3	APC-Cy7	BD Bioscience
CD5	53-7.3	BV421	Biolegend

Table 2: Antibodies used for ELISA

Panel 1: Primary

Target	Clone	Conjugate	Vendor
IgE	R35-72	-	BD Bioscience
IgG1	A85-3	-	BD Bioscience
IgM	R6-60.2	-	BD Bioscience
IgG2c	polyclonal	-	SouthernBiotech
IL-5	TRFK5	-	Biolegend
IL-13	eBio13A	-	ThermoFischer
IFN-γ	R4-6A2	-	BD Bioscience

Panel 2: Secondary

Target	Clone	Conjugate	Vendor
IgE	R35-118	Biotin	BD Bioscience
IgG1	A85-1	Biotin	BD Bioscience
IgM	II/41	Biotin	BD Bioscience
IgG2c	polyclonal	Biotin	SouthernBiotech
IL-5	TRFK4	Biotin	Biolegend
IL-13	eBio1316H	Biotin	ThermoFischer
IFN-γ	XMG1.2	Biotin	BioLegend

ISOLATION OF CELLS AND RNA ANALYSIS

B cells

For the isolation of B cell subsets, cells were sorted using a BD FACS Aria in the flow cytometry core facility at Northwestern University. Murine plasma cells ($CD138^+$) from *in vitro* B cell culture (described above) were isolated after 4 days of culture. Murine plasma cells ($DUMP^{neg}B220^+CD138^+$), germinal center B cells ($DUMP^{neg}B220^+GL7^+$), and T-follicular helper cells ($DUMP^{neg}CD4^+CXCR5^+$) were isolated from spleens of mice after OVA-alum immunization. To obtain human naïve B cells ($CD19^+IgD^+CD27^{neg}$), memory B cells ($CD19^+IgD^{neg}CD27^+CD38^{neg}$), and germinal center B cells ($CD19^+IgD^{neg}CD38^{mid}$), tonsil tissue was processed by overnight digestion at 4 °C in 1 mg/ml collagenase IV (Whitmann) and 30 μg/mL DNase I (Whitmann) followed by homogenization with a gentleMACS™ Dissociator (Miltenyi Biotec) [69]. Total RNA was extracted from sorted cells; for ≤100,000 cells or less Trizol was used according to the protocol by Immgen.org, for ≥100,000 cells the Qiagen kit was used (Qiagen). Real-time PCR was conducted on ABI 7500 using TaqMan probes.

RNAseq

Total RNA-seq analysis was performed on cultured $CD138^+$ cells from WT, H1R KO, H2R KO, and H1R/H2R DKO mice. Small RNA-seq library prep, sequencing, and analysis were conducted at the Northwestern University NUSeq Core Facility. To start, total RNA that retained small RNA species was used. The quality of total RNA samples was assayed on an Agilent 2100 Bioanalyzer and quantity determined by Qubit. The SMARTer smRNA-Seq Kit for Illumina (Takara, Mountain View, CA) was used to build sequencing libraries. The libraries were sequenced on an Illumina NextSeq 500 NGS System at NUSeq. Single 75 bp reads were generated from the sequencer. For bioinformatics analysis, the quality of the generated reads, in fastq format, was first evaluated using FastQC. Adapters were removed and reads of poor quality were filtered out. Trimmed reads were then aligned to the appropriate reference miRNAs found in miRBase (http://www.mirbase.org/) using Bowtie. The number of reads that aligned to each miRNA in miRBase was tabulated, and a differential miRNA expression analysis was performed using DESeq2. The targets for each differentially expressed miRNA were evaluated using TargetScan

(http://www.targetscan.org/vert_71/). Heat maps of significantly differentiated genes of H1R KO, H2R KO and H1R/H2R DKO plasma cells compared to WT plasma cells were derived with MORPHEUS (https://software.broadinstitute.org/morpheus/).

Single-cell RNAseq

To conduct single-cell analysis we used 10X genomics Chromium Single cell 3' assay. Isolated splenic plasma cells, germinal center B cells, and T follicular helper were pooled for each condition, resulting in 4 samples (WT PBS, WT OVA, DKO PBS, and DKO OVA). Pooled cells were prepared for single cell library preparation using a microfluidics chip and the 10X Chromium Controller, which partitions individual cells and combines each cell with a unique gel bead. Each gel bead is coated with oligo primers, a unique bardcode sequence, a unique molecular identifier (UMI) sequence, and a Poly(dT)VN that allows for hybridization to mature RNA for single strand cDNA synthesis. The barcode sequence is thus transferred to every cDNA molecule from that cell, allowing for discrimination of genes that originated in different cells. The UMI sequence provides and internal control for PCR duplication errors, and is used in the quality control filtering steps during the initial data analysis steps. cDNA synthesis occurs within each gel bead and the barcode serves to mark all newly synthesized cDNA from an individual cell. After this process newly synthesized cDNA from all cells can be combined, since the cDNA from each cell has a unique barcode corresponding to the cell it originated from that can be identified later. The cDNA is then processed into a library using PCR to add sequences required for the Illumina platform, and the UMI serves to correct for any errors in PCR amplification, as it is unique to each cDNA construct. cDNA and library quality were assessed using the Agilent Bioanalyzer, and Qbit measurements were used to quantify the final library concentration. The single cell RNAseq libraries were sequenced at the NU Genomics Core using the Illumina HiSeq 4000, with paired-end reads, according to the 10X Genomics instructions. The 10X Genomics Cell Ranger 3.0 pipeline was used to create FASTQ files from the raw BCL sequencing files, and to align the FASTQ files to the mouse genome. Sequencing quality metrics can be found in **Table 3**., and all samples were within standard ranges for high quality data.

Table 3: Sequencing quality metrics

Sample	# of Total Reads	# of Cells	Median Genes per Cell	Sequencing Saturation	Reads Mapped to Genome	Fraction of Reads in Cells
WT PBS	$3.5x10^8$	3,400	852	91.9%	91.6%	87.6%
WT OVA	$3.5x10^8$	3,454	1,107	88.8%	92.9%	90.5%
DKO PBS	$3.6x10^8$	5,794	883	89.0%	92.5%	89.8%
DKO OVA	$3.6x10^8$	6,034	773	88.6%	92.1%	89.1%

All data were then analyzed using the Seurat package in R [70]. Pre-analysis quality control included removal of cells with fewer than 200 genes, cells with high abundances of UMIs (likely doublets), and cells with a high percentage of mitochondrial genes (likely dead/dying cells). All data were log transformed, normalized, and scaled to regress out confounding effects due to UMI number or percent of mitochondrial genes. Principal component analysis (PCA) was used to identify distinct gene expression patterns, and the significant principal components were used to cluster cells with a resolution of 0.6. Clusters were then analyzed by t-stochastic neighbor embedding (tSNE) to identify unique cell clusters. Cell clusters within the tSNE were identified based on expression of canonical markers (CD3 for T cells, CD79a for B cells, and PRDM1 for plasma cells).

Gene expression differences were compared between the same cell types for WT vs DKO in the PBS group to assess baseline. Differentially expressed genes were identified using a Wilcoxon Rank sum test, and p values were adjusted for multiple comparisons using a bonferroni correction.

RNA analysis of Mast cells and Neuronal cells

Murine RNA was isolated from PC-12 (ATCC® CRL-1721™) neuronal cells, RBL-2H3 (ATCC® CRL-2256™) mast cells, MC/9 (ATCC® CRL-8306™) mast cells, and BMMC using RNeasy RNA isolation kit (QIAGEN). Human RNA was isolated from cultured iPSC-neuron cells (provided by Dr. John Kessler), ROSA$^{KIT\ WT}$ mast cells (provided by the Dr. Bruce Bochner), and cultured skin mast cells (provided by Dr. Bruce Bochner). Ambion RNAaqueous micro kit was used to isolate RNA from iPSC-neuron cells, and RNAeasy RNA isolating kit (QIAGEN) was used to isolate RNA from ROSA mast cells and isolated skin mast cells. Real-time PCR was conducted on ABI 7500 using TaqMan probes ([murine] *Nisch*, 4331182; [murine] *Actb,* 4331182 ; [human] NISCH, 4351372; [human] GAPDH, 4331182) (Applied Biosystems).

LIGAND-RECEPTOR BINDING ASSAY

In collaboration with the NIMH Psychoactive Drug Screening Program IAA and histamine were screened for receptor binding. Total binding and nonspecific binding were determined in the absence and presence of 10 μM of the appropriate reference compound, respectively. In brief, plates were incubated at room temperature in the dark for 90 min , reactions were stopped by vacuum filtration onto 0.3%

polyethyleneimine (PEI) soaked 96-well filter mats using a 96-well Filtermate harvester, followed by three washes with cold wash buffers. Scintillation cocktail was then melted onto the microwave-dried filters on a hot plate and radioactivity was counted in a Microbeta counter [71].

STATISTICAL ANALYSIS

Data are represented at mean ± SEM. Data was analyzed using Student's t test or one-way ANOVA where appropriate (GraphPad Prism 6 software).

CHAPTER 3 – Histamine-driven responses are sustained via a bioactive metabolite

INTRODUCTION

Significance: Food allergy

The prevalence of allergic diseases including eczema, allergic rhinitis, and food allergy has been increasing for serval decades [72]. Food allergy is most prevalent in westernized countries, however recent epidemiological data reports that the prevalence of food allergies is rising in developing countries [72,73]. Allergic responses to food can manifest in a variety of symptoms from minor hives, itching, and/or tingling of the mouth to one of the most severe and potentially life-threatening allergic responses, anaphylaxis [74]. Although fatalities have decreased in recent years due to increased awareness and use of epinephrine, there are no effective preventative treatments for food allergy or anaphylaxis [75].

In the last century there have been many proposed hypotheses to explain the development of allergic diseases, including the hygiene hypothesis [76,77], environmental factors [78], genetic predispositions [79], and the atopic (allergic) march [80]. To date, it has been appreciated that the development of specific allergic diseases can be explained by different combinations of these hypotheses. Nonetheless, these hypotheses ultimately serve to explain two major events that lead to the development of allergic diseases, (1) the break of immunological tolerance, which are the mechanisms that prevent adverse responses to non-pathogenic antigens, and (2) allergic sensitization, which is the process whereby the immune system generates a response to a non-pathogenic antigen that is characterized by type 2 (allergic) inflammation.

Food allergy is primarily diagnosed in children [72], and it is thought that the break in immune tolerance, or oral tolerance, is a result of a combination of early life events (prenatal and postnatal) [81] and genetic predispositions [82]. The mechanisms necessary to maintain oral tolerance are not fully established, however current literature strongly supports that key mechanisms occur in the gastrointestinal (GI) tract. The GI tract contains a variety of specialized sites that are important for immune responses. These include the gut-associated lymphoid tissues (GALT), the lamia propria (LP), and epithelium [83]. Cells identified to be important for oral tolerance include $CD4^+$ regulatory ($FoxP3^+$) T cells (Treg) and $CD103^+$ DCs. Studies have demonstrated that $CD103^+$ DCs residing in the LP of the GI tract continually take up antigen, traffic to the draining lymph nodes, and present that antigen to Tregs,

which then function to limit immune response to that antigen in the gut [84,85]. The importance of Tregs is further supported by studies on IPEX (immune dysregulation, polyendocrinopathy, enteropathym X-linked) patients that have misssense mutations in the FoxP3 locus [86]; IPEX patients are found to develop severe food allergy [87], amongst other disorders.

The second event that occurs in conjunction with a break in oral tolerance to promote development of food allergy, is allergic sensitization. In a simplified model, allergic sensitization is the exposure and processing of antigen that occurs in a type-2 inflammatory environment, ultimately resulting in the production of allergen-specific IgE. In food allergy, it is proposed that exposure of allergens is due to defects in barrier integrity of the skin [88] and intestine [89] and that this process likely play a key role in the development of allergic sensitization of foods in infants by increasing exposure to food antigens. In addition, mutations in genes that regulate skin integrity have been associated with food allergy and may provide the means for allergen exposure through the skin [90]. Production of the cytokines IL-33, IL-25, and TSLP by epithelial cells is also associated with initiating type-2 responses against food allergens [91].

As previously mentioned, there are a range of responses in food allergic patients, the most severe of which is anaphylaxis. There is much that is unknown about the biology of anaphylaxis, but we were most intrigued by a simple question, why is the response so robust? There is a general understanding of cell types and mediators that are involved, which will be discussed below. But the nature of anaphylaxis suggests that there is still much to learn. For example, the amount of antigen necessary to initiate the response can vary widely, and in some patients even the inhalation of tiny amounts of food particles can initiate an anaphylactic response [92]. This opens up the discussion about the nature of not only which cells are involved, but how is this response propagated so quickly and immensely? Again, the key mediators of anaphylaxis are known, but it is clear that there is much to discover regarding the potential of these known mediators during the anaphylaxis. Therefore, we aimed to fill in a piece of the puzzle by focusing on one of the most potent mediators of anaphylaxis, histamine.

Mechanism of Anaphylaxis

IgE-mediated anaphylaxis is a life-threatening robust allergic response that can affect multiple organ systems, including the skin, respiratory system, and GI tract [93]. The series of events that results

in anaphylaxis starts with sensitization, or the production of antigen-specific IgE. Common food allergens in most countries include cow's milk, egg, peanut, tree nuts, fish, shellfish, wheat, and soy. However, the type of food antigen that ultimately results in anaphylaxis differs based on geographical locations [72]. In the United States, the most common food allergen associated with fatal or near fatal anaphylaxis is peanut [94]. Allergen specific IgE circulates in the plasma where it has a half-life of 2-3 days, unless it binds to its high-affinity receptor FCεRI on mast cells and basophils, where it can stay bound for weeks [95,96]. Studies have shown that binding of IgE to FCεRI alone upregulates FCεRI expression on mast cells [97], thereby increasing the potential for sensitization to other allergens and increasing the binding capacity for existing IgE. Upon re-exposure to the same allergen, cross-linking of IgE bound FCεRI must occur in order to trigger signaling events leading to mast cell degranulation.

Mast cells

Mast cells are derived from the common myeloid progenitor cell in the bone marrow. Unlike most other immune cells, mast cells leave the bone marrow and enter the circulation in an undifferentiated state were they mature and acquire effector functions in peripheral tissues [98]. Mast cells are highly "armed" resident immune cells containing a plethora of mediators ready to respond at host-environment interfaces including the peripheral tissues in the skin, respiratory mucosa, and GI tract [98]. Mast cells contain granules packaged with preformed mediators including histamine, tryptase, chymase, carboxypeptidase A, and proteoglycans that are available for immediate release upon cellular activation. In humans, two main types of mast cells have been described: those that express the enzyme tryptase alone, and those that express both chymase and tryptase [99]. Mast cells also produce other mediators including cytokines, chemokines, and lipid mediators, such as prostaglandins and leukotrienes [98].

Mast cell degranulation

Mast cell degranulation occurs in a 3-stepwise manner, within seconds of IgE crosslinking, first is the release of preformed granules and granule swelling, mediated by granule-to-granule fusions and granule-to plasma membrane fusion [100]. Next, newly produced (*de novo*) mediators are released minutes after cellular activation. These *de novo* mediators include eicosanoids, prostaglandins (PGD2),

leukotrienes (LTB4, LTC4, LTD4, LTE4), and platelet-activating factor (PAF). Finally, hours following mast cell degranulation, cytokines are released [101]. Because of the severe nature of anaphylaxis, studies on the mechanisms driving the pathophysiologic changes in human subjects are limited. However, several mast cell mediators, such as histamine, PAF, and leukotrienes, are known to play critical roles during anaphylaxis [102], we will further discussed how histamine modulates anaphylaxis.

Biology of histamine in anaphylaxis

Histamine is one of the most potent mediators of anaphylaxis, where it initiates pathophysiology in the skin, digestive system, respiratory tract, and the cardiovascular system [102]. Studies in mice and humans have demonstrated that intravenous injection of histamine elicits the symptoms of anaphylaxis [103-105]. In humans, low doses intravenous injection of histamine results in increased permeability of the capillaries [106], and low dose intradermal skin injection results in an itchy wheal and fare response. Higher doses administered by intravenous injection or through inhalation facilitate the cardiovascular and respiratory irregularities observed during anaphylaxis [103,104].

The major histamine receptors facilitating responses during anaphylaxis are H1R and H2R. Studies done to determine the effect of intravenous injection of histamine demonstrated that H1R and H2R antagonists individually did not ameliorate the effects of histamine, but, when administered together prior to histamine injection, could blunt the anaphylactic reaction [107]. Similarly, a study using a murine model of IgE-dependent anaphylaxis demonstrated that H1R KO and H2R KO mice had less significant ablation of anaphylaxis symptoms compared to mice lacking both receptors [108].

During IgE-mediated anaphylaxis, histamine is rapidly released upon antigen exposure and can be detected at elevated levels in the plasma [58]. Studies on experimental anaphylaxis demonstrate that histamine levels peak within 5 to 10 minutes after the onset of anaphylactic symptoms and return to baseline by 15-30 minutes which indicate that histamine acts early and fast [109]. This timeline is explained by the short half-life of histamine in plasma, about 1-2 minutes, due to its rapid degradation by histamine metabolites. However, it has also been shown that histamine can mediate “delayed” symptoms associated with anaphylaxis that include pruritus, erythema, reflux, and eczematous rashes since antihistamine are effective in treating these responses [110]. Collectively, taking into consideration the

time-line of histamine metabolism and the efficacy of anti-histamines hours after the initiation of anaphylaxis, it is clear that there are unknown aspects of the biology of histamine in this response.

Treatments of anaphylaxis and inconsistencies

The first line of treatment for anaphylaxis is epinephrine, an active sympathomimetic hormone from the adrenal medulla. Epinephrine targets the alpha and beta-adrenergic systems, α_1, β_1, and β_2, and mediates vasoconstriction, gastrointestinal relaxation, stimulates the heart, and dilates bronchi and cerebral vessels. During anaphylaxis, epinephrine can prevent or decrease upper airway mucosal edema, hypotensive shock, dilate airways, and has been suggested to block further mast cell mediator release [102,111]. Although H1R and H2R seem to be obvious targets for the treatment of anaphylaxis, antihistamines are not considered effective first line treatments for anaphylaxis. Antihistamines reach maximal plasma concentrations in 1-3 hours, while epinephrine takes less than 10 minutes [112]. Instead, antihistamines are considered adjunctive therapy after the administration of epinephrine [110,113]. Nonetheless, because antihistamines are effective to alleviate symptoms long after the release of histamine and the onset of anaphylaxis, it seems that histamine-associated biology persists during later phases of anaphylaxis. Yet, the mechanisms of how histamine is mediating these responses are not known.

Summary

Taken together, it is clear that histamine can mediate long-term mechanisms that contribute to the anaphylactic response. While it is possible that there is continuous activation of cells that release histamine that could continue to mediate responses, blood levels of histamine are generally undetectable at these later time points. It is also possible that histamine induces the release of another mediator that could signal through the histamine receptors. However, to date no other ligands for the histamine receptors have been identified. Interestingly, early work showed that a metabolite of histamine, imidazole acetic acid (IAA), could mimic the effects of histamine to induce eosinophil migration in mice [63]. IAA is relatively stable compared to histamine, and accumulates in tissues during anaphylaxis, before being

excreted into the urine [60]. Therefore, we aimed to determine whether IAA could mediate any other functions of allergic responses, including anaphylaxis, *in vivo*.

RESULTS

IAA induces histamine-biology through the same receptors

As previously mentioned, IAA was shown to act as an eosinophil chemotactic agent *in vitro,* similarly to histamine. It was also observed that the combination of IAA and histamine did not result in increased or synergistic effects on chemotaxis of eosinophils, instead cross-deactivation was observed between histamine and IAA. This study suggested that histamine and IAA were mediating chemotaxis of eosinophils through a similar mechanism [63]. A follow-up study by the same group demonstrated that histamine- and IAA-driven chemotaxis could both be inhibited by the H2R antagonist burimamide. The H1R antagonist mepyramine also showed inhibitory effects, however, only at a high concentration of 10^{-3} M, whereas burimamide had inhibitory effects at 10^{-5} M [114]. These studies demonstrated the potential for immune regulation by IAA in responses mediated by histamine, and that these responses may be functioning through histamine receptors. However, no further studies were done to determine the mechanism of IAA action nor whether these effects were observed *in vivo*.

Therefore, we first aimed to characterize whether IAA was sufficient to induce biological responses similar to those induced by histamine *in vivo,* such as eosinophil recruitment, pruritus, and anaphylaxis in mice. Histamine was previously shown to induce eosinophil recruitment via H2R, and so we injected IAA or histamine into the peritoneum at increasing doses of WT mice and H2R KO mice. 24 hours following injection, we observed that mice injected with IAA had a dose-dependent recruitment of eosinophils that was also dependent on H2R, and this mirrored the responses to histamine (**Figure 2.**).

Next, we wanted to assess an H1R mediated response, since an H1R antagonist (mepyramine) was show to have some inhibitory effect on IAA. We specifically tested histamine-induced pruritus, since it is primarily mediated through H1R [115]. IAA or histamine were administered by intradermal injection into WT and H1R KO mice in the back of the neck (shaved). We observed that IAA induced itch in an H1R-dependent manner, similar to histamine (**Figure 3.**).

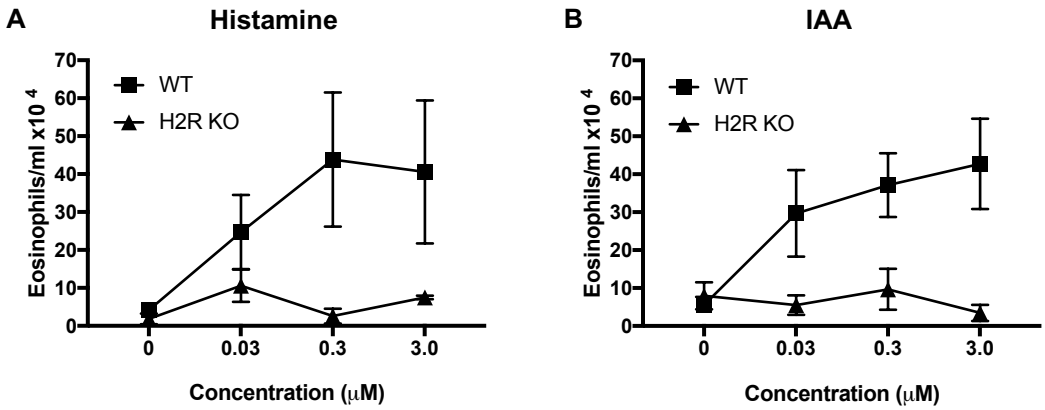


Figure 2: IAA induced eosinophil recruitment in a H2R-dependent manner

Histamine and IAA induced eosinophil recruitment to the peritoneum in WT mice and this response was ameliorated in H2R KO. **(A)** Histamine or **(B)** IAA were administered by i.p. injection at increasing doses, 24 hours following injection peritoneal lavages were collected, and eosinophils were quantified from. Data are representative of the mean ± SEM (*n* = 2-9).

(performed by Adam Bryne)

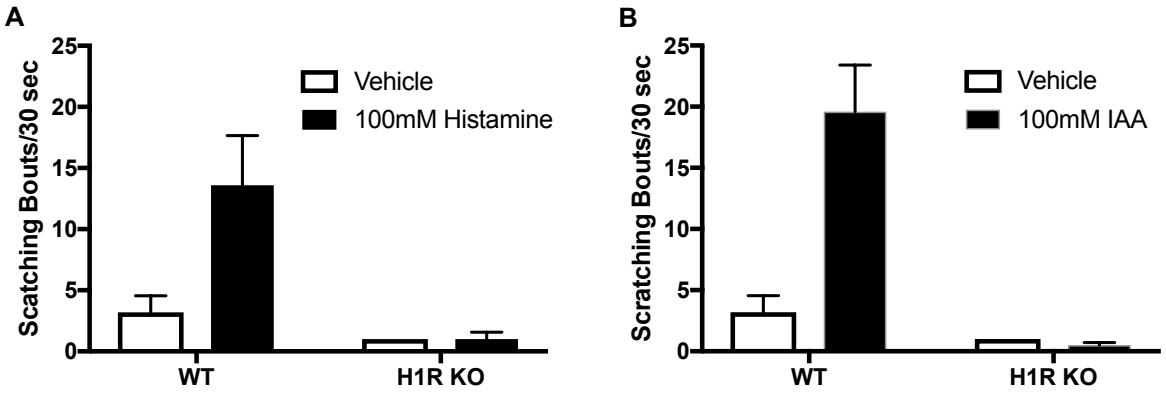


Figure 3: IAA induced pruritus in a H1R-dependent manner

Histamine and IAA induced scratching bouts in WT mice and this response was ameliorated in H1R KO mice **(A)** Histamine or **(B)** IAA were administered by i.d. injection into WT and H1R KO mice in the back of the neck (shaved). Itch was measured by counting the number of bouts of scratching in the 30-minute period immediately following injection. Data are representative of the mean $\pm$ SEM (n = 3-9).

