The Effect of Chronic Helminth Infection on Type I hypersensitivity

by

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DEDICATION

To my parents,

Thank you for encouraging me to pursue my education.

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ABSTRACT

The Effect of Chronic Helminth Infection on Type I hypersensitivity:

Laura E. Kropp, Doctor of Philosophy, 2018

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IgE-mediated anaphylaxis is a life-threatening condition. Binding of IgE with its cognate antigen causes basophils and mast cells to rapidly release pre-formed inflammatory mediators. While numerous animal studies have reported that helminths can prevent allergic sensitization, only 4 studies to date have evaluated the effects of infection on pre-existing allergy. Since chronic helminth infection suppresses basophil and mast cell function, we hypothesized that chronic infection would protect against the immunological and clinical signs of anaphylaxis in previously sensitized mice. Briefly, mice were sensitized by intraperitoneal injection of either ovalbumin (OVA)/alum or PBS/alum once per week for 3 weeks. At 4 weeks, mice were infected with *Litomosoides sigmodontis* (*L.s.*), a rodent filarial parasite, or administered a mock treatment. Twelve weeks post-infection, immunological and clinical parameters were measured before and after allergic challenge with OVA. Following challenge, serum levels of murine mast cell protease-1 (mMCP-1) were significantly lower in sensitized mice that were *L.s.*-infected as compared to mock-treated. However, chronic infection with *L. sigmodontis* did not

alter serum levels of OVA-IgE compared to mock infection. With respect to clinical signs, *L.s.*-infected mice that had been previously sensitized exhibited an average drop in core body temperature of 3.5°C after 30 minutes, which was significantly less than the 5.0°C drop observed in sensitized and mock-infected mice.

Anaphylaxis was dependent on IgE-signaling, as FcεRI knockout mice did not release mMCP-1 or exhibit a drop in core body temperature in response to intraperitoneal challenge with OVA. Further, anaphylaxis in our model was largely dependent on mast cells, with mast cell-deficient mice exhibiting a significantly impaired release of mMCP-1 and decrease in core body temperature compared to wild type- or basophil-depleted mice. Analysis by flow cytometry indicated that mast cells from mice chronically infected with *L.s.* were dramatically altered compared to mock controls. More specifically, mast cells were less granular and exhibited lower baseline levels of the activation markers CD200R and CD63. Furthermore, analysis of mast cells from chronically infected mice indicated that baseline levels of histamine in *L.s.*-infected mice were significantly lower than in mock-treated mice. Analysis of mast cells by proteomics showed that additional proteins were differentially expressed in the setting of chronic infection, including inflammatory meditators. Our results indicate that chronic infection with *L.s*. dramatically impacts the baseline status of mast cells, rendering them less responsive to allergic challenge. We hypothesize that chronic activation from *L.s.* infection leads to an exhausted state for mast cells, whereby they contain lower basal levels of inflammatory mediators. Our results are the first to describe the impact of chronic *L.s.* infection on mast cells in the context of allergic disease and have important implications for translation of helminths and helminthic products into therapeutics.

TABLE OF CONTENTS

LIST OF TABLES x

LIST OF FIGURES xi

CHAPTER 1: Introduction 1

Allergy 1
Type I hypersensitivity 3
Anaphylaxis 4
Treatments for type I hypersensitivity 6
Epidemiology of type I hypersensitivity 8
Role of mast cells in type I hypersensitivity 9
T helper type 2 response 10
Helminths and allergy 15
Murine studies of allergy and helminths 16
Lymphatic filariasis 19
Litomosoides sigmodontis/ BALB/c model 20
Methods to study allergy effector cells 21
Purpose of research and proposed specific aims 27

CHAPTER 2: Materials and Methods 30

Animals 30
Model of sub-lethal anaphylaxis 30
Infection of mice with *Litomosoides sigmodontis* 31
Mast cell purification 31
Enzyme-linked immunosorbent assay 32
Cytokine assay 33
Histology 33
Electron Microscopy 34
Flow cytometry 34
Proteomics of mast cells 36
Mass spectrometry analysis of lipids 37

CHAPTER 3: Results 39

Specific aim 1: Establish and characterize a model of type I hypersensitivity 39
Specific aim 2: Determine if chronic helminth infection protects against pre-existing type-I (immediate) hypersensitivity. 52
Specific aim 3: Evaluate potential mechanisms of *L.s.* protection against anaphylaxis at 12 weeks post-infection. 64

CHAPTER 4: Discussion 94

REFERENCES 120

LIST OF TABLES

Table 1. Proteins up-regulated in Infected mast cells 87
Table 2. Proteins down-regulated in Infected mast cells 88
Table 3. Proteins unchanged between Infected and Mock mast cells 89