(Performed by Dr. Adam Bryne)

Finally, we wanted to assess the role of IAA during anaphylaxis. As previously mentioned, histamine is a key mediator of anaphylaxis, and it has been shown that H1R and H2R are both necessary to induce anaphylaxis in mice [108]. Theoretically, the biological function of IAA would also be most potent during anaphylaxis, where vast quantities of histamine are released. IAA or histamine were administered by i.v injection into WT and H1R/H2R DKO mice. As expected, both histamine- and IAA-induced anaphylaxis and the responses were blunted in H1R/H2R DKO mice (**Figure 4.**).

Taken together, these data establish that IAA induces physiological responses similarly to histamine, and histamine receptors are necessary for these IAA-initiated responses. A potential mechanism of action for IAA may be through the direct binding to histamine receptors, since biological functions of IAA were ameliorated in histamine receptor KO mice. However, another potential mechanism could be that IAA is inducing further histamine release by mast cells or other cells, thereby acting indirectly though histamine receptors.

IAA requires histamine but does not bind histamine receptors

We first sought to determine whether IAA was binding histamine receptors directly. We performed a binding assay in collaboration with the NIMH Psychoactive Drug Screening Program [71]. We tested binding of both IAA and histamine to a panel of 51 receptors. The receptors that were screened included Ca^{2+} and cAMP signaling-associated receptors, as well as all 4 histamine receptors. Data obtained showed that IAA did not bind to any of the receptors, including the histamine receptors (**Figure 5.**). As expected however, histamine bound to all 4 histamine receptors. Therefore, we next tested the hypothesis that IAA was inducing histamine release.

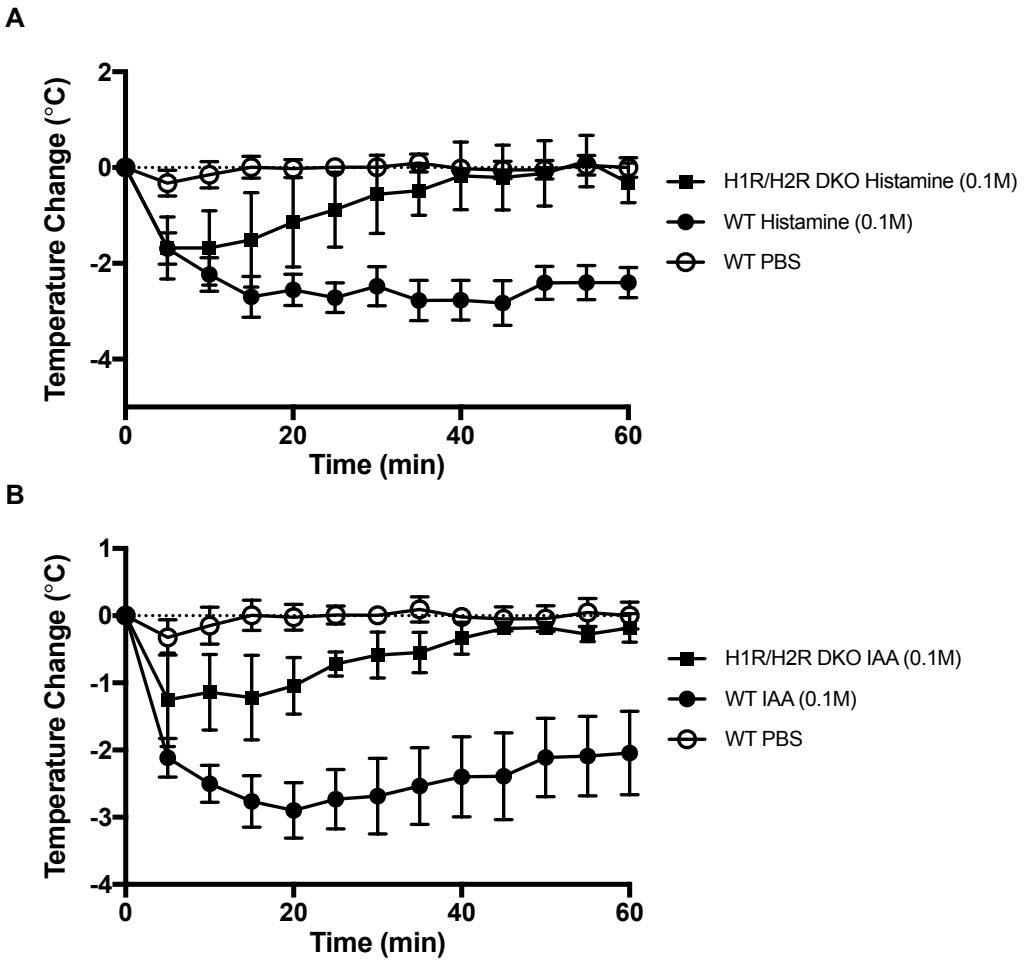


Figure 4: IAA induced anaphylaxis through H1R and H2R

Histamine and IAA induce anaphylaxis in WT mice and this response was ameliorated in H1R/H2R DKO mice. **(A)** Histamine or **(B)** IAA were administered by retro-orbital injection into WT and H1R/H2R DKO mice and rectal temperatures were recorded to quantify anaphylaxis. Data are representative of the mean ± SEM (*n* = 3-10 from 3 independent experiments).

(Performed by Dr. Adam Bryne)

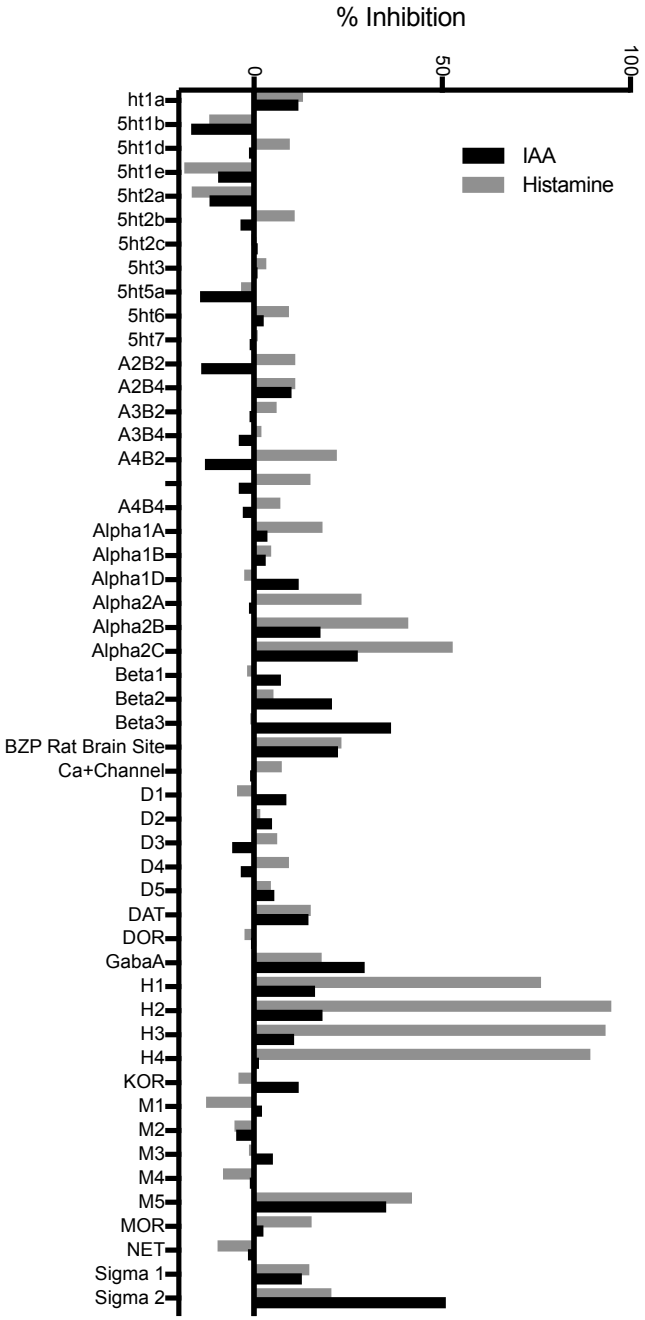


Figure 5: IAA did not bind histamine receptors

Histamine bound to all four receptors (H1-H4), but IAA did not. IAA and histamine were screened for receptor binding.

To test whether IAA could directly induce histamine release, IAA or PBS was administered, and serum histamine was quantified at 1 minute, 3 minutes, 10 minutes, and 30 minutes after injection. We observed that injection of IAA resulted in a significant increase in histamine levels compared to PBS after 1 minute, and histamine levels diminished thereafter (**Figure 6.**). These data clearly indicated that IAA could directly induce histamine release *in vivo*.

Mast cell derived-histamine is necessary for initiating a response to IAA

We next wanted to definitively determine whether the increased histamine we observed was the indirect mediator of IAA responses. To do this, we induced IAA-driven anaphylaxis in WT and HDC KO mice, which lack the HDC enzyme that is necessary for histamine production. As expected, IAA induced anaphylaxis in WT animals but not in HDC KO animals (**Figure 7.**), establishing that the effects of IAA require endogenous histamine sources.

We next wanted to determine which cells were releasing histamine in response to IAA. Mast cells were the obvious first candidate since they are known to produce large amounts of histamine, and our data showed that IAA was mediating functions in the skin, peritoneum, and systemically, where mast cells are known to reside. Thus, we focused on determining whether mast cell-derived histamine was necessary for IAA-driven anaphylaxis. We used a method of mast cell reconstitution into mast cell deficient mice (SASH, $Kit^{W\text{-}sh}$). Using this model we reconstituted mice with bone marrow-derived mast cells (BMMC) from WT or HDC KO animals. In the absence of mast cells, IAA did not induce anaphylaxis (**Figure 8.**), indicating that mast cells were necessary for this response. Further, $Kit^{W\text{-}sh}$ animals reconstituted with WT BMMCs had a significantly larger temperature change after IAA injection than $Kit^{W\text{-}sh}$ mice reconstituted with HDC KO BMMCs (**Figure 8.**). However, there was a small temperature drop in mice reconstituted with HDC KO BMMC. These data indicated that mast cell-derived histamine was important for the induction of anaphylaxis after IAA injection. Importantly however, mast cells may not be the only source of histamine, as in the induction of anaphylaxis by IAA in $Kit^{W\text{-}sh}$ animals reconstituted with HDC KO mast cells were not completely ameliorated.

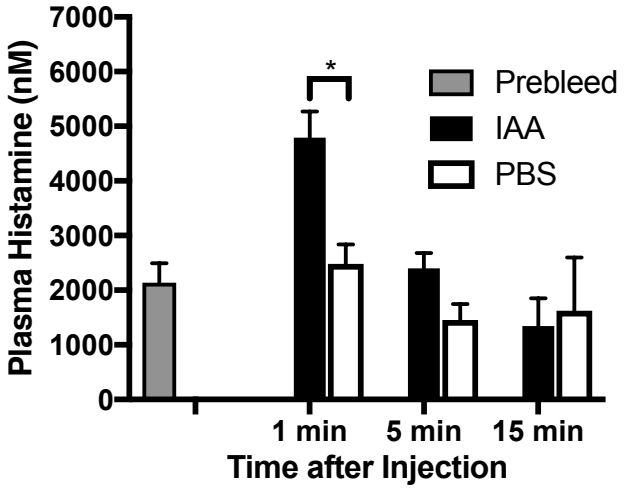


Figure 6: IAA induced histamine release into the plasma

Histamine levels significantly increased at 1 minute after IAA injection. Histamine was measured in the serum at baseline, or at 1 minute, 5 minutes, and 15 minutes after injection. Data are representative of the mean ± SEM (n = 3-4). *$p < 0.05$ by Student's t-test.

(Performed by Dr. Adam Bryne)

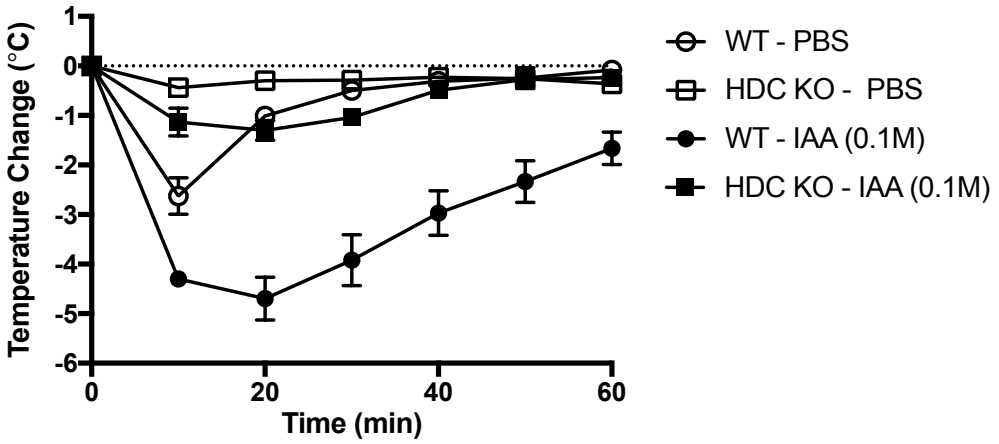


Figure 7: Endogenous histamine was necessary to initiate IAA induced anaphylaxis

IAA did not induce anaphylaxis in HDC KO mice. Histamine or IAA were administered by retro-orbital injection into WT and HDC KO mice and rectal temperatures were recorded to quantify anaphylaxis. Data are representative of the mean ± SEM (*n* = 4-6 from 2 independent experiments).

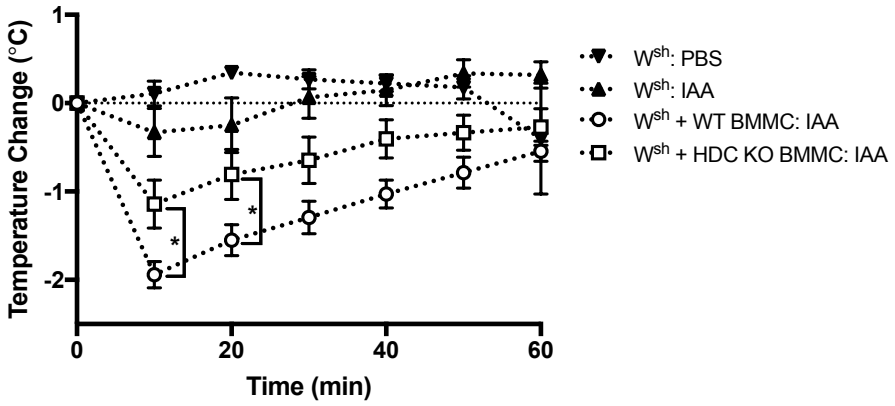


Figure 8: Mast cell-derived histamine was necessary for the IAA-induced anaphylaxis

Mast cell deficient mice reconstituted with WT mast cells had IAA-induce anaphylaxis while reconstitution with HDC KO mast cells had significantly ameliorated IAA-induced anaphylaxis response compared to WT. Mast cell deficient mice ($Kit^{W\text{-}sh}$) were reconstituted with mast cells from WT or HDC KO animals. PBS or IAA was administered to $Kit^{W\text{-}sh}$ and $Kit^{W\text{-}sh}$ reconstituted mice by i.v. injection and rectal temperatures were recorded to quantify anaphylaxis. Data are representative of the mean $\pm$ SEM (n = 5-12 from 5 individual experiments). *$p < 0.05$ by Student's t-test.

Efaroxan inhibits IAA response

Because we found that mast cells provided significant amounts of histamine in response to IAA, we next examined which receptors on mast cells were responsible for the IAA-induced release of histamine. There are limited studies on IAA, however a few suggest it functions through imidazole receptors [62]. There are 3 characterized Imidazole receptors I_1, I_2, and I_3, however IAA is only known to facilitate functions through I_1 and I_3 [116]. We focused on the most well described imidazole receptor, I_1, also known as Nischarin. Although imidazole receptor are functions are primarily defined in neurobiology, Nischarin has been shown to promote arachidonic acid release and S1P-mediated calcium flux, both of which are critical pathways for mast cell activation during anaphylaxis [116].

To determine whether Nischarin could potentiate histamine release in response to IAA, we first characterized the expression of Nischarin mRNA in mast cells. We found that Nischarin mRNA was expressed by both murine and human mast cells (**Figure 9. A, B**). To determine whether Nischarin was necessary for the effects of IAA, we pre-treated mice with efaroxan, a reported Nischarin inhibitor, at 10 mgs/kg, 30 minutes before challenging mice with IAA. IAA did not induce anaphylaxis in mice pretreated with efaroxan (**Figure 10.**), suggesting that IAA may function via Nischarin to induce histamine release from mast cells.

Nischarin enhances the anaphylactic response

To definitively determine whether Nischarin was initiating the response to IAA, we used a Nischarin LacZ-reporter mouse that can be modified become a cell-specific KO (**Figure 11.**). In this mouse, the LacZ gene disrupts the Nischarin gene resulting in a non-functional protein, therefore upon activation of the Nischarin promoter, LacZ will be expressed instead of Nischarin. Mice that contain both modified gene alleles will be Nischarin KO, whereas heterozygous (het) mice are reporters. In our initial study we challenged Nischarin KO, Nischarin het, and WT mice with IAA. We hypothesized that in the absence of Nischarin IAA would not be able to initiate the anaphylaxis response. However, our data demonstrated that Nischarin KO and Nischarin het animals had an even more robust anaphylaxis response compared to WT mice (**Figure 12. A**) .

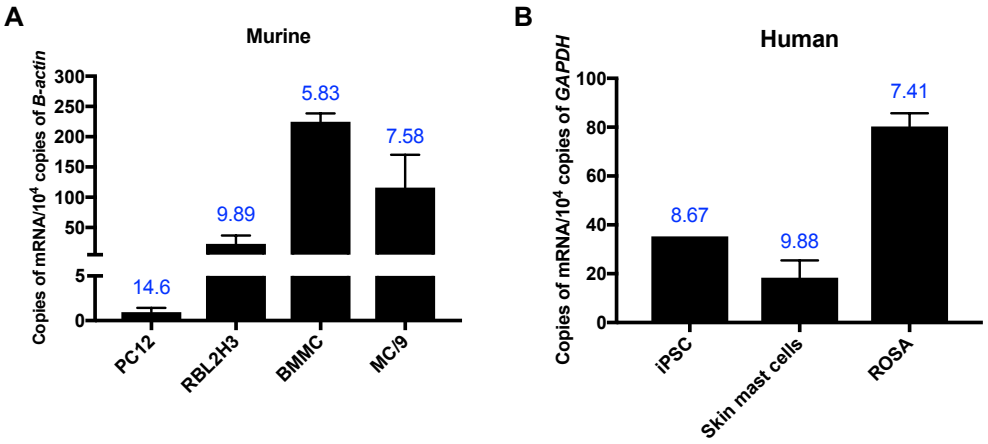


Figure 9: Nischarin was expressed in human and murine mast cells

Nischarin was expressed on murine and human mast cells. **(A)** Murine RNA was isolated from PC-12 neuronal cells, RBL-2H3 mast cells, MC/9 mast cells, and BMMC. Values above graph bars represent the average delta Ct value for Nischarin verus β-actin for each cell type. Relative values of Nischarin (NISCH) mRNA represent number of NISCH mRNA copies per 10^4 copies of β-actin. **(B)** Human RNA was isolated from cultured iPSC-neuron cells, $ROSA^{KIT\ WT}$ mast cells, and cultured skin mast cells. Values above graph bars represent the average delta Ct value versus GAPDH. Relative values of NISCH represent number of NISCH mRNA copies per 10^4 copies of GAPDH. Data are representative of the mean ± SEM (**[A]** *n* = 3 and **[B]** *n* = 1-3 of individual wells for each).

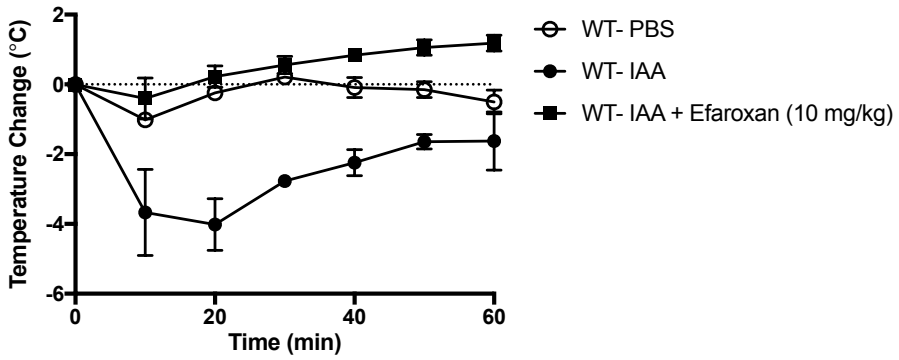


Figure 10: IAA-induced anaphylaxis response was abrogated with pre-treatment of Efaroxan

IAA induced anaphylaxis was abrogated with pre-treatment of Efaroxan. Efaroxan was administered by i.v. injection 30 minutes prior to i.v injection of IAA and rectal temperatures were recorded to quantify anaphylaxis. Data are representative of the mean $\pm$ SEM (n = 2-3).

Figure 11: Nischarin null allele mouse construct.

A LacZ gene was placed between the fourth and fifth exon resulting in a null allele. The expression of LacZ allow for characterization of protein expression by Beta-galactosidase staining. The loxP sites allow for cell-specific mutations.

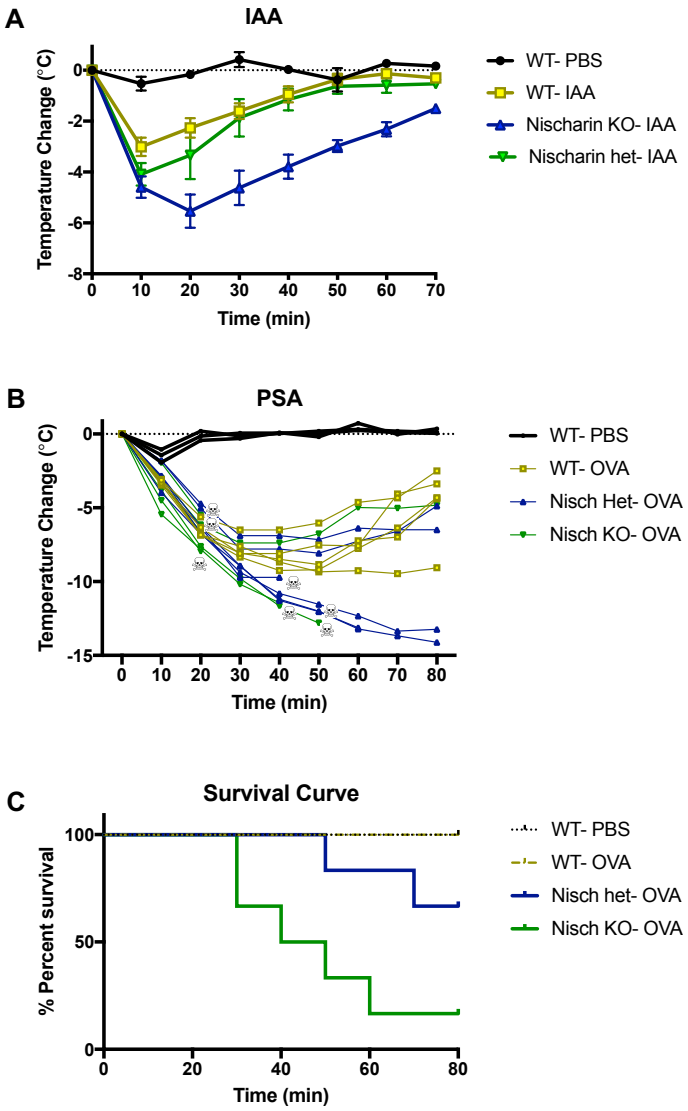


Figure 12: The absence of Nischarin resulted in more severe anaphylaxis

IAA-induced anaphylaxis and PSA responses were elevated in Nisch het and Nisch KO mice compared to WT mice. Survival from anaphylaxis responses of Nisch het and Nisch KO was lowered compared to WT mice. **(A)** Mice were challenged with IAA and rectal temperatures were recorded to quantify anaphylaxis. **(B)** Passive systemic anaphylaxis (PSA) was performed by administering OVA-specific IgE by i.v. overnight and challenged the next day with OVA. Rectal temperatures were recorded to quantify anaphylaxis. **(C)** Survival curve of PSA from experiment in panel **(B)**. Data are representative of the mean ± SEM (*n* = 3-6 from 2 individual experiments).

In addition, we induced passive systemic anaphylaxis (PSA) to determine whether the enhanced anaphylaxis response in these mice was due specifically to the effect of IAA. Again, Nischarin KO and Nischarin het mice had more robust anaphylaxis compared to WT mice (**Figure 12. B**). In addition the survival rate of mice during PSA decreased drastically in the absence of Nischarin compared to WT mice (**Figure 12. C**). Although unexpected, these results indicated that Nischarin is an important protein for dampening or regulating the anaphylactic response. Because we did not generate any cell specific Nischarin KO mice, we cannot conclude which cells are mediating this robust response. However, it is clear that there is a strong phenotype in the absence of this protein, and further studies are needed to clarify the role of Nischarin in the development of anaphylaxis.

Finally, we wanted to test whether Nischarin was important for other allergic responses, and we used a classical model of airway hypersensitivity where mice are immunized with ovalbumin (OVA) protein and the adjuvant alum to induce IgE, followed by challenges of aerosolized OVA to induce local inflammation in the lung (**Figure 13. A**). The resulting readouts of this model are increased inflammation in the lung, which can be measured by increased cellular infiltration, specifically eosinophils in the bronchial alveolar lavage (BAL) fluid, as well as increased total IgE antibody levels in the sera. We did not observe any significant changes between IgE levels or cell infiltration into the lung in Nischarin KO mice compared to WT (**Figure 13. B**). These data suggest that Nischarin plays a key role in the regulation of anaphylaxis, but may not be important for the development of allergic airway disease.

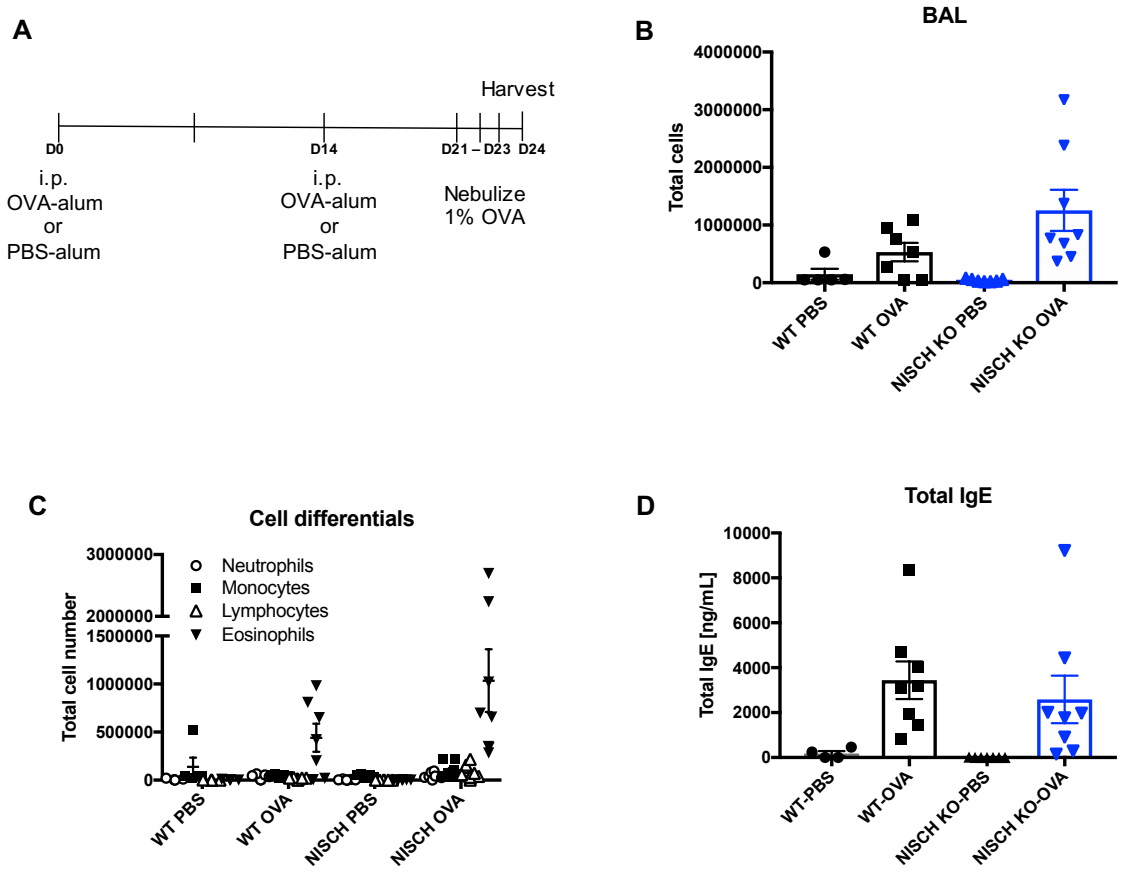


Figure 13: Nischarin did not affect Th2 driven response in a model of OVA-alum

Th2 responses were initiated in Nischarin KO mice similarly to WT mice. **(A)** OVA-alum model: Nischarin KO and WT mice were administered OVA-alum by i.p. injection OVA conjugated with alum on day 0 and day 14 followed by challenge with aerosolized OVA inhalation on days 21, 22, and 23. On day 24 mice were euthanized, BAL and serum was collected to quantify. **(B)** Total cells and **(C)** differential cell counts from BAL of cytospun cells. **(D)** Total and IgE antibody by ELISA. Data are representative of the mean $\pm$ SEM (n = 4-8).

DISCUSSION

Food allergy is life-long disease, that can manifest in one of the deadliest allergic reactions, anaphylaxis. Currently, there is a general understanding of the mechanisms, cell types, and mediators involved in anaphylaxis. However, there is much that is not explained regarding why the response is so potent. The clinical effectiveness of antihistamines long after the release and degradation of histamine indicates that histamine is indeed potentiating these responses. However, until now it has been overlooked that histamine seemed to be functioning well beyond the limits of its known kinetics.

We have found that the histamine metabolite IAA recapitulates several key functions of histamine, including recruitment of eosinophils, induction of itch, and induction of anaphylaxis, through H1R and H2R. We determined that IAA was facilitating these responses through histamine receptors indirectly by initiating histamine release, rather than binding directly to histamine receptors themselves. We further confirmed that IAA-induced histamine release was necessary for these responses, and that mast cell deficient mice did not respond to IAA, which strongly indicates that mast cells are necessary for IAA-driven anaphylaxis. Further, we were able to show that there was a significantly greater anaphylaxis response in mice reconstituted with WT mast cells compared to reconstitution with HDC KO mast cells, indicating that histamine from mast cells was also key to these responses.

While we did show that in the absence of mast cells IAA could not induce anaphylaxis, we did not test how mast cell degranulation specifically affects IAA-driven histamine release. In the context of IgE-mediated anaphylaxis, mast cells rapidly degranulate and are able to regranulate, however this process takes time (up to an hour), and it is not clear whether regranulated mast cells have recovered enough in that time to continue to secrete histamine [117,118]. One explanation for the long-lasting effects of IAA on histamine release could be that IAA targets the mast cells that did not undergo degranulation during the initial activating event. Nonetheless, It is important to acknowledge that other cells have inducible histamine release, such as T cells, DCs, and macrophages [7]. Therefore, it is possible that IAA could also target these cells. In support of this hypothesis, going back to the early studies that demonstrated IAA could induce chemotaxis of eosinophils, it was also found that this mechanism could be blocked by histamine receptors antagonist [63,114] which mirrors our findings of IAA functions that were abrogated in histamine receptor KO mice. This would indicate that in these early studies there was release of

histamine that induced chemotactic activities, however these studies were done *in vitro*, which would indicate that histamine release had to be occur from the eosinophil itself. Therefore IAA was potentially inducing histamine release of eosinophils that was then acting in an autocrine manner to initiate chemotaxis through its own histamine receptors.

IAA is known to function through the imidazoline receptors I_1 and I_3. We focused on I_1, also known as Nischarin, since previous studies indicated that Nischarin mediated signaling pathways similar to those in mast cells. We initially observed that IAA functions could be inhibited by efaroxan, an imidazole I_1 receptor antagonist, indicating that I_1 could be the receptor for IAA in our model system. We also found Nischarin mRNA expression on murine and human mast cells. Using a Nischarin KO mouse, we unexpectedly observed that the absence of Nischarin resulted in more severe anaphylaxis compared to WT mice.

There are different possible explanations for this response including: **(1)** Nischarin is not the receptor for IAA, despite the fact that a Nischarin inhibitor (efaroxan) blunted the effects of IAA. Although efaroxan blocks Nischarin receptor it is also known to inhibit the α2 adrenergic receptor. However, the role of α2 adrenergic receptor is focused in the central and peripheral nervous systems [119] and agonists induce physiological symptoms such as sedation, analgesia, and euphoric effects [120] instead of anaphylaxis. There are studies on asthma that demonstrate that an α2 adrenergic receptor agonist could be beneficial however, the route of administration greatly impacts the functions of the agonist as to whether it will reduce allergen induced bronchial responses or exacerbate them [121,122]. Overall it does not seem that α2 adrenergic receptors are mediating IAA responses in our model. **(2)** Nischarin is playing distinct roles in different cell types. Nischarin has also been identified to also exist as a cytosolic scaffolding protein [123], and in our analysis we only verified the mRNA expression of Nischarin in mast cells, and not its cellular localization. Interestingly, the cytosolic form of Nischarin has been shown to inhibit AMP-activated protein kinase (AMPK) [124], and AMPK is known to suppress FcεRI-driven mast cell activation and anaphylaxis in mice [125]. Given that we found exacerbated anaphylactic responses in Nischarin KO animals, it is unlikely that the Nischarin was expressed as this suppressive cytosolic form. Future studies utilizing cell-specific KOs are needed to further define the roles of Nischarin in anaphylaxis.

In conclusion, this work has characterized an unrecognized mechanism that may play a key role during allergic and anaphylactic responses. We show for the first time that IAA could mimic the effects of histamine *in vivo*, and that this was dependent on the histamine receptors and histamine release from mast cells. Further, we were able to show that that IAA could induce anaphylaxis in a histamine-dependent manner that could be blocked by the inhibitor efaroxan. Altogether, these studies provide valuable insights into the mechanisms that may promote and amplify allergic responses.

CHAPTER 4 – Histamine Regulates Antigen-Specific Antibody Production

INTRODUCTION

Significance: Allergy and IgE

Allergy describes chronic inflammatory diseases that result due to a break in immune tolerance to non-pathogenic antigens such as house dust mite, pollens, and food antigens. Throughout recent decades there has been a reported increase in the prevalence of individuals that develop allergies [126,127], and it has been documented that up to 40% of the population has IgE against a non-pathogenic foreign antigen [126]. Allergic diseases are generally categorized based on pathological manifestations; however, it is known that these diseases are heterogenous and can be further categorized as being mediated by IgE-dependent or independent mechanisms; IgE-dependent mechanisms driven by type 2 responses are the most prevalent. Although not all manifestations of IgE-mediated allergic responses result in fatality, such as anaphylaxis (see Chapter 3), chronic inflammation does lead to increased susceptibilities to other diseases and overall lower quality of life. Evolutionarily, type 2-driven responses are appropriate and necessary for the clearance of helminth infections [128]. Unfortunately, even though there are similarities between the effector phases of helminth infections and allergic responses, IgE-mediated allergic responses lack appropriate mechanisms for resolution of the inflammatory response. Therefore, understanding factors that sustain antigen-specific IgE antibody production during allergic disease is important for the development of new therapeutic targets for treatment of these diseases.

Currently there are therapeutics that target IgE, either directly or indirectly. Dupilumab is a monoclonal antibody that blocks the IL-4 receptor alpha chain, which prevents both IL-4- and IL-13-driven mechanisms [129]; IL-4 is an important mediator of IgE production, and will be discussed in more detail below. Omalizumab is a monoclonal antibody against the Cε3 domain of the Fc region of IgE that prevents IgE from binding to the α-chain of FCεRI [130]. However, its use is currently limited to patients suffering from severe asthma and chronic idiopathic urticaria with circulating IgE levels of >30 IU/ml [130], and it has not been approved for the treatment of food allergy. Patients with severe asthma treated with omalizumab have reduced asthma exacerbations, improved quality of life, and reduced use of systemic corticosteroids and rescue bronchodilators [131]. Although omalizumab does not target the underlying

causes of allergic inflammation, it does work well to alleviate symptoms, and long-term use is associated with reductions in the levels of IgE in these patients. Interestingly, multiple studies have also demonstrated that patients with low circulating levels of IgE benefit from treatment with omalizumab [132,133]. This suggests that circulating levels of IgE may not be a good predictor of response. Local production of IgE at sites of allergic inflammation may be one mechanism to explain this.

Currently, the most convincing evidence for a role for local IgE production is in allergic rhinitis (AR) and chronic rhinosinusitis with nasal polyps (CRSwNP) [134]. This is probably due to a lack of overall data in other atopic diseases with local inflammation like asthma, since collection of samples from AR and CRSwNP patients is relatively non-invasive compared to sampling the lung. Nonetheless, local production of IgE at sites of allergic inflammation likely plays a key role in the activation of mast cells, and the majority of IgE produced at these sites is likely immediately bound to FCεRI on mast cells, and therefore, would not be detected in the systemic circulation. Given the efficacy of omalizumab in patients with low levels of circulating IgE, it is likely that production of IgE in the tissues plays a critical role during allergic inflammation.

Despite the key role that IgE plays in all allergic disease, little is known about the mechanisms that regulate and sustain its production in atopic diseases. Thus, it is important to consider other locations of antibody production and potential non-canonical mediators that may contribute to the induction of IgE responses. We focused this study on the role of a potent mast cell mediator, histamine, in the induction of IgE responses. While it is well established that IgE bound to mast cells is critical for the release of histamine, a potential role for histamine in the induction of IgE production has not been investigated.

The atopic march

As mentioned in Chapter 3, IgE-mediated allergic response starts with a break in tolerance and is followed by allergic sensitization where the ultimate outcome is the production of antigen-specific IgE. Asthma and food allergy are known to be driven by antigen-specific IgE, and there are known allergens that initiate these responses. Although the development of atopic dermatitis (AD) is not thought to be directly driven by IgE, there are some important aspects of this disease that contribute to sensitization as well as provide knowledge about IgE biology. AD is often the first allergic disease to develop in infants,

and this disease can persist into adulthood in some patients. While levels of circulating IgE do not always corelate with severity of disease in patients with AD [135,136], there is evidence that AD patients have increased levels of IgE with age and disease progression [137]. Furthermore, there is a wide variety of antigen specific IgE in AD patients, including IgE against microorganisms, such as staphylococcal super antigens [138,139], fungal species [140], and auto-antigens [141]. AD patients have also been reported to have IgE against food antigens that correlate with the later development of food allergy [142,143]. This specific phenomenon of allergic diseases developing from preexisting allergic disease is called the atopic (allergic) march [144,145]. There is also a high percentage of patients with AD that later go on to develop allergic rhinitis and asthma. Though not all food allergy or asthma cases start with AD, the progression from AD to other allergic diseases provides insights into the mediators and the immune environment that induces sensitization. We were interested in the effector mediators that could influence antibody production such as histamine, however we will first describe the mechanism of antibody production.

Antibody production and allergic sensitization

Antigen-specific antibody production

There are two major types of antibody responses that are determined by the type of antigen encountered, T cell-dependent or T-cell independent. The immune system has three mature B cell subsets that can induce a humoral response: follicular B cells, marginal zone (MZ) B cells, and B1 B cells [146,147]. These different subsets are designed to respond to the two different types of antigens. Follicular B cells are the most abundant subset of the three and are located in the lymphoid follicles of the spleen and lymph nodes. These are the classical B cells that are important for responding to protein antigens and require simultaneous $CD4^+$ T helper cell activation, hence the term T-cell dependent. The other two subsets are considered more innate responders, since they are able to rapidly respond to bacterial components and do not generally require T cell help for their antibody production [148]. We are most interested in the process of antibody production to T-cell dependent antigens, since this is the major mechanism for the production of antigen-specific IgE.

The maturation of B cells into antibody-secreting cells (ASC) that produce antibodies specific for T cell-dependent antigens requires germinal center formation. Germinal centers are structures within

lymphoid tissues that facilitate the differentiation of B cells into ASC and promote production of high-affinity antigen-specific antibodies [149]. Prior to the establishment of the germinal center response, B cells can be active by an extrafollicular mechanism. Once a B cell has encountered its cognate antigen it migrates to the interface between the B cell follicles and T cell areas of secondary lymphoid organs to interact with T-helper (Th) cells [150]. Th cells provide two key signals to B cells at this site: CD40L and cytokines, which B cell activation and differentiation pathways. Early in this response, B cells remain in the extrafollicular space, differentiate into short-lived plasma cells, and produce low-affinity antibodies that are important for controlling infections at early phases. Some of these extrafollicular B cells and T cells then traffic back to the follicle to seed the germinal center response. Within the germinal center, B cells receive additional signals through CD40 ligation and cytokines to initiate class-switch recombination (CSR). This is the process whereby a B cell changes the antibody isotype it is expressing (from IgM to either IgG, IgA, or IgE) by the activation of intracellular enzyme activation-induced cytidine (AID). In the allergic response the signal would come from $CD4^+$ T cells that provide IL-4, which is necessary for CSR to IgE [151]. In the germinal center B cells go through multiple cycles of proliferation and somatic hypermutation that leads to the development of high-affinity antibodies. These cycles occur in areas designated the dark zone, followed by selection in an area designated the light zone. The light zone contains important cells that are necessary for the selection of ASC, including follicular DC (FDC) and follicular T helper (Tfh) cells. FDC are important for the selection process and Tfh are important for the survival of GC B cells [149]. Ultimately there are two different types of B cells that exit the GC, memory B cells or long-lived plasma cells.

In conjunction to what is happening the GC to generate ASC, there are important mechanism of in the B cell that are necessary for survival. Due to the large amounts of antibodies they produce, plasma cells increase their ER network and reduce mitochondrial content. Plasma cells have large amounts of misfolded proteins as a by-product of their antibody production, and this leads to cellular stress. There are multiple responses to this phenomenon to prevent plasma cell death. One mechanism is autophagy, which is the process to degrade cytoplasmic contents through lysosomal degradation, and this is important for reducing the ER [152]. There are several identified proteins that are important for the autophagy response, such as Atg5 [153]. Another response triggered by the large amounts of protein

generated by plasma cells, the unfolded protein response (UPR), is driven by X box binding protein 1 (XBP1). XBP1 functions to relieve cellular stress caused by the accumulation of mis-folded proteins, which lead to ER expansion. The efficiency of these processes can enhance survival and antibody production of plasma cells, which in the case of IgE in allergy, is not beneficial. For example, IL-4 has been found to induce autophagy in B cells leading to exacerbating asthma in mice [154]. This is only one example of a mediator of allergic disease enhancing and potentially sustaining the allergic response by regulating IgE production. It is important to understand how mediators of the allergic response could be modulating ASC.

Allergic sensitization

In allergy there are specific signals that lead to the production of IgE, through a process known as allergic sensitization. The classical model of sensitization starts with the processing and presentation of antigen by an antigen-presenting cell (APC). Studies have shown that DCs are the major APC involved in allergic sensitization in the lung and skin [155-157]. There are different classifications of dendritic cells in mice and humans. In mice they include the plasmacytoid DCs (pDC), type 1 conventional DCs (cDC1), type 2 conventional DCs (cDC2), and monocyte-derived dendritic cells (moDC) [158]. In humans there are parallel classifications including pDCs, myeloid DCs (mDCs) that are either classical or conventional, and $CD14^+$ DC (monocyte derived) [159]. The subtype that has been shown to be important for sensitization through the lung and the skin in murine models of sensitization is cDC2s [158]. In the course of sensitization DC antigen presentation and IL-4 lead to skewing of $CD4^+$ T cells towards a Th2 cell phenotype. It remains unclear which cells are providing this initial IL-4, but it has been postulated that basophils could be the initial source of IL4 [160]. Th2 cells then interact with B cells to induce differentiation into IgE^+ plasma cells through additional production of IL-4, along with other co-stimulatory factors. Newer data demonstrate that other cells are also important for the development of long-lived IgE responses. T follicular helper cells (Tfh) play a critical role in the development of antibody responses in general, due to their role during the germinal center reaction. However, recent studies have also shown that Tfh cells are necessary for IgE production, especially for the development of long-lived IgE responses [161]. In the course of allergic sensitization there is potential for mechanisms to be enhanced

or regulated. As mentioned before, AD provides a foundation for sensitization where there are components that increase IgE throughout the course of disease, similarly in other allergic diseases there are areas of local inflammation that can recapitulate the same inflammatory environment. Understanding how inflammatory mediators influence IgE production is important for understanding IgE biology.

Local IgE production

There is abundant evidence on the importance of local IgE production. Studies have shown that levels of IgE are more concentrated in the tissue where responses are initiated, rather than the serum, such as the lung [162] and nasal cavity [163], suggesting that there is local production of IgE at these sites. As shown by Richard Locksley's group, mast cells quickly take up IgE in a form of active surveillance by extending cell processes across vessel walls to capture luminal IgE [164]. Therefore any IgE that is present in the serum may be the "left over" IgE that did not bind to mast cells, and thus serum IgE levels do not indicate true IgE levels. In line with these findings, serum IgE levels do not always indicate the severity of allergic disease in AD [165], allergic asthma [166], and food allergy [167]. Additionally, as mentioned above, treatment of IgE-mediated diseases with omalizumab, a humanized monoclonal anti-IgE antibody, has shown efficacy both in patients with high serum IgE levels and patients with low/undetectable levels of serum IgE [130,168]. Finally, there is evidence indicating that class-switching to IgE occurs in the bronchial mucosa [169], nasal tissues [170], and nasal polyps[171]. Altogether these studies suggest that perhaps local production of IgE at sites of inflammation may be more relevant to disease severity where IgE is taken up and concentrated in the tissues.

In this model of local IgE production, IgE is produced and binds to mast cells that are subsequently activated leading to local inflammatory responses that contribute to IgE production where IgE is quickly taken up by mast cells in these tissues to start the cycle again. Moreover, in these local inflammatory sites there is a cellular interface between adaptive cells, such as T cells and B cells, and innate effector cells, such as mast cells that are present in the tissue. Consequently, mast cells have been shown to sustain and regulate antibody production directly through cell contact and indirectly through secreted mediators *in vitro* [172,173]. However, the role of the most abundant and potent mast cell mediator, histamine, in regulating antibody production is not well understood.

Novel mediators of antibody production in allergy

IgE and histamine

Previous work conducted by our lab and others, has investigated the *in vivo* role of histamine in the production of IgE. Histidine decarboxylase knockout (HDC KO) mice, which lack the enzyme necessary for the production of histamine, have significantly reduced levels of IgE in a house dust mite model of allergic airway inflammation [174]. Similarly, H2R KO mice have significantly less IgE in an OVA-alum model of allergic disease [53,54]. In addition, studies have demonstrated that histamine can act directly on cells that are critical for the induction of IgE, including T cells, DCs, and B cells. One study found that exposure of murine splenic T cells to histamine prior to CD3/CD28 activation resulted in increased production of IL-4, IL-13, and IFN-γ *in vitro* [54]. Histamine has also been shown to regulate dendritic cell functions *in vitro*, including differentiation, endocytosis, antigen presentation, and T-cell polarization capacity [175]. Finally, an early study showed that histamine inhibited antibody production by the human B cell lines IM-9 and CLB in an H2R-dependent manner. It was also demonstrated that histamine selectively enhanced IgE and IgG4 production by purified tonsil B cells after stimulation with IL-4 or IL-13 and anti-CD58. Interestingly, histamine regulated antibody production through different histamine receptors depending on the stimuli. An H3R antagonist, thioperamide, inhibited histamine-enhanced antibody production induced by IL-4 and anti-CD58, while an H2R antagonist, dimaprit, inhibited histamine-enhanced antibody production by IL-13 and anti-CD58 [176]. While these studies demonstrate the complexity of histamine effects, they suggest that histamine may play an important role in the production of IgE. However, no studies have described a mechanism for how histamine may be directly regulating antibody production *in vivo*. As such, we sought to investigate the role that histamine plays during the development of IgE responses.

Summary

The production of antigen-specific IgE is critical for the induction of allergic disease. Antibody production itself is a highly regulated process that can occur in both secondary lymphoid organs and at peripheral sites during inflammatory disease. Despite the key role that IgE plays during allergic disease, little is known about its regulation and induction in disease. Classically, IL-4 is known to play a key role in

the induction of IgE class-switching in B cells, but the mechanisms in allergy that regulate production and secretion of IgE by B cells and plasma cells are yet to be fully characterized. We focused on the role of a key mediator in the allergic response, histamine, to further characterize the regulation of IgE production. Histamine has established roles in the effector phases of allergic inflammation, but recent work suggests that it may also be important for the production of IgE in allergic disease. The work described in this chapter aims to further investigate the potential role for histamine in the production of antigen-specific IgE during allergic disease. We show for the first time that histamine can act directly on B and T cells to promote antigen-specific IgE responses, which elucidates a new layer of regulation of IgE responses and histamine biology in allergy.

RESULTS

Histamine receptors 1 and 2 regulate antibody production in a model of allergic airway inflammation

While histamine receptors are known to be expressed on a variety of inflammatory cells, including T cells [51], their expression on B cells has not been previously studied. As such, we first wanted to characterize the expression of the histamine receptors on murine and human B cell subsets. While there was little to no detectible expression of either H3R or H4R in any of the B cell subsets examined, both H1R and H2R were expressed by most of the B cell subsets examined (**Figure 14.**). In mice, naïve B cells expressed only H1R, while plasma cells expressed both H1R and H2R (**Figure 14. A**). Stimulation of naïve B cells with either IL-4 and anti-CD40 or IL-4 and LPS, which both induce activation and maturation of naïve B cells into IgE producing plasma cells, resulted in increased expression of both H1R and H2R in culture (**Figure 14. B**). We found similar expression patterns of H1R and H2R on B cell subsets isolated from adult human tonsil tissue, in naïve, memory, and CG B cell subsets. H2R had the highest expression in human naïve and memory B cells (**Figure 14. C**). These data indicate that murine and human B cells are capable of responding directly to histamine through both H1R and H2R. Additionally, in both murine and human B cell subsets, H2R was expressed at higher levels compared to H1R in more mature B cell subsets, which may be important for the regulation of B cell responses by histamine.

We and others have previously shown that mice that undergo the OVA-alum model of allergic airway inflammation produce significantly reduced levels of OVA-specific and total IgE in the absence of H2R [53,54]. Given our results and previous findings, we sought to determine the role of H1R and H2R during antibody production. In order to determine the effects of histamine on B cells and assess which receptors were required, we first measured total and antigen-specific antibody production in WT, H1R KO, H2R KO, and H1R/H2R DKO mice using the OVA-alum model of allergic airway inflammation (**Figure 15. A**).

In this model, alum is an adjuvant that promotes Th2-driven antibody responses, likely through the formation of multivalent alum-Ag complexes that are preferentially taken up by APCs for presentation to T cells [177]. Additionally, Alum alone is also known trigger immune responses through the activation of the inflammasome, which leads to the release of IL-1β [178]. In the antibody response of the OVA-alum

model, IgG1 and IgE are commonly measured as markers of sensitization and inflammation, however we also quantified IgM and IgG2c levels to determine whether histamine was affecting antibody production globally. While levels of total IgG1, IgM, and IgG2c were not altered in the serum of either the single or H1R/H2R DKO animals immunized with OVA-alum, the levels of total IgE were significantly reduced in the H2R and H1R/H2R DKO mice ($p < 0.001$; **Figure 15. B**). Moreover, we found that levels of OVA-specific antibodies of all isotypes were significantly reduced in both the H2R KO and H1R/H2R DKO animals compared to WT ($p < 0.0001$; **Figure 15. C**). This suggests that histamine plays a key role in the production of IgE in general, as well as in the generation of antigen-specific antibody responses, of all isotypes.

Interestingly, we also found that levels of total IgG1 were significantly reduced in PBS-alum-treated H1R/H2R DKO animals compared to WT ($p < 0.05$**; Figure 15. B**)**.** These data suggests that there is an defect in naïve mice that lack H1R and H2R.

There is no defect in B cell development in the absence of histamine receptors 1 and 2

Next we wanted to address whether the defects in antibody production observed above may be due to defects in B cell development in the histamine receptor KO animals, since there are many antibody deficiencies that are due to B cell development defects [179]. B cells undergo a well-defined series of maturational steps in the bone marrow before they exit to the periphery and traffic to the spleen [180], where they rapidly differentiate through two transitional stages before becoming either mature follicular or marginal zone B cells [181]. To determine whether there was a defect in B cell development in these animals, we characterized the frequency of distinct B cell subsets in the bone marrow, spleen, and peritoneal cavity of WT and H1R/H2R DKO KO mice by flow cytometry. The flow cytometry gating strategy excluded cells stuck together that result in doublets, debris, and dead cells (**Figure 16. A**). Depicted in **Figure 16. B,C,D** are the gating strategies for bone marrow, spleen, and peritoneal cavity.

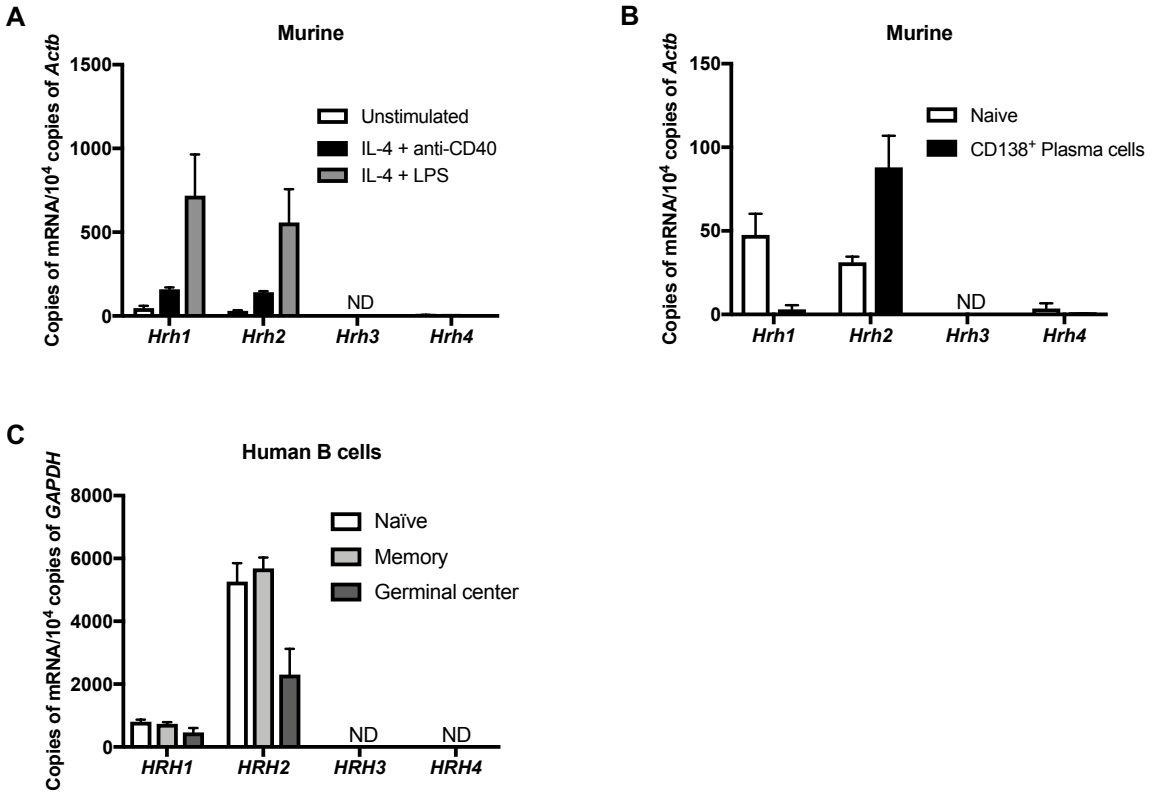


Figure 14: Murine and human B cells differentially expressed histamine receptors 1 and 2

B220^{+} cells were purified from the spleen and cultured with either rIL-4 or LPS and anti-CD40 for 4 days. Cells were collected and **(A)** mRNA was isolated from bulk culture to quantify histamine receptor expression. **(B)** CD138^{+} plasma cells were FACs sorted and mRNA was collected to quantify histamine receptor expression. **(C)** Human tonsil tissue was digested overnight and cells were collected and FACs sorted for naïve (CD19^{+}IgD^{+}CD27^{neg}), memory (CD19^{+}IgD^{neg}CD27^{+}) and germinal center (CD19^{+}IgD^{neg}CD38^{mid}) B cells, mRNA was collected to quantify histamine receptor expression. Data are representative of the mean $\pm$ SEM (n = 3-6; ND = not detectable)

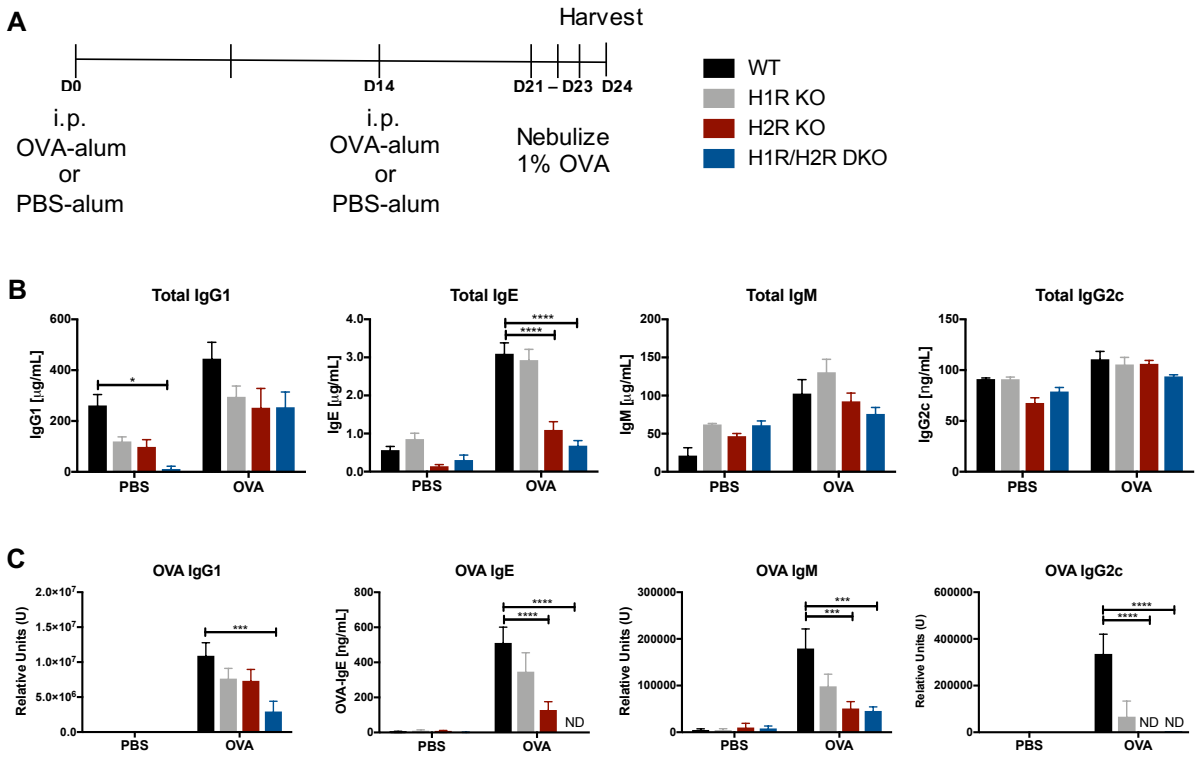


Figure 15: Histamine regulated IgE and antigen-specific antibody responses through H1R and H2R

Total and OVA-specific antibodies were quantified in WT, H1R KO, H2R KO, and H1R/H2R DKO mice. In the absence of H2R there was a defect in IgE and OVA-specific antibody production, there was a greater decrease in the absence of H1R and H2R. **(A)** OVA-alum model. H1R KO, H2R KO, H1R/H2R DKO, and WT mice were administered OVA-alum by i.p. injection on day 0 and day 14 followed by nebulization with OVA on day 21-23. On day 24 mice were euthanized and serum was collected to quantify **(B)** Total antibody and **(C)** OVA-specific antibody by ELISA. Data are representative of the mean ± SEM ([**B**, **C**; IgE and IgG1] n = 8-33 from 6 individual experiments, [**B**, **C**; IgM and IgG2c] n = 3-5 from 2 individual experiments). *$p < 0.05$, ***$p < 0.0001$, ****$p < 0.0001$ by One-way ANOVA.

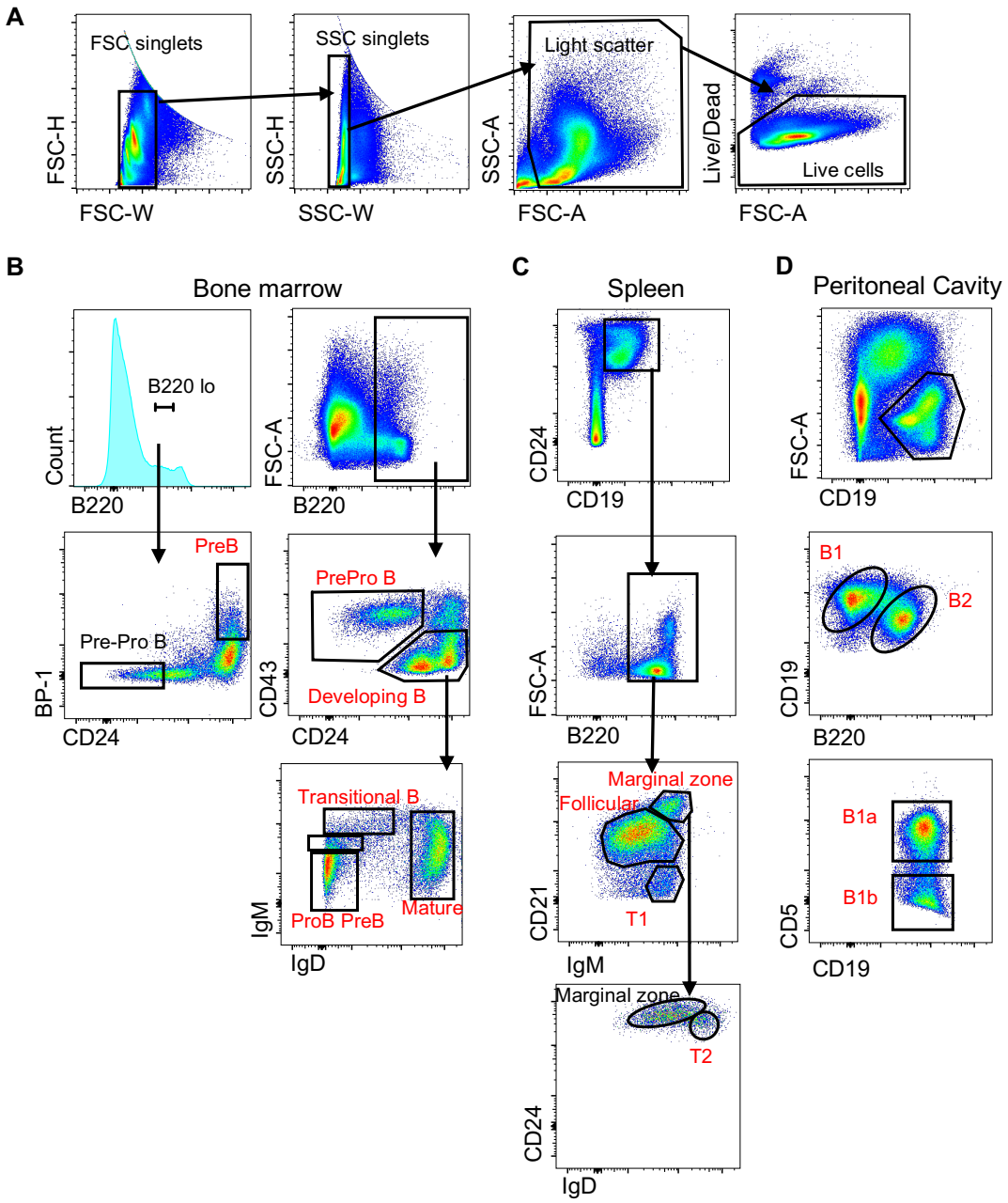


Figure 16: B cell development gating strategy

(A) All B cells were gated to eliminate doublets, debris, and dead cells. **(B)** Bone marrow B cells were gated off two different $B220^{+}$ gates **(C)** Splenic B cells were gated off $CD24^{+}CD19^{+}B220^{+}$ cells. **(D)** Peritoneal cavity B cells were gated off $CD19^{+}$ cells. Populations in red were quantified for B cell development analysis.

While there was a small, but significant, decrease in the frequency of pre-pro B cells in the bone marrow of H1R/H2R DKO compared to WT mice, there was no difference in the frequency of more differentiated subsets in these animals, indicating that the small decrease in the frequency of the early B cell lineages did not affect development of more mature subsets (**Figure 17. A**).

Similarly, in the spleen, the frequency of transitional type 2 (T2) B cells was slightly, but significantly, lower compared to WT (**Figure 17. B**). However again, this did not affect the downstream development of marginal zone or follicular B cells, as the frequencies of these cells were not altered in the spleens of H1R/H1R DKO animals compared to WT (**Figure 17. B**). Finally, we also characterized the frequencies of B cell populations in the peritoneal cavity, including B1a, B1b and B2 cells, and observed that they were unaltered in H1R/H2R DKO mice compared to WT (**Figure 17. C**). Altogether, these data indicated that there was no defect in the development of B cells in H1R/H2R DKO animals.

We also quantified the levels of antibodies in naïve mice to determine whether production of natural antibodies was altered in histamine receptor KO animals. Natural antibodies are produced in the absence of antigen and have been shown to be important in a variety of processes from immune regulation to resistance to infection [182]. While naive H1R KO mice had significantly increased levels of circulating antibodies ($p < 0.01$; **Figure 18**), which has been observed upon OVA-alum immunization of H1R deficient mice previously [54], H2R KO and H1R/H2R DKO mice had significantly lower levels of circulating IgG1 ($p < 0.0001$; **Figure 18**). Altogether, these data indicated that B cell development was normal in histamine receptor KO animals, but there was a defect in production of natural antibodies in H2R and H1R/H2R DKO mice. This data suggested that there may be a more global defect in antibody production or secretion and that there is probably and intrinsic defect in B cells in the absence of histamine signals through H1R and H2R.

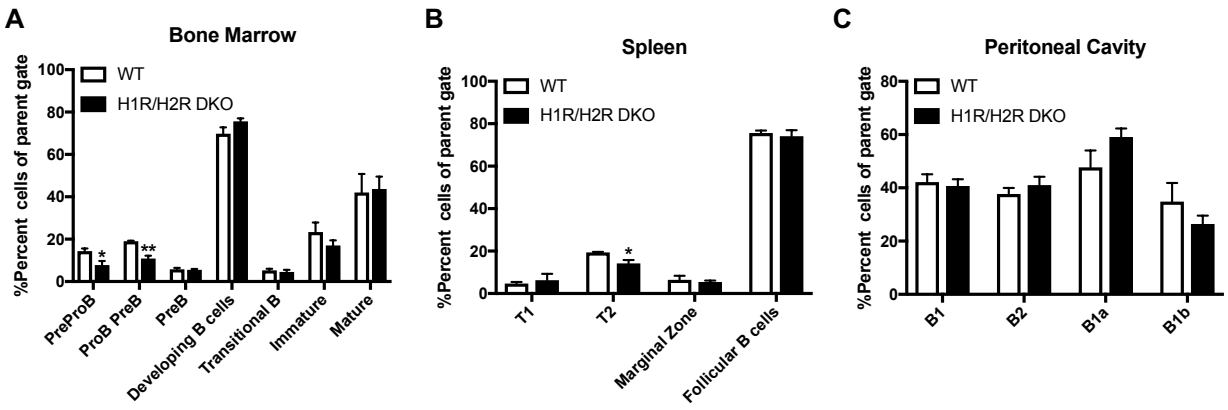


Figure 17: B cell development was unaltered in the absence of H1R and H2R

All quantified B cell populations were present in H1R/H2R DKO similarly to WT mice. **(A)** Bone marrow was isolated from tibia and femur and fluorescently labeled to identify PreBProB ($B220^{lo}BP1^{neg}CD24^{lo}$), proB preB ($B220^{+}CD43^{+}CD24^{lo}$), preB ($B220^{lo}CD24^{+}BP1^{+}$), developing ($B220^{+}CD43^{+}CD24^{+}$), transitional ($B220^{+}CD43^{+}CD24^{+}IgM^{hi}IgD^{lo}$), immature ($B220^{+}CD43^{+}CD24^{+}IgM^{lo}IgD^{neg}$), and mature ($B220^{+}CD43^{+}CD24^{+}IgM^{+}IgD^{+}$) B cells. **(B)** The splenic cells were isolated and fluorescently labeled to identify T1 ($CD24^{+}CD19^{+}B220^{+}CD21^{neg}IgM^{+}$), T2 ($CD24^{+}CD19^{+}B220^{+}CD21^{+}IgM^{+}$), marginal zone ($CD24^{hi}CD19^{+}B220^{+}CD21^{+}IgM^{+}$), and Follicular ($CD24^{+}CD19^{+}B220^{+}CD21^{med}IgD^{lo}$) B cells. **(C)** Cells of the peritoneal cavity were isolated and fluorescently labeled to identify B1($CD19^{hi}B220^{neg}$), B2($CD19^{+}B220^{+}$), B1a ($CD19^{hi}B220^{neg}CD11b^{+}CD5^{+}$), and B1b ($CD19^{hi}B220^{neg}CD11b^{+}CD5^{neg}$) cells. Data are representative of the mean ± SEM (n = 3 from individual experiments). *p < 0.5 by Student T test.

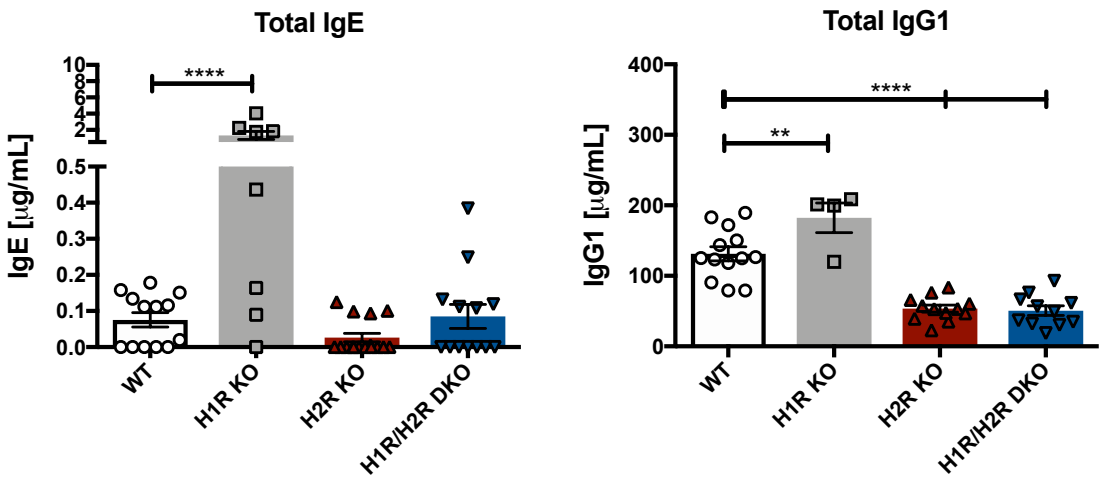


Figure 18: Natural antibody levels were decreased in the serum of H1R/H2R DKO mice when compared to WT mice

There was a significant decrease in IgG1 natural antibodies in H2R KO and H1R/H2R DKO mice compared to WT mice. Serum was collected from naïve WT, H1R KO, H2R KO, and H1R/H2R DKO mice, Total IgE and Total IgG1 was quantified by ELISA. Data are representative of the mean ± SEM (n = 4-16).**$p < 0.01$, ****$p < 0.0001$ by One-way ANOVA.

Histamine receptors 1 and 2 are not necessary for Th2 priming events

We next wanted to address one of the limitations of the above experiments, since they were performed in global KO animals and the results we obtained could be due to the loss of the effect of histamine on a variety of cell types. Importantly, previous work on H2R KO mice suggested that histamine regulates Th2 cytokine production [54], which is important for the induction of IgE responses [183].

Thus, it was possible that the defect in production of IgE in the histamine receptor KO animals was due to defects in T cell skewing towards a Th2 phenotype in these animals. As mentioned above, there are several factors that are necessary for Th2 skewing of T cells, including antigen presentation by dendritic cells and IL-4. To determine whether there was a Th2 priming defect, we restimulated splenic and mediastinal lymph node (LN) cells from OVA immunized mice with anti-CD3/CD28 or OVA *in vitro* and measured production of the type 2 cytokines IL-5, IL-13 and IL-4. In this experiment, the restimulation of splenocytes with OVA results in OVA uptake, processing, and presentation by APCs in the context of MHCII molecules to $CD4^+$ Th2 cells, which are also present in the splenocyte population. Production of Th2 associated cytokines after *in vitro* restimulation would confirm proper Th2 skewing occurred *in vivo*. Anti-CD3/CD28 was used as a positive control, since it non-specifically activates T cells, and splenocytes plated in media alone served as our negative control.

We first assessed T cells from H1R KO, H2R KO, and H1R/H2R DKO spleens and observed that there were no significant differences in levels of IL-5 and IL-13 in either the positive control group or antigen-specific (OVA) stimulation (**Figure 19. A**). IL-4 levels were not detectable in any experimental group by means of ELISA, and so we analyzed intracellular IL-4^+ CD4 T cells by flow cytometry. Likewise, the frequency of IL-4^+ T cells was not different between WT and H1R/H2R DKO $CD4^+$ T cells in the spleen (**Figure 19. B**), indicating that T cells in all the KO animals were appropriately skewed to Th2 cells and could respond to OVA. As an additional control, we quantified IFNγ, which is known to downregulate Th2 responses and promote Th1 responses. IFNγ levels were normal in restimulated splenic cultures of KO animals compared to WT animals, indicating that the reduced levels of IgE in H2R KO and H1R/H2R DKO animals was not due to altered skewing of T cells towards a Th1 phenotype **(Figure 19. C).** Next, we assessed restimulation in mediastinal LN cells, which represent the population of T cells involved in the local inflammatory response in the OVA-alum model. As in the spleen, we found no significant

differences between IL-5 or IL-13 production in positive control groups or antigen-specific (OVA) stimulation in cells from LN **(Figure 20)**.

These data show that there was no defect in Th2 skewing in the absence of H1R and H2R in our model. Thus, antigen presentation, IL-4 secretion, and Th2 $CD4^+$ cells were all functional. Contrary to previous findings that demonstrated elevated levels of IL-4, IL-13 and IFNγ in H2R KO T cells [54], these data demonstrated that H1R and H2R were not necessary for Th2 cytokine production, and therefore, not necessary for the priming events during the OVA-alum immunization. The discrepancies in our data and previous findings may be due to the type of experiment conducted. We examined the role of T cells *ex vivo* after OVA-alum immunization, whereas the previous work assessed T cell skewing by non-specifically activating naïve T cells from H2R KO mice that were previously primed with a high concentration of histamine (10^{-3} M) [54].

Antibody regulation by histamine through H1R and H2R depends on the type of antigen and adjuvant

In addition to the OVA-alum immunization driving a Th2 response, it also promotes antibody production in a T-cell dependent manner. Antibody production can be triggered in B cells through either T cell-dependent (TD) or T cell-independent (TI) antigens. While TD antigens are generally protein antigens that can induce production of all antibody isotypes, TI antigens are highly repetitive structures, like poly-saccharides, that can act directly on B cells. There are two different types of TI antigens, designated type 1 and type 2, based on the necessity for the B cell-receptor (BCR) to initiate an antibody responses [184,185]. Type 1 TI (TI-1) antigens include B cell mitogens, such as LPS, that can activate naïve and mature B cells independently of their BCR and subsequently induce antibody production. Type 2 TI (TI-2) antigens include bacterial capsular polysaccharides that contain highly repetitive motifs that bind directly to BCRs to induce antibody production. Because the BCR is necessary for TI-2 antigens, only mature B cells are able to mount a response, and these responses are generally dominated by production of antigen-specific IgM by B1 B cells and marginal zone B cells [186].

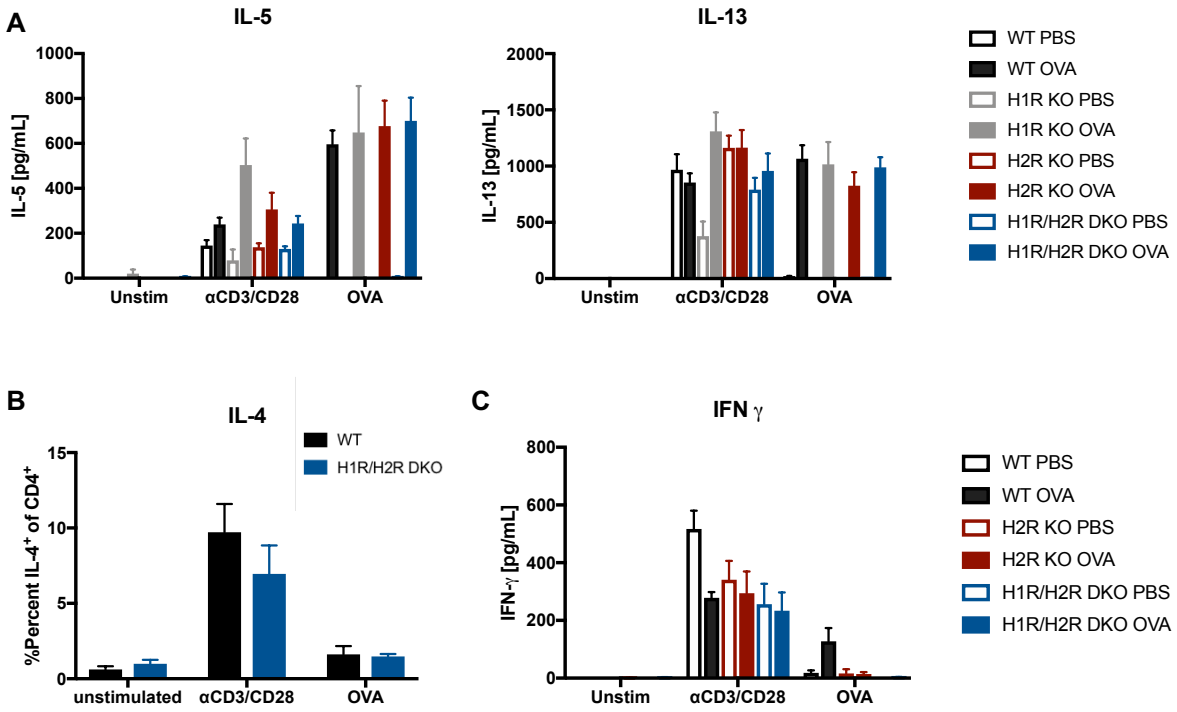


Figure 19: T cells from the spleen did not require H1R or H2R for Th2 skewing and cytokine production

Splenic T cells from H1R KO, H2R KO, and H1R/H2R DKO mice were restimulated in cultures and secreted IL-5 and IL-13 similarly to WT mice. IL-4 was also produced in splenic $CD4^+$ T cells from H1R/H2R DKO mice similarly to WT mice. Splenocytes were isolated from OVA-alum or PBS-alum immunized mice, plated in 24 well plates, and stimulated with either media, αCD3/CD28, or OVA for 72 hours. Supernatants from culture were collected to quantify **(A)** IL-5, IL-13, and **(C)** IFN-γ by ELISA **(B)** Cells from culture were fluorescently labeled to quantify the frequency of intracellular $IL\text{-}4^+$ in $CD4^+$ T cells. Data are representative of the mean ± SEM from (**[A]** *n* = 4-18 from 3individual experiments, **[B]** *n* = 3-5, **[C]** *n* = 3-6 from 2 individual experiments)

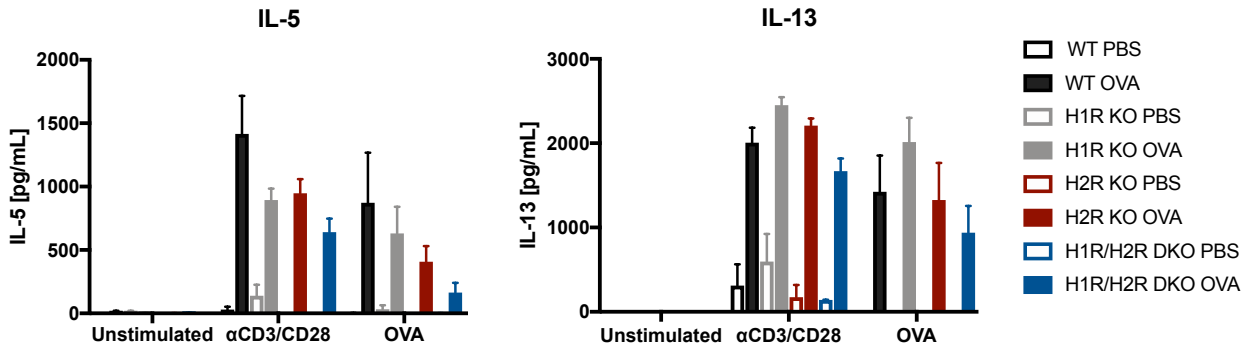


Figure 20: T cells from draining lymph nodes did not require H1R or H2R for Th2 skewing and cytokine production

Lymph node cells from H1R KO, H2R KO, and H1R/H2R DKO mice were restimulated in cultures and secreted IL-5 and IL-13 similarly to WT mice. Lung draining lymph nodes (mediastinal) were isolated, plated in 24 well plates, and stimulated with either media, αCD3/CD28, or OVA for 72 hours. Supernatants from culture were collected to quantify IL-5 and IL-13 by ELISA. Data are mean ± SEM (*n* = 3-5)

In order to assess the role of histamine in both TD and TI antigen-specific antibody production, we used a hapten-carrier protein immunization. Haptens are small molecules that do not elicit an antibody response alone, but they do elicit an antibody response when conjugated to a protein carrier. This model is useful because it allows us to quantify the hapten-specific antibody production from two different types of immunization. For a TI response we used the hapten NP (4-hydroxy-3-nitrophenyl) conjugated to ficoll, a highly branch polysaccharide. For a TD response, we used NP conjugated to CGG (chicken gamma globulin) with alum as the adjuvant to promote Th2 responses.

We found no differences in Total or NP-specific IgM levels in the serum of KO mice compared to WT after stimulation with the TI antigen (**Figure 21.**), indicating that histamine was not necessary for TI responses involving a TI-2 antigen. For the NP-CGG immunization we used the adjuvant alum where NP-antibodies of isotypes IgM, IgG1, and IgE can be quantified. There were no differences in total IgM and IgG1 levels in the serum of H1R/H2R DKO mice compared to WT (**Figure 22. A**). There was a decrease in total IgE levels in the serum of H1R/H2R DKO mice compared to WT, however this did not reach statistical significance ($p = 0.075$; **Figure 22. A**). We also measured NP-specific antibody and found a small, but not significant, decrease in NP-IgM levels, and no difference in NP-IgG1 levels in the serum of H1R/H2R DKO compared to WT. Due to the short nature of this model, IgE levels were too low to quantify (**Figure 22. B**). Importantly, this model involves a single immunization and is shorter than our OVA-alum model, and this may not be a sufficient time frame to see a strong effect of histamine on antibody responses. Altogether, it is very clear that H1R and H2R are not necessary for antibody responses to TI antigens. However, our findings on NP-CGG alum immunization indicate that H1R and H2R may be necessary for early IgE responses.

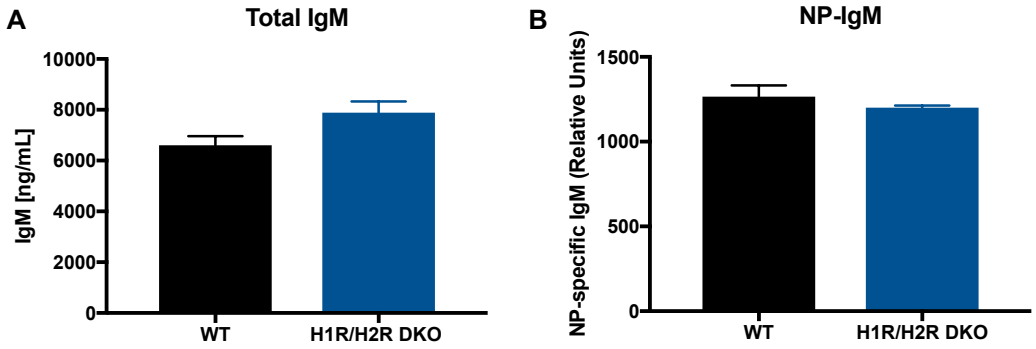


Figure 21: H1R and H2R were not necessary for T cell-independent antigen antibody production

There was no difference in Total IgM or NP-IgM in H1R/H2R DKO mice compared to WT immunized with NP-Ficoll. WT and H1R/H2R DKO mice were administered NP-Ficoll by i.p. injection on day 0. Serum was collected on day 14 to quantify **(A)** Total IgM and **(B)** NP-specific IgM by ELISA. Data are representative of the mean ± SEM (*n* = 3).

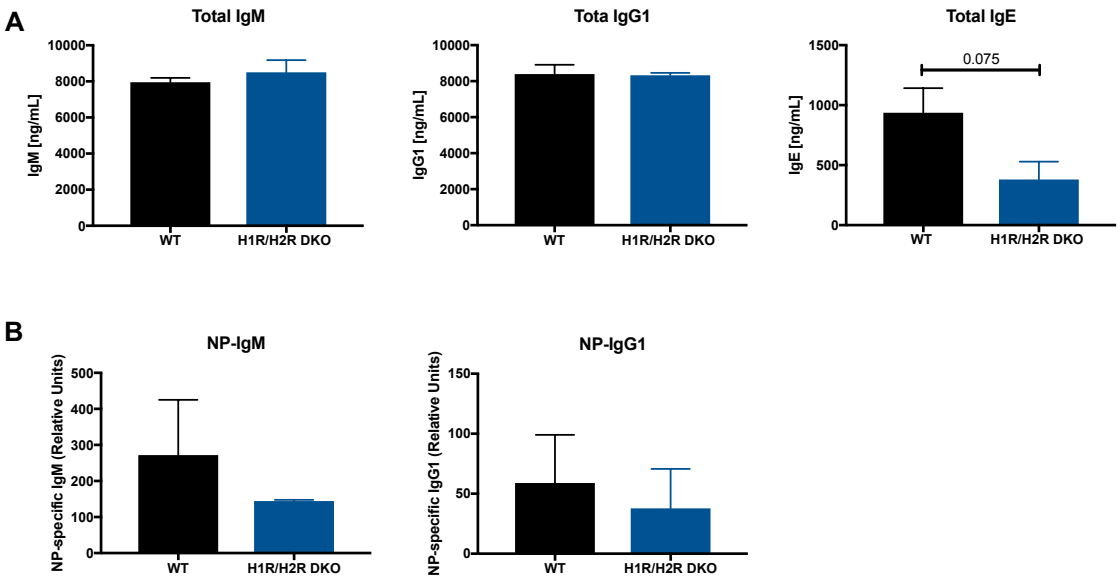


Figure 22: There was no significant difference in levels of total IgE in H1R/H2R DKO mice immunized with NP-CGG-alum compared to WT

There were no significant differences in total or NP-specific antibody levels in H1R/H2R DKO mice compared to WT mice immunized with NP-CGG-Alum. WT and H1R/H2R DKO mice were administered with NP-CGG conjugated with alum by i.p. injection on day 0. Serum was collected on day 14 to quantify **(A)** Total IgM, IgG1, and IgE and **(B)** NP-specific IgM, and IgG1 by ELISA. Data are representative of the mean ± SEM (n = 3-5). P = 0.075 by Student's T test.

Thus far, our data suggests that histamine regulates antibody production through H1R and H2R in strong IgE responses (**Figure 15.)**. This hypothesis is supported by the nature of Th2 responses where histamine is available and known to drive inflammation. In order to determine whether the effects of histamine were indeed restricted to Th2-associated inflammatory responses, we next immunized mice with NP-CGG in complete Freund's adjuvant (CFA). CFA contains inactivated Mycobacterium tuberculosis that drives Th1 responses and results in the production IgM. We found that H1R/H2R DKO mice had a slight reduction in NP-specific IgM antibody production after immunization with NP-CGG in CFA (p = 0.039**, Figure 23.**)**,** indicating that histamine may also regulate non-Th2 driven antibody responses. Further experiments using longer models of immunization are needed to confirm these results.

B cells that lack histamine receptors 1 and 2 have an intrinsic defect in vitro

It is clear that B cell development and Th2 skewing are intact in the absence of histamine receptors in our model. However, it is still not clear whether histamine is acting directly on B cells in promote antibody production. In order to determine whether the defects in antibody production seen in histamine receptor KO animals was due to a direct effect of histamine signaling on B cells, we conducted *in vitro* experiments to characterize B cell differentiation, antibody production, and antibody secretion. The design of these *in vitro* experiments was based on our *in vivo* results, which demonstrated that H1R/H2R DKO mice had the greatest reduction in total and antigen-specific antibodies compared to WT mice. More importantly, IgE was the most affected isotype compared to IgG1, IgM, and IgG2c. Therefore, we chose an *in vitro* model that induces IgE^+ B cells by culturing $B220^+$ B cells in the presence of IL-4 and anti-CD40, where anti-CD40 induces clonal expansion and differentiation [187,188] and IL-4 promotes isotype switching and antibody secretion [189]. We also focused on comparing responses from H1R/H2R DKO B cells and WT B cells.

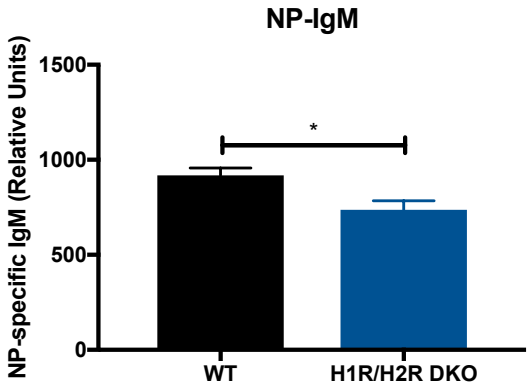


Figure 23: Th1 antibody production is regulated by histamine through H1R and H2R

There was a significant decrease in NP-IgM antibody in H1R/H2R DKO mice compared to WT mice immunized with NP-CGG/CFA. WT and H1R/H2R DKO mice were administered NP-CGG emulsified with CFA by i.d. injection at flanks behind legs and scruff of neck on day 0. Serum was collected on day 14. Data are representative of the mean ± SEM (n = 3). *p < 0.5 by Student's t-test.

First, we characterized B cell differentiation throughout culture by flow cytometry by staining for the plasma cell marker CD138, and IgG1 and IgE. Although IgG1 is not the main focus of these experiments, it is an important precursor of IgE secretion. Typically, there are two known mechanisms of IgE class-switching: (1) sequential class-switching from IgG1 to IgE, or (2) direct IgE class switching from IgM. Studies have demonstrated that sequential class-switching from IgG1 is most common and gives rise to high affinity IgE [190,191]. Thus, in our studies dysregulation of IgG1 class-switching may account for lower levels of IgE.

We observed that $CD138^+$ plasma cells start populating the culture around day three and peak on day five before they start dying on day six. The percent of $CD138^+$ plasma cells and MFI of CD138 were quantified on day 4 and were similar in the H1R/H2R DKO mice compared to WT (**Figure 24.**). We further characterized $CD138^+$ plasma cells on day 5 of culture by gating on surface IgE^+ and $IgG1^+$ cell to quantify the frequency and mean fluorescence intensity (MFI) of IgE^+ and $IgG1^+$ cells, and found that H1R/H2R DKO and WT B cells expressed had similar frequencies and MFI of both cell types. (**Figure 25.**)**.** These data suggest that histamine receptors 1 and 2 are not necessary for the development of $CD138^+$ plasma cells, IgE^+ cells, or $IgG1^+$ cells, and therefore are also not necessary for class-switching events.

Next, we wanted to determine whether antibody production in the cell was affected by the absence of histamine receptors. We conducted intracellular flow cytometry analysis of IgG1 and IgE after four days of culture. There was no difference in the frequency of intracellular $IgG1^+$ or IgE^+ cells in H1R/H2R DKO B cells compared to WT B cells (**Figure 26.**). Thus, in the absence of histamine receptors 1 and 2 $B220^+$ cells differentiated into $CD138^+$ plasma cells and were able to induce class-switching events that ultimately resulted in the expression of IgE and IgG1. Since we observed lower levels of antibody in the serum of H1R/H2R DKO mice *in vivo*, it is possible that B cell antibody secretion is defective in the absence of histamine signals, and so we measure antibody secretion in the culture supernatants by ELISA after 4 days of culture. Interestingly, H1R/H2R DKO B cells secreted significantly less IgE compared to WT cells, but there was no difference in secretion of IgG1 ($p < 0.001$; **Figure 27.**).

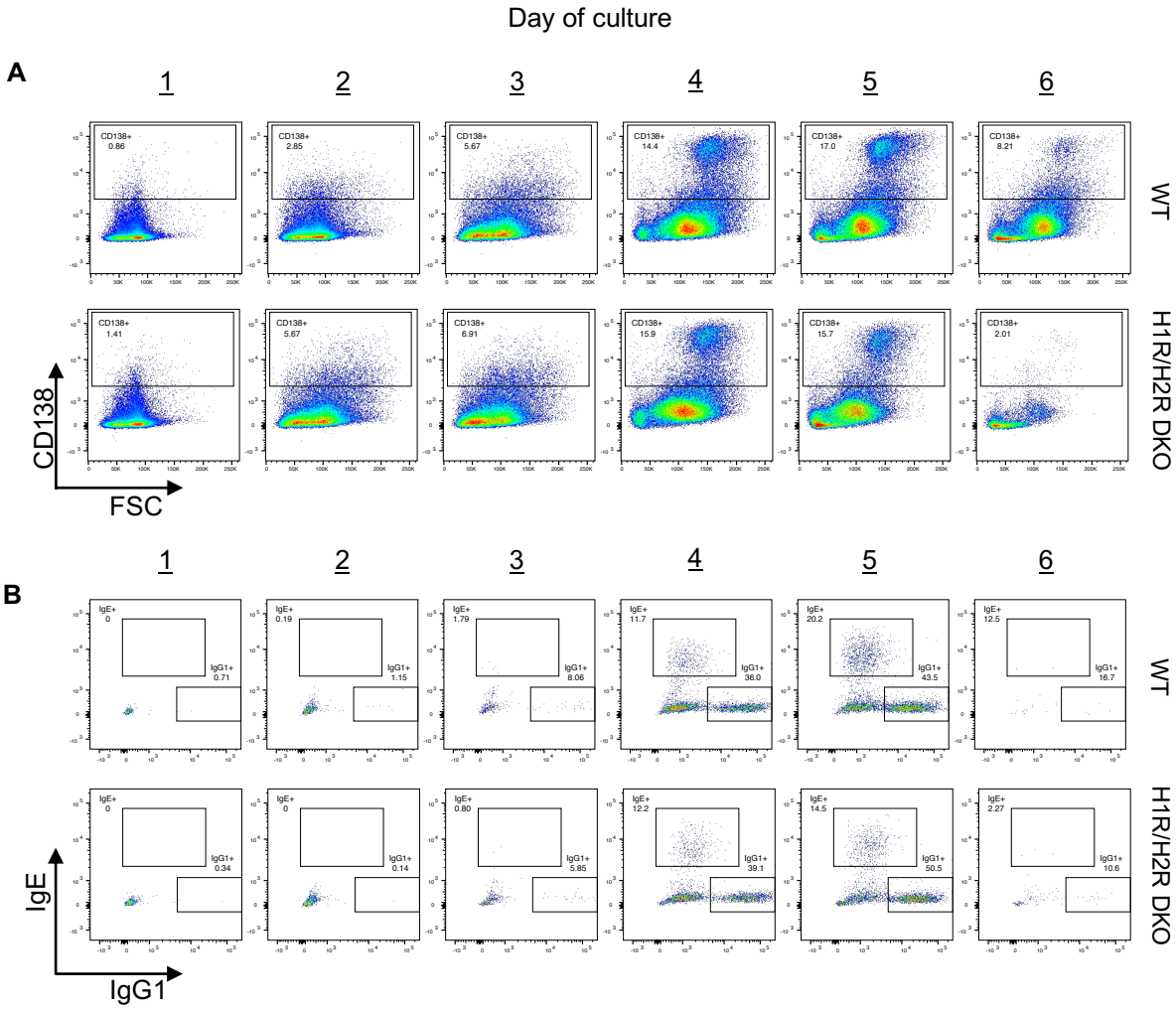


Figure 24: Plasma cell (CD138⁺) development was unaltered in H1R/H2R DKO B cells

Plasma cell, IgE⁺, and IgG1⁺ populations were present in WT and H1R/H2R DKO B cells *in vitro*. B220⁺ cells were purified from the spleen and cultured with rIL-4 and αCD40 for 6 days. **(A)** Cells were collected daily and fluorescently labeled to quantify plasma (CD138⁺), IgE⁺ and IgG1⁺ cells. Data are representative of the mean ± SEM (*n* = 4 from 4 individual experiments).

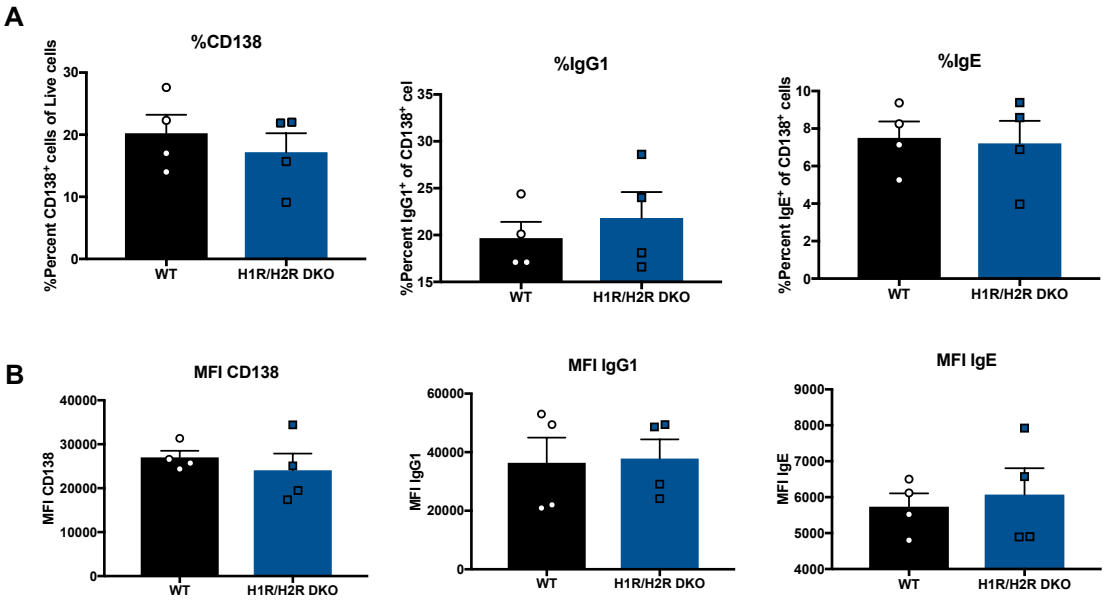


Figure 25: B cells did not require H1R or H2R for the development of IgE or IgG1 positive plasma cells *in vitro*

There was no difference in the frequency or MFI of IgE$^+$ and IgG1$^+$ of H1R/H2R DKO plasma cells (CD138$^+$) quantified at day 5. B220$^+$ cells were purified from the spleen and cultured with rIL-4 and αCD40 for 5 days. **(A)** Percent and **(B)** MFI of plasma cells (CD138$^+$), IgE$^+$ and IgG1$^+$ cells. Data are representative of the mean ± SEM (*n* = 4 for 4 individual experiments).

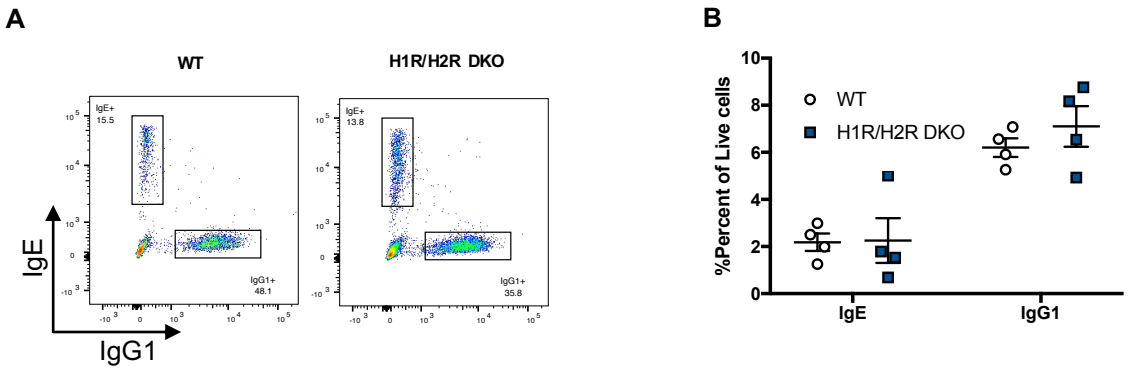


Figure 26: H1R and H2R were not necessary for intracellular production of IgE an IgG1 in B cells

There were no significant differences between intracellular IgG1 or IgE expression in H1R/H2R DKO B cells compared to WT B cells. $B220^{+}$ cells were purified from the spleen and cultured with rIL-4 and αCD40 for 4 days. Cell were collected, trypsinized and permeabilized to selectively fluorescently stain for intracellular IgE and IgG1 and analyzed by **(A)** flow cytometry and **(B)** quantified by percent of live cells. Data are representative of the mean $\pm$ SEM (n = 4 for 4 individual experiments).

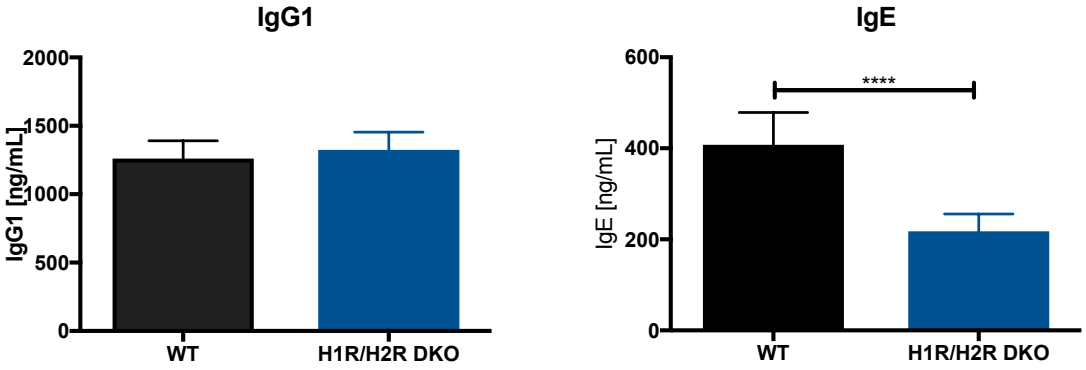


Figure 27: H1R and H2R were required for IgE secretion of B cells *in vitro*

H1R/H2R DKO B cells secreted significantly less IgE compared to WT B cells but secreted IgG1 at similar levels compared WT B cells. $B220^{+}$ cells were purified from the spleen and cultured with rIL-4 and αCD40 for 4 days. Supernatants were collected and IgG1 and IgE were quantified by ELISA. Data are representative of the mean $\pm$ SEM of (n = 8 from 3 individual experiments). ***$p < 0.0001$ by Student's T test.

A phenotype where IgE secretion is affected but IgG1 is not has also been reported in a study of the effects of cell density in B cell *in vitro* cultures [192]. In this study it was shown that higher B cell densities resulted in reduced IgE secretion but had no effect on IgG1. In addition, high B cell density did not affect germline transcription or expression of mature IgE transcript on the cell surface, indicating that there was no defect in class-switching events or the production of IgE in the cell. High B cell density was shown to affect proliferation and increase apoptosis, which altered the terminal differentiation of IgE-committed B cells, resulting in lower IgE secretion [192].

In our studies, we were able to find an optimal cell density for WT B cells in culture of 250,000 cells/well, while seeding at 125,000 or 500,000 cells per well resulted in lower IgE production (**Figure 28.**). Unlike WT B cells, we did not observe the same trend in H1R/H2R DKO B cells. Rather, all seeding densities resulted in IgE secretion that were significantly lower compared to WT, and none of the densities tested in DKO cells was superior for the production of IgE (**Figure 28.**). Given the similarities to these results, and those from the study on cell density, we next characterized cell survival, proliferation, and apoptosis in our cultured cells. To determine whether we were observing lower levels of IgE due to cell survival we counted total cells at the end of culture and proliferation. We found that cultures of H1R/H2R DKO B cells had reduced numbers of cells at the end of the culture period compared to WT cells (**Figure 29.**). It was possible that there were lower numbers at the end of culture do to defective proliferation, therefore we next investigated whether there was a defect in proliferation in H1R/H2R DKO cultures. We used CFSE staining and flow cytometry to assess proliferation after 4-day culture. B cell differentiation in culture occurs in a cell-by-cell independent way, meaning not all B cells in culture differentiate to become IgE^+ cells. Therefore, we also performed surface staining of IgM, B220 and CD138 in addition to CFSE to fully characterize proliferation of different populations. Ultimately, there was no difference in the proliferation of B cells in H1R/H2R DKO and WT cultures (**Figure 30.**). Similarly, we did not observe increased apoptosis or necroptosis in H1R/H2R DKO cultures compared to WT. **(Data not shown)** Collectively, these *in vitro* data indicate that there is an intrinsic defect in B cells that lack histamine receptor 1 and 2 in their ability to secrete IgE. This defect is not attributed to a defect in class-switching events, proliferation, intracellular antibody production, or apoptosis. However, we did observe fewer cells at the end of H1R/H2R DKO culture compared to WT.

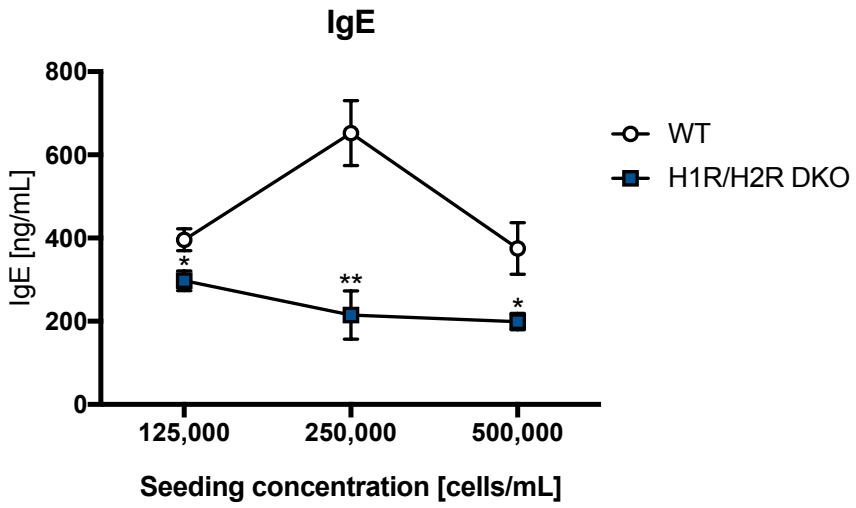


Figure 28: Optimal seeding concentration phenotype was absent in H1R/H2R DKO B cells

Optimal seeding density was found with WT B cells at a concentration 250,000 cells/mL while H1R/H2R DKO B cells did not have an optimal seeding density at either of the three concentrations. $B220^{+}$ cells were purified from the spleen and cultured with rIL-4 and αCD40 for 4 days at concentrations 125,000 cells/mL, 250,000 cells/mL, and 500,000 cells/mL. Supernatants were collected and IgG1 and IgE were quantified by ELISA. Data are representative of the mean $\pm$ SEM of (n = 6 from 2 individual experiments). *p < 0.01,**p < 0.001 by Student's T test.

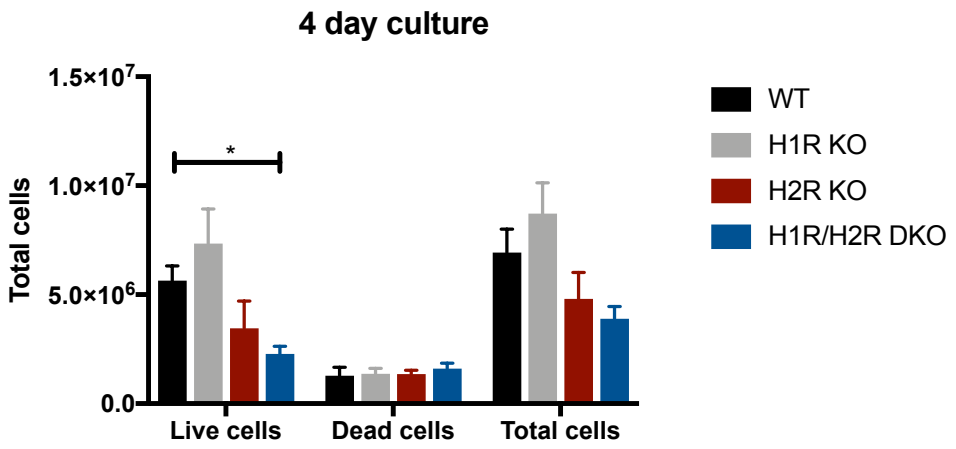


Figure 29: There was a defect in B cell survival at the end of culture in the absence of H1R and H2R

There were fewer cells in H1R/H2R DKO B cell culture at day 4 compared to WT B cell culture. $B220^+$ cells were purified from the spleen and cultured with rIL-4 and αCD40 for 4 days. Cells were stained with trypan blue and counted. Data are representative of the mean ± SEM (n = 3). *p < 0.01 by Student's T test.

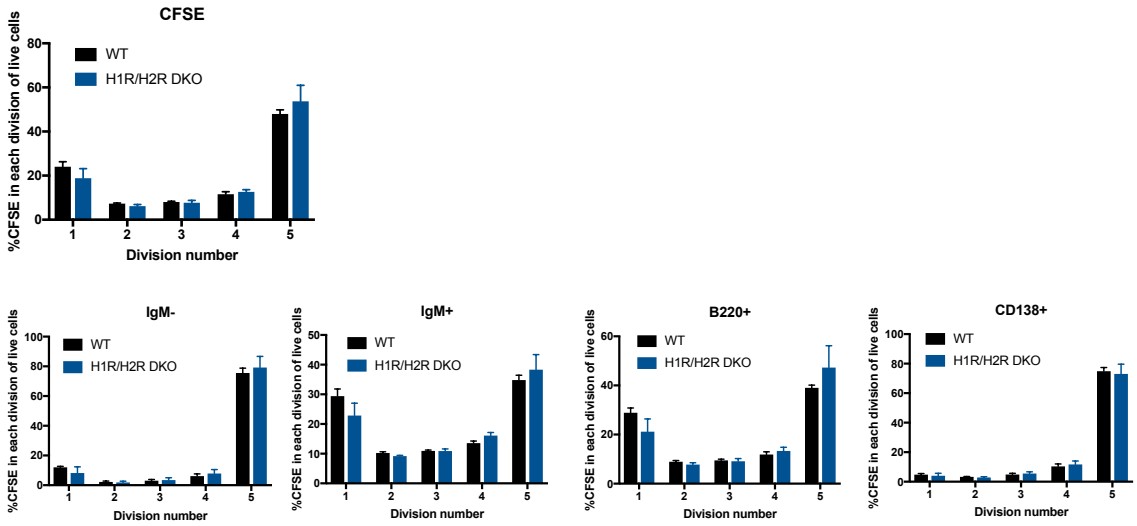


Figure 30: There was no defect in cell proliferation of B cells in the absence of H1R and H2R *in vitro*

There was no difference in cell proliferation of H1R/H2R DKO B cell cultures compared to WT B cell cultures. $B220^{+}$ were stained with CFSE and cultured with rIL-4 and αCD40 for 6 days. Cell were collected daily for 6 days and fluorescently labeled to identify IgM^{+}, IgM^{neg}, $B220^{+}$, and $CD138^{+}$. Data are representative of the mean $\pm$ SEM of (n = 3-4).

There is a defect in GC B cell formation in the absence of histamine receptors 1 and 2

Given all the data, it is clear that histamine affects IgE secretion through H1R and H2R. However, it not clear which pathways of B cell signaling are affected by histamine signaling. Therefore, we performed an RNA sequencing experiment on $CD138^+$ cells isolated from culture. To fully characterize how the absence of histamine receptors affects plasma cells we isolated $CD138^+$ cells from WT, H1R KO, H2R KO, and H1R/H2R DKO B cells by FACs (**Figure 31.**)**.** Analysis of the RNA-seq data revealed that compared to WT, H1R KO B cells had 6 differentially expressed genes (**Figure 32. A**), H2R B cells had 11 differentially expressed genes (**Figure 32. B**), and H1R/H2R DKO B cells 17 differentially expressed genes (**Figure 32. C**). There was only one gene that came up in all KO B cells as differentially expressed, SPIB, a gene that is expressed in B cells up until GC status and is later downregulated in ASC by BLIMP1. There was only one other gene whose expression was similar between H2R KO and H1R/H2R DKO B cells, PLAC8, which has not been studied previously in B cells. These data show that even though H2R KO mice demonstrated a similar antibody phenotype to H1R/H2R DKO mice *in vivo,* there is a completely different gene expression signature in plasma cells, and this could account for H1R/H2R DKO mice having a greater antibody defect. We further validated expression of some of the identified genes in purified WT and H1R/H2R DKO $CD138^+$ cells by qPCR. We were able to verify altered expression of two genes (**Figure 33.**), PLXND1 and PLAC8. Although SPIB came up in all KO mice, we were unable to confirm its altered expression by qPCR (data not shown).

PLXND1 and PLAC8 have been reported to be involved in GC formation, which is a critical step in B cell activation that results in the development of antibody-secreting plasma cells and memory B cells. Similar to H1R/H2R DKO mice, PLXND1 KO mice did not have defects in B cell maturation or development, but instead had a defect in production of IgG1 and IgG2b in a model of immunization with NP-CGG. The defect in antibody production in these animals was attributed to defects in GC reactions, where fewer GC B cells were present in the spleens of NP-CGG immunized mice compared to WT. Additionally, PLXND1 was shown to regulate GC B cell migration to chemokines CXCL12, CXCL13, and CCL19 [193]. Similarly, it has been reported that PLAC8 was upregulated in animals with defects in GC formation, although the mechanisms by which PLAC8 may be regulating GC formation were not clear [66].

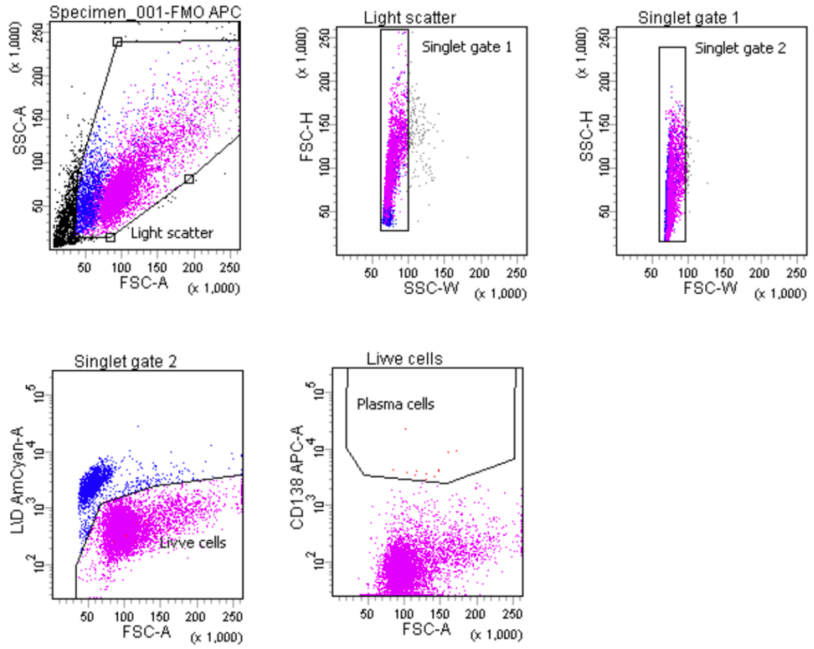


Figure 31: RNAseq gating strategy for isolating CD138^{+} cells

Cells from B cell cultures of H1R KO, H2R KO, and H1R/H2R DKO were gated based on side-scatter and forward-scatter to eliminate debris and doublets. We further gated out dead cells and used a fluorescent minus one (FMO) to appropriately set gate for CD138^{+} plasma cells.

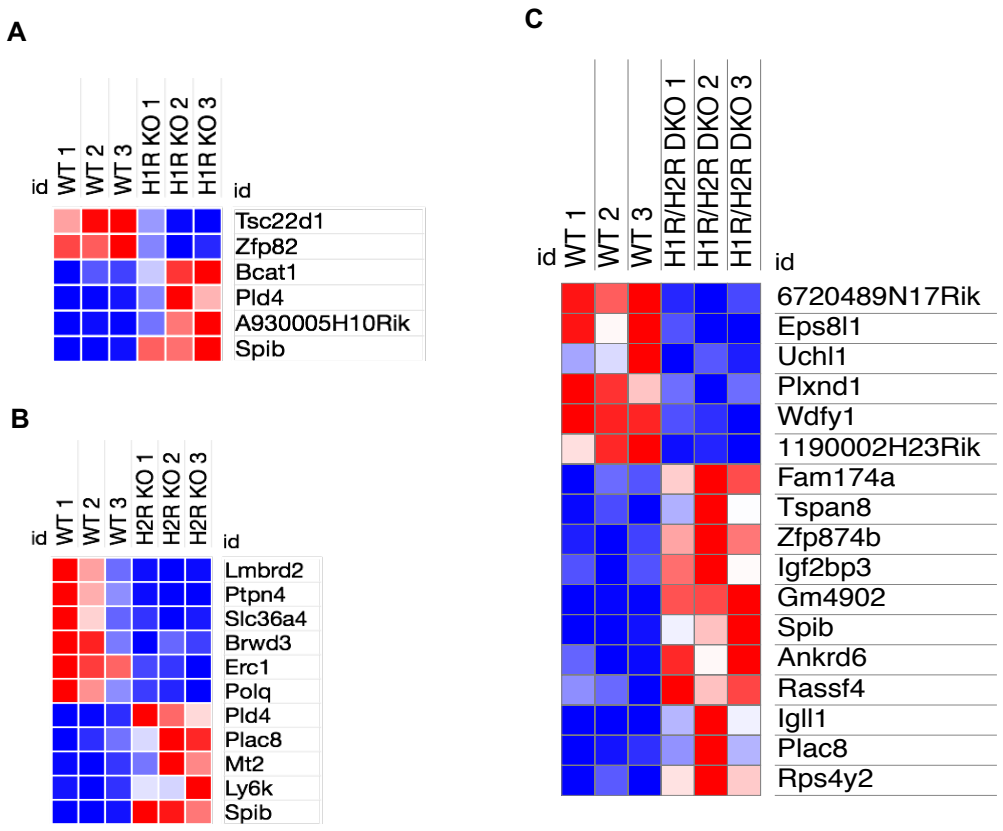


Figure 32: There were distinct signatures of differentially expressed genes in the absence of H1R, H2R, or both H1R and H2R compared to WT CD138^{+} cells

Heat maps of significantly differentiated genes between WT and **(A)** H1R KO **(B)** H2R KO, and **(C)** H1R/H2R DKO plasma cells (CD138^{+}).

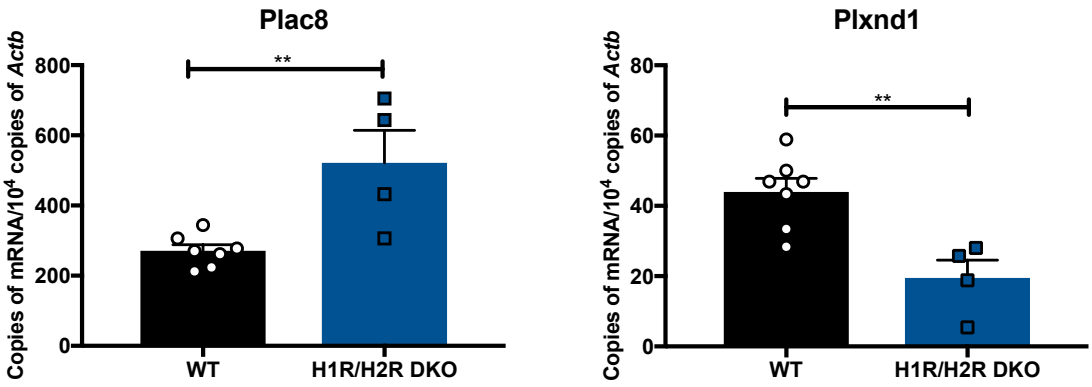


Figure 33: CD138$^+$ cells from H1R/H2R DKO mice have altered expression of genes important for germinal center biology

Plac8 and Plxnd1 were significantly differentially expressed in H1R/H2R DKO plasma cells compared to WT plasma cells. B220$^+$ cells from WT, H1R KO, H2R KO, and H1R/H2R DKO mice were purified from the spleen and cultured with rIL-4 and αCD40 for 4 days. CD138$^+$ cells were FACs sorted and mRNA was isolated to quantify expression by RT PCR. Data are representative of the mean $\pm$ SEM (n = 4-7). **$p < 0.001$ by Student's T test.

Altogether, our data demonstrate that there is differential expression of PLXND1 and PLAC8 in H1R/H2R DKO B cells compared to WT and suggest that histamine may play a role in the ability of B cells to undergo activation and maturation in GCs. Therefore, we next wanted to determine whether there were any defects in GC formation *in vivo* in histamine receptor KO animals that may explain the defect in antibody production. Using flow cytometry, we observed significantly lower frequencies of GC B cells in the spleen after OVA-alum immunization in H1R/H2R DKO mice compared to WT ($p < 0.5$; **Figure 34. A)**.

We previously showed that histamine receptors do not affect the induction Th2 responses, however, due to the reduced numbers of GC in DKO animals, we wanted to characterize the effects of histamine on development of Tfh cells. As discussed above, Tfh cells play an important role in the development of the GC response, and they have also been shown to play a key role in the induction of IgE-expressing B cells [161]. Moreover, $CD4^+$ T cells are known to express histamine receptors 1 and 2 [51], thus it is likely that Tfh cells can also directly respond to histamine. We performed flow cytometry analysis of the spleen and observed no significant differences in the frequency of $CD4^+$ T cells in OVA immunized H1R/H2R DKO mice compared to WT. However, there was a trend towards decreased frequencies of Tfh cells in H1R/H2R DKO mice compared to WT mice after ova-alum immunization compared to WT (**Figure 34. B**). To determine whether there was a defect in plasma cells in the spleen, we also analyzed $CD138^+$ cells in the spleen by flow cytometry. We found no significant differences in the frequency of splenic plasma cells in H1R/H2R DKO mice compared to WT (**Figure 34. C**). From these data, we are able to conclude that there is dysregulation of GC formation in the absence of histamine signals that may be in part due to effects on Tfh cells and GC B cells.

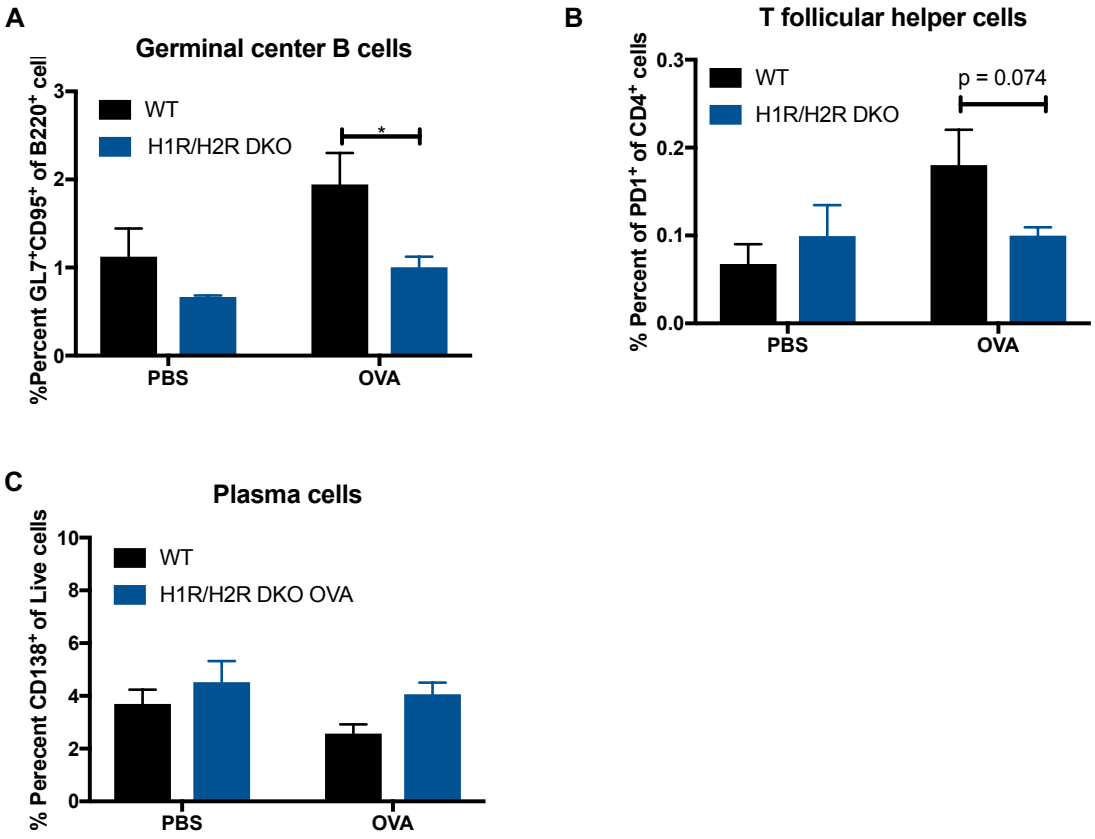


Figure 34: H1R and H2R were necessary for the development of GC B cells

There are fewer GC B cells in the spleen of H1R/H2R DKO mice compared to WT mice. Spleens from OVA-alum immunized mice were isolated, homogenized, RBC lysed and fluorescently labeled for **(A)** GC B cells ($B220^+GL7^+CD95^+$) **(B)** T follicular helper cells ($CD4^+$ $PD1^+$) and **(C)** plasma cells ($B220^+$ $CD138^+$) Data are representative of the mean ± SEM (n = 4-10 from 2 individual experiments). p = 0.074, *$p < 0.01$ by Student's T test.

Loss of H1R and H2R results in intrinsic defects in both T cells and B cells in vivo

All of our preceding *in vivo* experiments have been conducted using global histamine receptor KO mice. To determine whether there was an intrinsic defect in B cell and/or T cell function *in vivo* in the absence of histamine receptors, we next used models of cellular reconstitution in Rag1 KO animals, which lack T and B cells, to specifically determine the role of histamine receptors 1 and 2 on B cells and T cells [194]. We reconstituted mice with varying combinations of WT or H1R/H2R DKO T and B cells for 8 weeks, then sensitized with OVA-alum and challenged with OVA (**Figure 35.**). Importantly, cellular reconstitution of WT and H1R/H2R DKO T and B cells was similar in these experiments **(data not shown).** As expected, mice reconstituted with H1R/H2R DKO T cells and H1R/H2R DKO B cells had significantly lower total and OVA-specific IgE compared to mice reconstituted with WT T and B cells ($p < 0.001$, $p < 0.01$; **Figure 36. A, B)**. Total IgE was also significantly lower in Rag1 KO mice reconstituted with WT T cells and H1R/H2R DKO B cells compared to mice reconstituted with WT B and T cells($p < 0.01$; **Figure 36. B**), indicating that there was an intrinsic defect in B cells lacking H1R and H2R that resulted in reduced production of total IgE. Interestingly, OVA-specific IgE levels were similar between animals reconstituted with WT T cells and H1R/H2R DKO B cells compared to those reconstituted with WT T and B cells (**Figure 36. B**), suggesting that histamine may support the production of antigen-specific IgE through its functions on T cells. Mice that were reconstituted with H1R/H2R DKO T cells and WT B cells had similar levels of total and OVA-specific IgE compared to animals reconstituted with WT T and B cells (**Figure 35. B**), indicating that histamine acts directly on B cells to induce IgE production *in vivo*. Finally, there were no significant differences between any of the experimental groups for total or OVA-specific IgG1 levels.

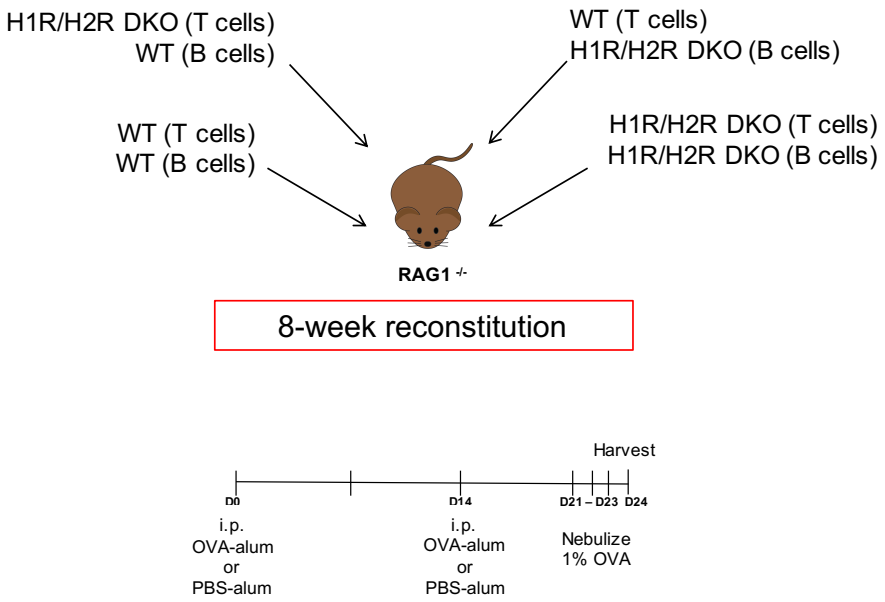


Figure 35: Experimental schematic of Rag1 KO T and B cell reconstitution

Rag1 KO ($Rag1^{-/-}$) Mice were reconstituted with different combinations of T and B cells from WT and H1R/H2R DKO mice. 10^7 T and B cells were administered into mice by i.v. injection. After 8 weeks mice were immunized with OVA-alum.

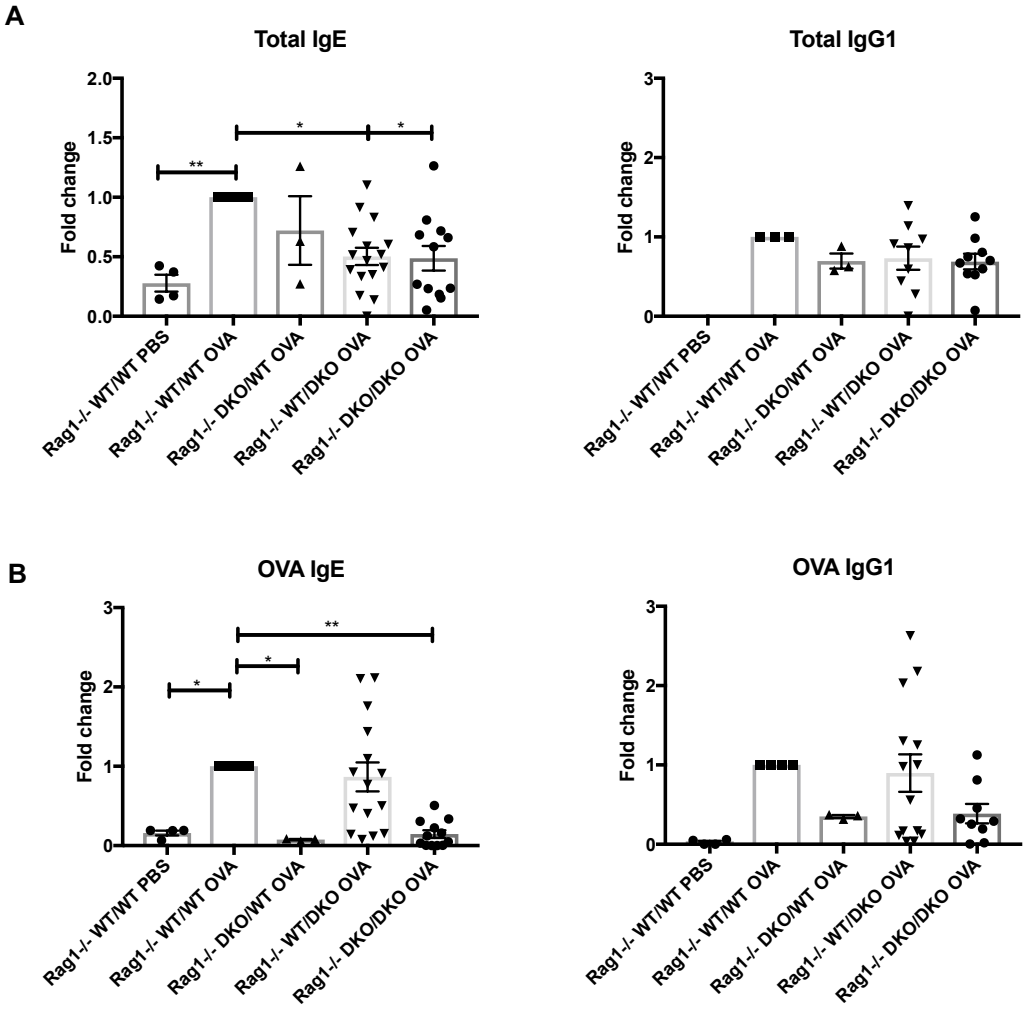
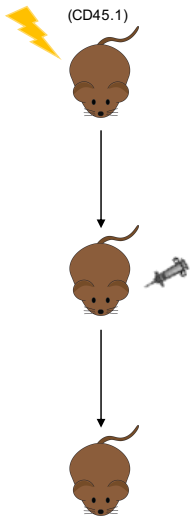


Figure 36: There was an intrinsic defect in T and B cells that lack H1R and H2R that resulted in decreased IgE production of mice

Rag1 KO ($Rag1^{-/-}$) mice reconstituted with H1R/H2R DKO B and T cells produced significantly less IgE compared to $Rag1^{-/-}$ mice reconstituted with WT B and T cells. Serum was collected after OVA-alum immunization to quantify **(A)** Total antibody **(B)** OVA antibody. DKO = H1R/H2R DKO. Data are representative of the mean ± SEM. $Rag1^{-/-}$ WT/WT OVA values were averaged from each experiment to calculate fold change of remaining experimental groups. (n = 4-15 from 5 individual experiments). *p < 0.01, **p < 0.001 by One-way ANOVA.

To further verify these results, we generated mixed bone marrow chimeras using μMT mice, which lack B cells but have normal T cells, Rag1KO mice, and H1R/H2R DKO mice. Our recipient mice were congenic CD45.1 mice and our donor bone marrow mixes were CD45.2, which served to verify the levels of chimerism. We lethally irradiated donor mice and reconstituted chimeras by retro orbital injection. 12 weeks following reconstitution we immunized mice with OVA (**Figure 37.**). Reconstitution was successful for all experimental groups, and all hematopoietic cells were from the donor cells (**data not shown**). Additionally, the frequencies of B cells and T cells were similar between the different experimental groups (**Figure 38.**). Quantification of antibodies demonstrated that mice reconstituted with mixed bone marrows from Rag1KO : H1R/H2R DKO (H1R/H2R DKO T and B cells) had significantly less total IgE compared to the control group reconstituted with mixed bone marrows from Rag1KO : WT ($p < 0.1$; **Figure 39**)**.** These results support our findings in the Rag1KO reconstitution experiments. Interestingly however, there was no significant differences in total antibody production between mice reconstituted with mixed bone marrows from μMT : H1R/H2R DKO (H1R/H2R DKO B cells) and control mixed bone marrows from μMT : WT (WT control) (**Figure 39**). These data indicate that histamine receptors on both T and B cells are necessary for the production of total IgE. Analysis of OVA specific IgE antibodies demonstrated that there was a significant different between animals reconstituted with mixed bone marrows from Rag1 KO : H1R/H2R DKO and μMT : H1R/H2R DKO, but not between their appropriate control groups. Finally, as we saw with Rag1KO reconstitutions, there were no differences in serum antibody levels of total IgG1 and OVA specific IgG1. Altogether these experiments verified that histamine signaling in both T and B cells is necessary for optimal total IgE production *in vivo*. Moreover, both experiments demonstrated a significant decrease in OVA-specific IgE in mice reconstituted with H1R/H2R DKO B and T cells compared to mice reconstituted with WT T cells and H1R/H2R DKO B cells, indicating that histamine acts directly on B cells to promote IgE production.



CD45.2	Rag1 KO (80)	μMT (80)	WT (20)	H1R/H2R DKO (20)
H1R/H2R DKO T and B cells				
H1R/H2R DKO B cells				
WT (Rag1 KO control)				
WT (uMT control)				

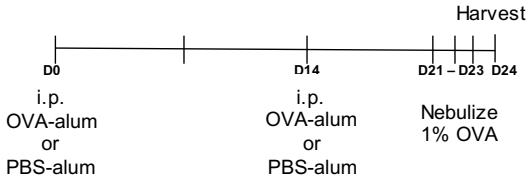


Figure 37: Experimental schematic of mixed bone marrow chimeras

CD45.1 mice were lethally irradiated and reconstituted with bone marrow from CD45.2 mice at a chimera of 80:20 ratio as indicated above. Each row represents an experimental group; there were four experimental groups: H1R/H2R DKO T and B cells, H1R/H2R DKO B cells, WT(Rag1 KO control), WT(μMT control). Each experimental group is composed of either Rag1KO or μMT and WT or H1R/H2R DKO, the combination of each group is indicated by a blue highlighted box. After 12 weeks of reconstitution mice underwent OVA-alum model.

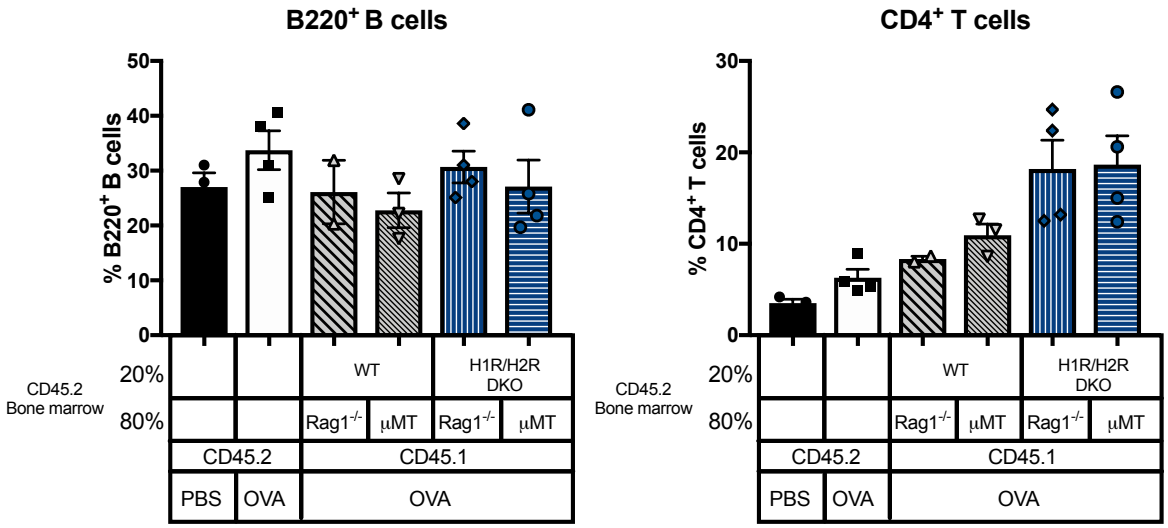


Figure 38: There were no differences in chimera reconstitutions between experimental groups of T and B cells

The spleen was homogenized, RBC lysed, and fluorescently labeled to identify B cells (B220+) and T cell (CD4+). Data are representative of the mean ± SEM (*n* = 3-4).

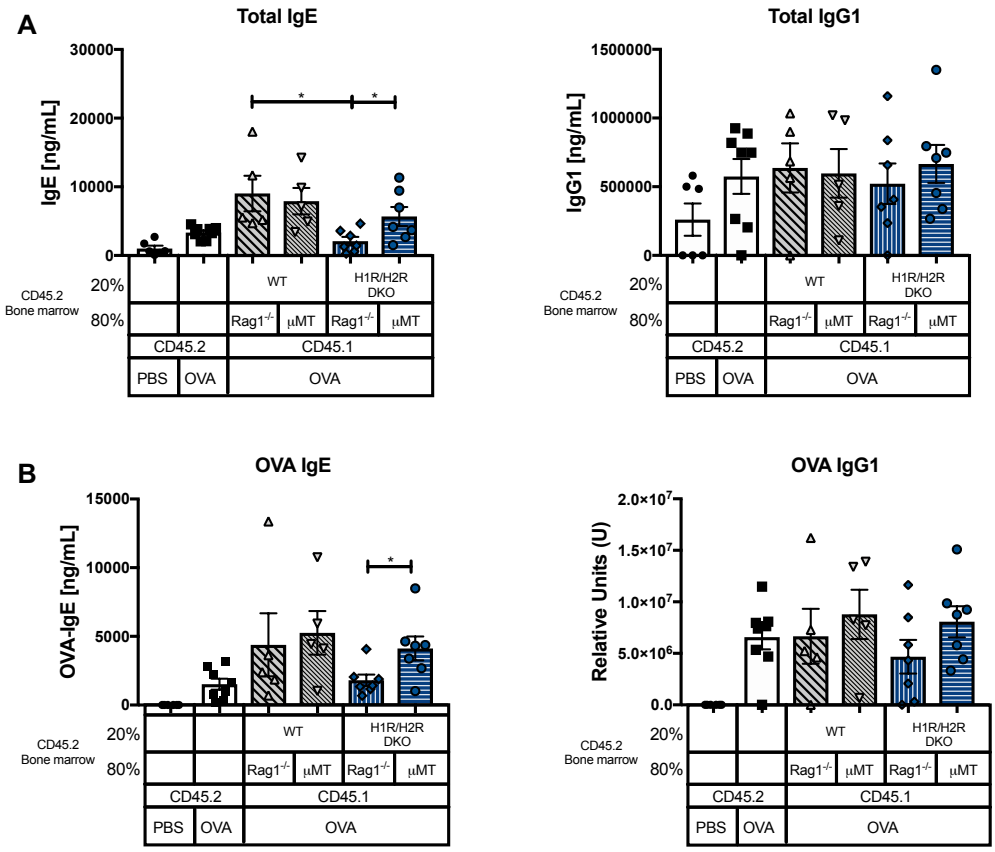


Figure 39: H1R and H2R on B and T cells were necessary for IgE production

Bone marrow chimera reconstitutions with Rag1KO and H1R/H2R DKO bone marrow demonstrated a significant decrease in Total IgE production compared to WT control group. Following immunization with OVA-alum serum was collected. The first two white bars are WT mice controls. **(A)** Total and **(B)** OVA-specific antibody was quantified by ELISA. Data are representative of the mean ± SEM (*n* = 5-8 from 2 individual experiments). **p* < 0.05 by Student's T test.

There is a dysregulation in cellular stress response in the absence of histamine receptors 1 and 2

It is clear that histamine plays an important role in multiple cell types during the induction of IgE responses *in vivo*. However, it is not clear how the lack of histamine signals is affecting these cells or which subset within these cells. In order to more fully characterize the functional effects that result from the lack of histamine signaling, we isolated splenic Tfh cells ($CD4^+CXCR5^+$), GC B cells ($B220^+GL7^+$), and plasma cells ($CD138^+$) from PBS-alum or OVA-alum immunized WT or DKO animals for analysis by single-cell RNA-seq. Unlike bulk RNA sequencing which was conducted for analysis of *in vitro* derived $CD138^+$ plasma cells (**Figure 30.**), single cell RNA-seq allows us to characterize gene expression signatures on a cell-by-cell basis from small numbers of cells. Moreover, traditional RNAseq analysis provides insights into the average gene expression in a mixed population of cells, whereas single cell RNAseq allows us to assess the gene expression profiles of each cell in a heterogenous mix. This is particularly important for our studies because we know that histamine receptors 1 and 2 are differentially expressed on different subsets of B cells that could have distinct expression signatures, which would be lost in a bulk RNAseq analysis from all $CD138^+$, or $GL7^+$, or $CD4^+$ $CXCR5^+$ cells.

Gene expression differences were compared between the same cell types for WT vs DKO in the PBS group to assess baseline differences in expression, or in the OVA group to assess differences after induction of an immune response. Importantly, cells from different experimental groups separated into similar clusters, indicating that all groups have the same cell subsets present (**Figure 40**). We found that in WT PBS vs DKO PBS groups significantly differentiated genes included Fcer1g, Pgls, and Uba52 (**Figure 41.**). We also observed significant differential expression of genes in the OVA groups, including Il4i1, Tnfrs17, and Erdr1 (**Figure 42.**) Overall, these preliminary data suggest a general signature of dysregulation of cellular stress response genes in cells from DKO animals, both at baseline and after immunization. Further studies are needed to fully characterize what these gene signatures mean in terms of IgE antibody production.

Histamine receptors are necessary for local IgE responses

Lastly, we wanted to assess the role histamine in local IgE responses. Studies have shown that in allergic airway inflammation, the induction of IgE occurs in draining lymph nodes [195]. We performed the OVA immunization model using H1R/H2R DKO and WT and compared the systemic response, in the spleen, to the local response, in the draining lymph nodes (mediastinal). As shown above, we did not find any differences in the frequencies of plasma cells in the spleen between H1R/H2R DKO and WT OVA immunized mice, and we also did not find any differences in the frequency of IgE+ cells. In mediastinal lymph nodes, we also found no differences in the frequency of plasma cells however, there was a significant decrease in IgE^+ cells in H1R/H2R DKO mice compared WT mice ($p = 0.0049$; **Figure 43. A)**.

We did not see any significant differences between $IgG1^+$ cells in the H1R/H2R DKO mice compared to WT (**Figure 43. B**). We also performed ELIspot assays using cells isolated from the spleen or mediastinal lymph nodes to quantify the frequencies of IgE-secreting cells. We plated the same number of lymph node cells and splenocytes in the presence of anti-CD40 and IL-4, and we were able to quantify significantly fewer IgE secreting cells in H1R/H2R DKO lymph nodes compared to WT ($p < 0.001$; **Figure 43. C**). Together, these data suggest that defects in IgE production in the absence of histamine signals may be localized to sites of inflammation, and further studies are needed to more fully characterize the histamine-dependent mechanisms that promote local production of IgE.

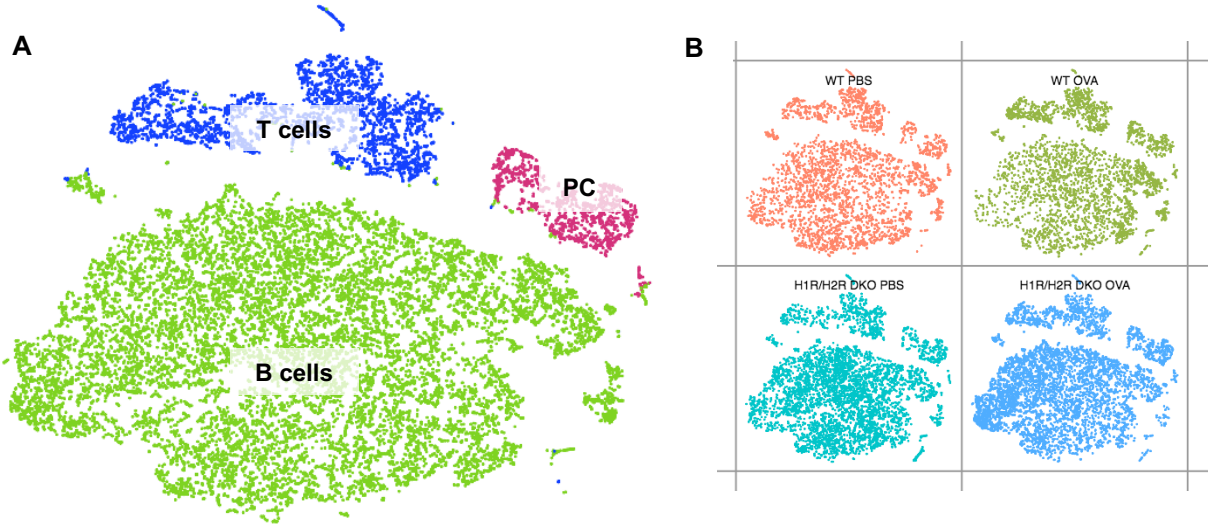


Figure 40: Clustering of GC B cells, Tfh, and plasma cells from PBS or OVA-treated WT H1R/H2R DKO mice

Single cell RNAseq data were clustered based on differential gene expression using the 10X Genomics Loupe Browser. tSNE dimensionality reduction was used to generate the clusters, and each dot represents 1 cell. **(A)** tSNE plot of cell clusters from all samples pooled together. (**B)** tSNE plots of cells from each experimental group.

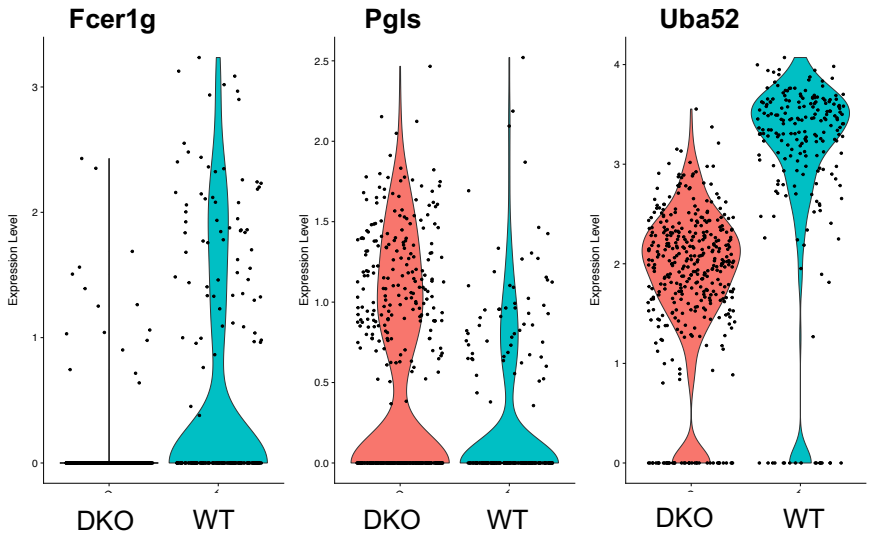


Figure 41: There were distinct gene expression signatures at baseline between WT and DKO PCs

Analysis was conducted using Seurat package in R. Gene expression was compared between PCs from WT and DKO PBS-treated animals. Violin plots display the distribution of gene expression levels for each cell. Fcer1g and Uba52 were significantly upregulated, while Pgls was significantly downregulated, in PCs from WT animals compared to DKO (adjusted $p < 10^{-6}$ for each). Differentially expressed genes were identified using a Wilcoxon Rank sum test, and p values were adjusted for multiple comparisons using a bonferroni correction.

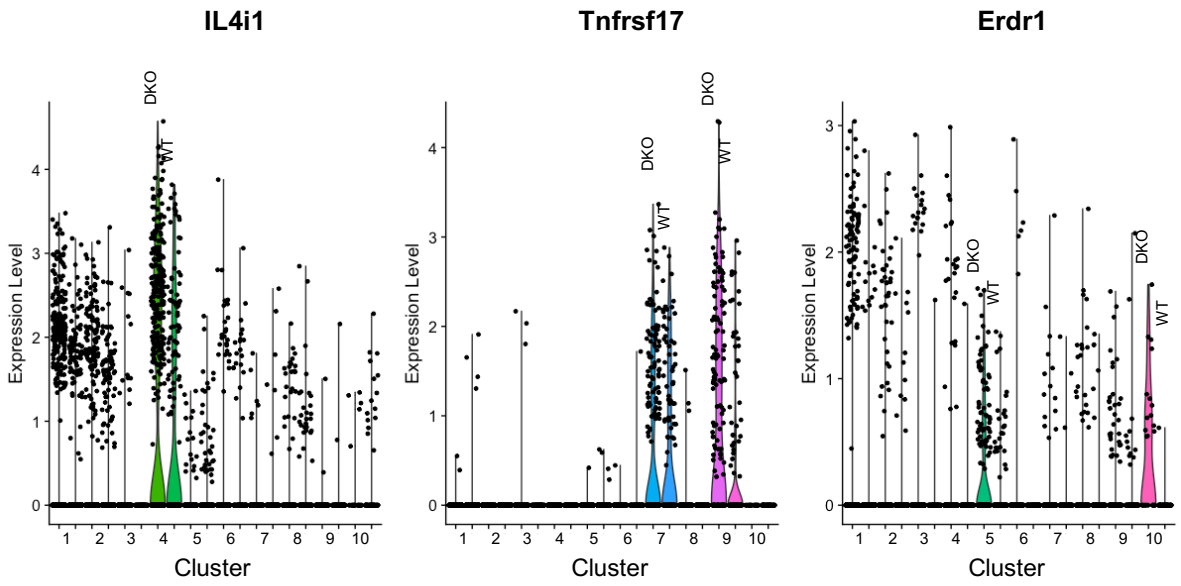


Figure 42: Distinct gene expression patterns in B cells from WT and DKO mice after OVA immunization

Analysis was conducted using Seurat package in R. Gene expression was compared between WT OVA and DKO OVA B cells. Violin plots display the gene expression level distribution of differentially expressed genes (adjusted $p < 10^{-6}$ for each). Differentially expressed genes were identified using a Wilcoxon Rank sum test, and p values were adjusted for multiple comparisons using a bonferroni correction.

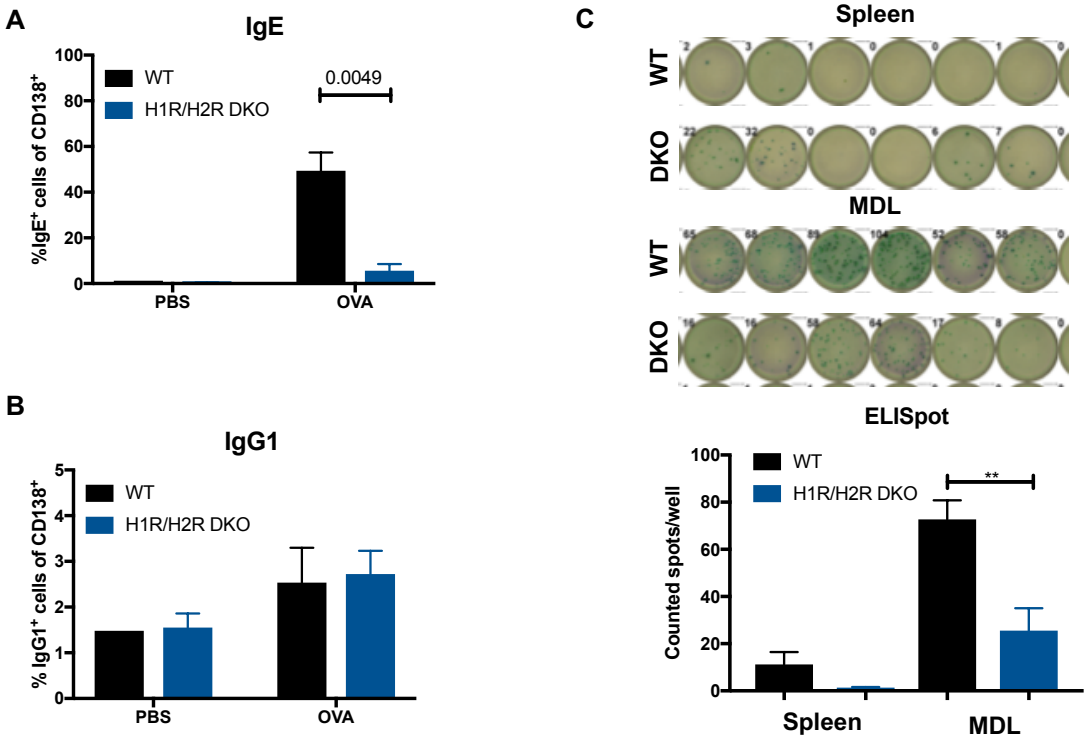


Figure 43: IgE$^+$ cells were elevated in the draining LN and are regulated by histamine through H1R and H2R

Following immunization with OVA-alum the draining LN (mediastinal lymph nodes) were collect and prepared for fluorescently labeling of the markers CD138, IgE, and IgG1 to quantify **(A)** IgE and **(B)** IgG1 ASC. **(C)** lymphoid cells were plated at 10^6 cells/well in the presence of rIL-10 and anti-CD40. Data are representative of the mean ± SEM (*n* = 2-4). ***p* = 0.049 by Student's T test.

DISCUSSION

In this chapter we showed for the first time the complex roles that histamine plays in antibody production through H1R and H2R on T cells and B cells. We found that a lack of histamine signals had no effect on B cell development or maturation, nor did it alter Th2 skewing. But, it did lead to significant decreases in IgE production, and altered the formation GCs. Thus far, histamine has primarily been known as an effector mediator in the allergic response, but our data demonstrate that histamine also has roles in antibody production, predominantly of IgE. This is an important finding since there is much that is unknown about how IgE is sustained or initiated in IgE-mediated allergic disease. In addition to providing insight on the cellular processes that are affected by histamine through H1R and H2R, our data also spotlights important molecular regulation of histamine receptors that are not currently appreciated in allergy.

Using a classical model of allergic airway hypersensitivity, we show that there were significant decreases in total and antigen-specific IgE production in the absence of H2R but not H1R. Interestingly this phenotype was even stronger when both H2R and H1R were absent. Although the majority of our studies focused on determining the role of both receptors in antibody production, it is important to note this initial finding. In previous studies using this model in H1R KO mice, antibody levels were either similar to WT or greater, specifically for IgE or IgG1 [52,54]. In the current study, we did not observe any significant differences in antibody levels in the OVA model, but we did see enhanced levels of natural IgG1 in H1R KO mice. These findings combined would suggest that the absence of H1R should either have no effect, or neutralize the H2R phenotype. But instead, we found an even stronger phenotype of decreased antibody production than the H2R KO. A potential explanation for this phenotype stems from GPCR biology. Studies have shown that H1R and H2R interfere with activity of other GPCRs and even each other, since they are typically co-expressed [196,197]. Specifically, it has been shown that over stimulation of both these receptors results in desensitization. Although, this specific mechanism may not explain what is going on in our model. Instead, it is possible that other signaling pathways are altered in the absence of each individual receptor. GPCRs, including H1R and H2R, have been shown to experience ligand bias, where the state of a GPCR alters the binding of ligand leading to induction of differential signaling pathways. This phenomenon was potentially seen in our RNAseq experiment, where

there was little over-lap in differential expression of genes between H1R KO, H2R KO, and H1R/H2R DKO $CD138^{+}$ plasma cells. This finding is even more supported by our data showing differential expression of H1R and H2R on mature B cell subsets, where H2R was more highly expressed in both murine and human B cells cell subsets. This may also explain why the absence of H2R had a profound effect on the production of antigen-specific antibodies in our model.

Our data also highlights the importance of investigating both local and systemic antibody responses. We found that the reduction in the frequency of IgE-secreting cells in the absence of histamine signals was much more pronounced in plasma cells from the lung-draining LN compared to plasma cells from the spleen. This suggests that the effects of histamine may be more important, or perhaps more potent, at local sites of inflammation, where mast cells are present and may be secreting large amounts of histamine into the local inflammatory environment. Consequently there is evidence of mast presence in local inflammatory locations such as the nasal polyps, sinus mucosa, and nasal turbinate mucosa [198,199]. It is important note that mast cell expansion has also been observed in the spleen, specifically in a model of murine food allergy, and has been shown to be important in the pathology of disease [200,201]. Collectively there is strong potential for mast cells as an important source of histamine during the establishment of GC reactions both systemically and locally.

Another significant finding was the antibody response in H1R/H2R DKO mice in the NP-CGG/alum model. We showed that total IgE was decreased in H1R/H2R DKO mice compared to WT. In this model there is only one injection of NP-CGG/alum, which indicates that the histamine that is regulating the IgE response must be coming from an IgE-independent mechanism that would include cells that can respond to alum. It would be interesting to determine if alum could initiate histamine release either directly or indirectly. The role for histamine in sensitization and sustainability of IgE could be studied in longer models of allergic disease that include allergen challenges.

In our OVA alum model, we also saw a defect in antigen-specific antibody production of different isotypes, indicating that histamine may be regulating global antibody production that is not specific to Th2 responses. Although, it was clear that IgE was the most affected isotype. Nonetheless, we used a Th1 model of immunization with NP-CGG and CFA. CFA contains inactivated *Mycobacterium tuberculosis*, a bacteria that has a cell wall characteristic of both Gram-positive and Gram-negative bacteria. Based on

genome tree analysis *M. tuberculosis* is genetically more Gram-negative [202]. These features are important since the immune response varies based on the type of bacteria [203]. Using this model, we saw significant decreases in NP-specific IgM. Interestingly however, a study on murine infection by *Yersinia enterocolitica*, a Gram-negative bacillus-shaped bacterium, showed that HDC was significantly upregulated upon infection. And interestingly, when H2R antagonists were given, there was a decrease in bacterial clearance [204]. However, whether this was due to antibody responses was not addressed in this work.

Finally, our data suggest that histamine may be promoting antibody production through the regulation of cellular stress processes. Our single cell RNA-seq data indicated that DKO B cells had decreased expression of molecules associated with regulation of cellular stress, and this may contribute to their decreased ability to secrete antibodies. Upon the production of antibody, plasma cells produce vast amounts of antibody proteins, and this leads to the development of cellular stress. The most well-characterized effect of this is the upregulation of the unfolded protein response, which helps the cell deal with the large amounts of cytoplasmic protein being produced [146,153]. One gene that was consistently upregulated in WT B cells was Uba52, which is one of 4 mammalian ubiquitin genes [205]. Ubiquitin plays a key role in tagging proteins that are targeted for degradation, and thus, the decreased expression of this gene may result in a block in protein degradation in DKO cells and increased cellular stress. Future studies are needed to verify the gene expression changes identified in our single cell-RNA seq experiments, and to determine whether control of cellular stress responses is important for proper antibody secretion.

Altogether, these data provide novel insights into an uncharacterized role for histamine in the induction of antibody responses, especially IgE responses. It is clear that histamine plays a complex role in both T cells and B cells to promote antibody production, and it may function to regulate cellular stress processes that are important for the survival of plasma cells. Future studies aimed at elucidating the specific histamine-dependent mechanisms that regulate these responses will be critical for advancing our understanding of this potent inflammatory mediator, and may highlight pathways that could be targeted therapeutically for the treatment of allergic disease.

Chapter 5: Overall Summary and Conclusion

Histamine was discovered over a century ago, and the earliest studies demonstrated that it could mediate the physiological manifestations of anaphylaxis [206]. Since then, scientific advances have continuously unveiled new mechanism regulated by histamine in the allergic response. In our work we have demonstrated important new facets of histamine biology. We described the potential for a histamine metabolite, IAA, to sustain histamine responses long after its metabolism. This is a key finding that has grand implications for understanding why the anaphylactic response are so robust. Second, we demonstrated a new role for histamine in allergic responses in the production of IgE, which provides a means for sustaining IgE and therefore chronic disease.

FUTURE DIRECTIONS

Characterization of IAA

Given the potential for IAA to augment histamine-driven responses, it is important to fully characterize its kinetics. Thus far, there is one frequently referenced model on the percentages of different histamine metabolites that arise through the course of histamine metabolism; it has been shown that roughly 42-47% is *N*-Methyl Imidazole Acetic acid, 4-8% is *N*-Methyl histamine, 2-3% remains histamine, 9-11% is imidazole acetic acid, and 16-23% is imidazole acetic acid riboside. These percentages were derived mainly from an early study where [^{14}C] histamine was administered by i.d injection and metabolites were monitored 12 hours later [57]. This model demonstrates that a small percentage of IAA is present, however, IAA riboside may be the true active metabolite. In, studies not shown, we were not able to induce histamine release from mast cells upon IAA stimulation *in vitro*. We attributed this to using a non-ribosylated form of IAA since, the only study that has shown function of IAA *in vitro* used the ribosylated form [62]. In our animal studies we also used the non-ribosylated form of IAA but we were able to get responses *in vivo*. It is possible that IAA gets ribosylated in the animals upon administration since in the normal course of histamine metabolism IAA gets ribosylated after it is derived. More importantly the current model indicates that there is a higher percentage of ribosylated IAA compared to IAA.

To move forward from our studies, characterization of histamine metabolite content in urine of anaphylaxis patients could provide insight on whether there are varying levels of IAA that may correlate with severity of anaphylaxis. These studies could potentially also be conducted in mice to determine when IAA levels peak. More importantly, it is necessary to determine the receptor for IAA. Our preliminary studies on the Nischarin KO mice suggest that Nischarin is not the receptor mediating the effects of IAA in our model. However, it is also possible that Nischarin is expressed in different sub-cellular locations, since It has also been shown to as a cytosolic scaffolding protein [123]. We verified by qPCR that Nischarin was expressed in human and murine mast cells, however based on online databases, Nischarin is shown to be expressed by most cells in mice and humans (http://biogps.org/#goto=genereport&id=64652) (http://biogps.org/#goto=genereport&id=11188). The cytosolic form of Nischarin has specific roles in metabolism and binding to integrin α5 to inhibit cell migration [123,124], which may impact the manifestation of anaphylaxis. It would be interesting to determine whether cell-specific KO of Nischarin, such as in mast cells, would result in ameliorated IAA responses.

Sources of histamine

Our studies in Chapter 4 did not characterize the specific sources of histamine that may be contributing to antibody production. In addition, in Chapter 3, we mainly focused on the source of histamine necessary to drive IAA-induced anaphylaxis. Therefore, it would be beneficial to fully characterize where histamine is coming from in all of these processes.

In the context of the source of histamine for the regulation of antibody production, the obvious candidates are mast cells and basophils since they have vast amounts of histamine, and studies have shown that mast cells can activate and drive B cells to differentiate into ASC [172,173]. Mast cells would be a potential source during the elicitation of allergic responses, since there would be IgE available to mediate mast cell activation. However, during the sensitization phase of the allergic response there must be other sources of histamine. It is possible that multiple sources are important through the different phases of the allergic response. Current development of HDC cre mice may serve as a useful tool to pinpoint specific cells that are required to produce histamine and facilitate antibody production. In

addition, the expression of HDC can be monitored in the spleen and lymphoid tissues through a model of allergic sensitization and elicitation to characterize sources of histamine. Interestingly, there was also a strong indication that histamine was coming from non-canonical sources, based on our observations in Th1 antibody production. We saw that H1R and H2R were necessary for the production of WT levels of IgM. Previous studies have shown that HDC is induced upon infection of *Yersinia enterocolitica* in secondary lymphoid tissues; although antibody levels were not measured and it is not known if the upregulation of HDC was relevant in this model [204]. Nonetheless, this study suggests that histamine could regulate non-Th2 antibody responses. It would be interesting to characterize the role H1R and H2R in antibody production in other contexts, such as viral and autoimmunity.

In the context of sources of histamine in our studies on IAA, we show definitively that mast cell-derived histamine is necessary for IAA-induced anaphylaxis, which could suggest that they are the sole source of histamine in this system. However, it is also important to consider that during anaphylaxis many mast cells undergo degranulation and release their histamine contents. Therefore IAA could only act on the remaining, inactivated mast cells. Although this may be the case, it would be important to know if elicitation of anaphylaxis and mast cell activation leads to upregulation of the IAA receptor responsible for inducing histamine release on mast cells or other cells that could potentially secrete histamine. These studies could potentially be conducted in humans but would be limited to peripheral blood monocular cells (PBMC). In mice these studies are more feasible and would also allow us to determine how the receptor for IAA is regulated during anaphylaxis. In addition, it would be interesting to determine whether IAA has similar effects on other allergic responses, such as airway hyperreactivity.

Histamine and IgE in other diseases

There are non-allergic diseases that have been associated with high levels of histamine, multiple sclerosis and rheumatoid arthritis [44,45]. In addition, there are IgE has been implicated in the pathogenesis of autoimmune disease [207]. However, it is not known how histamine may be regulating antigen-specific IgE in this context. It is known that IgE can potentiate disease in patients with systemic lupus erythematosus (SLE) by inducing secretion of IFN-α from pDCs [208]. It would be interesting to determine whether histamine levels corelated with IgE or severity of disease. In addition, studies

investigating the role for the induction of histamine in autoimmune disease would provide additional insights into the mechanisms that regulate expression of this potent antibody isotype in distinct disease contexts. These studies could utilize the histamine receptor KO animals in models of autoimmune disease to assess the effects of the loss of histamine signals on antibody production and disease severity.

Histamine receptor expression and local inflammation

In our studies we were able to demonstrate that there was differential expression of histamine receptors on B cells in WT animals. However, based on our data, other cells were also important for the regulation of IgE production through histamine receptors, including T cells. It would be interesting to characterize how histamine receptor expression is regulated on different cell types throughout disease. Studies could assess the expression patterns of histamine receptors on distinct T cell subsets, including Th1, Th17, Tfh, and Treg cells, in naive and challenged animals. Changes in the expression patterns of these receptors on different T cell subsets or during disease may provide further insights into the role that histamine may play in the development of disease in specific inflammatory contexts.

Our data also demonstrated that there were more IgE^+ plasma cells in the draining LN compared to the spleen, which supports the hypothesis that local IgE production is important for development of allergic diseases. We could also hypothesize that histamine receptor expression is also different in the draining LN compared to the spleen, which would have important implications for the regulation of IgE production by histamine in the LN. It would be interesting to fully characterize the regulation of histamine receptor expression in both the draining LN and in the lung tissue itself to determine whether local histamine production is important for local IgE production. Moreover, even though we did not see H4R expression on B cells, it is possible that H4R may be regulating other processes important for B cell function. Studies using a murine model of AD showed that there was a defect in IgE production in H4R KO mice [209], suggesting that signaling through H4R is indirectly important for IgE production. Identification and characterization of the $H4R^+$ cells in the lung and draining LN would provide additional insights into the other cells that may be important for histamine-driven IgE responses.

Understanding mechanisms of resolution of allergic disease

The understanding of how mediators induced during the elicitation phase of allergic disease can affect the mechanisms that sustain disease is key. It is well known that patients with onset of allergic disease at an early age have the possibility to “out-grow” their disease. This is true with asthma and atopic dermatitis, and the mechanisms that are involved are not known. Studies from AD patients clearly show correlations between increased disease severity and subsequent development asthma and/or food allergy [210]. An important component of this is the levels of IgE, since higher IgE levels correlate with the onset of other allergic diseases. Given that histamine plays a key role in the development of IgE responses through effects on both B and T cells, it will be useful to determine how these effects can be counter-acted in order to try to prevent further production of IgE and restore immunological tolerance to specific allergens.

Management of elicitation phase of allergic disease may also play a role in controlling levels of IgE. As mentioned previously, allergic elicitation results in the release of a plethora of meditators, including histamine. Reduction in histamine availability could be an important therapeutic strategy to break the cycle of allergic inflammation and IgE production. Antihistamine are primarily used to treat minor allergic responses, such as rhinitis, but it would be interesting to investigate whether long-term use has any effect on IgE levels.

Overall conclusions

Collectively, this work has identified and characterized novel roles for histamine in allergic responses. We identified a mechanism whereby histamine can potentiate its own response through one of its metabolites and a role for histamine in regulating antibody responses. These findings highlight the non-canonical roles that histamine may play in the development of allergic disease, and may provide insights into the identification of targets for the development of improve therapeutics for the treatment of these diseases. This work also paves the way for future studies aimed at further characterizing the mechanisms that facilitate the functions of histamine and its metabolites in a wide variety of disease states and sites within the body.

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