LIST OF FIGURES

Figure 1. Initiation and propagation of type 2 response 14
Figure 2. OVA/alum-sensitized mice undergo antigen-specific anaphylaxis in response to allergic challenge. 41
Figure 3. FcεRI is required for anaphylaxis following challenge. 44
Figure 4. FcεRI is required for mMCP-1 release following challenge. 45
Figure 5. Anaphylaxis is primarily dependent on mast cells in OVA/alum model of sensitization 49
Figure 6. OVA-IgE is present in the serum of OVA-sensitized C57BL/6 and Sash mice 50
Figure 7. Mast-cell deficient mice release less histamine and mMCP-1 following allergic challenge 51
Figure 8. Timeline for evaluating the therapeutic effect of helminths on allergy 54
Figure 9. *L. sigmodontis* protects against anaphylaxis at 12 weeks post-infection 56
Figure 10. Chronic infection with *L. sigmodontis* does not affect OVA-IgE levels, but decreases OVA-IgG1 at 12 weeks post-infection 57
Figure 11. *L. sigmodontis* impairs mast cell degranulation, but does not protect against hypothermia at 7 weeks post-infection 59
Figure 12. *L. sigmodontis* increases OVA-IgE, but decreases OVA-IgG1 at 7 weeks post-infection 60
Figure 13. *L. sigmodontis* protects against anaphylaxis at 2 weeks post-infection 62
Figure 14. *L. sigmodontis* does not significantly alter OVA-IgE at 2 weeks post-infection 63
Figure 15. IL-10 is not required for protection against anaphylaxis at 12 weeks post-infection with *L. sigmodontis* 66
Figure 16. OVA-IgE levels in the serum were not significantly different in IL-10 knockout mice infected with *L. sigmodontis*, but OVA-IgG1 levels were decreased compared to mock controls 67
Figure 17. Stimulation of splenocytes from sensitized mice results in cytokine secretion. 70
Figure 18. Stimulation of splenocytes from sensitized mice results in cytokine secretion. 71
Figure 19. Cytokine profile of *L.s.*- and mock-infected mice at 12 weeks. 72
Figure 20. Cytokine profile of *L.s.*- and mock- infected mice at 12 weeks. 73
Figure 21. Chronic *L. sigmodontis* infection alters the appearance of peritoneal mast cells 75
Figure 22. *L. sigmodontis* infection does not alter the number of mast cells in the peritoneum at 12 weeks 76
Figure 23. Mast cells from *L.s.*-infected mice contain significantly less histamine at 12 weeks post-infection than mock-infected mice 78
Figure 24. *L.s.* infection does not alter total histamine in the blood at 12 weeks post-infection 79
Figure 25. Mast cell gating strategy 81

Figure 26. Mast cells from Infected comprise less of the total cell population that from Mock mice and their median expression of IgE is higher 82
Figure 27. Baseline expression of activation markers is lower on mast cells from chronically infected mice 83
Figure 28. Network of proteins differentially regulated in mast cells from chronically infected versus mock-infected mice 90
Figure 29. Proteins differentially regulated in mast cells from chronically infected mice 91
Figure 30. Chronic infection with *L. sigmodontis* did not alter sphingolipid composition of mast cells at 12 weeks post-infection 93

CHAPTER 1: Introduction

ALLERGY

Allergic diseases are medical conditions in which the immune system mounts a deleterious response to a foreign substance (33). The substance, or allergen, that elicits the response is itself innocuous, but the pathology it elicits is often damaging. Allergy is similar to autoimmunity in that both are hypersensitivity responses and are unwanted. However, allergy differs from autoimmunity in that autoimmune responses occur when the immune systems attacks "self" or endogenous antigens, while allergic responses occur when the immune system responds against "non-self" or exogenous antigens. In the context of allergic disease, antigens are referred to as *allergens*. Common examples of allergic diseases include seasonal allergies, medication allergies, food allergies, allergic asthma, and atopic dermatitis.

There are four different types of hypersensitivity, defined by the immunological mechanism by which they act during their effector stage (110). The effector stage is the phase of the immune response that occurs following re-exposure to an allergen to which an individual is sensitized. In type I hypersensitivity the primary immune reactant in the effector stage is the immunoglobulin IgE, the production of which is tightly regulated by the immune system. Types II and III are also antibody mediated, and the primary immune reactants in these hypersensitivities are IgG and/or IgM, but their mechanisms are different from one another. Finally, type IV hypersensitivity is the only of the four types of hypersensitivity that is cell-mediated.

Type I hypersensitivity, which is also called *immediate hypersensitivity*, progresses quickly. As its alternate name implies, it typically occurs between 5-30 minutes following re-exposure to an allergen. The effector stage in a type I hypersensitivity reaction occurs quickly because the mechanism of action is through IgE-signaling of mast cell and basophils, which are granulocytes containing pre-formed inflammatory mediators (112). Thus, when an allergen encounters IgE bound to these cells, the cells are prepared to respond with inflammatory mediators such as histamine, cytokines, chemokines, and lipids. Common clinical manifestations of type I hypersensitivity include hay fever and allergic asthma.

Like type I hypersensitivity, type II hypersensitivity is also antibody-mediated, primarily by either the IgG or IgM isotypes. The antibodies are actually autoantibodies that cause destruction of cells by reacting with cell surface or matrix antigens (54). The range of onset for Type II hypersensitivity is several minutes to hours. Examples of type II hypersensitivity include pemphigus and drug-induced hemolytic anemia. In drug-induced hemolytic anemia, individuals with sensitivity to a certain drug react to that drug binding to the surface of a red blood cell. The drug becomes a target for IgG antibody, and the cell undergoes destruction and clearance by phagocytic cells.

Type III hypersensitivity is mediated by IgG and IgM antibodies that react with soluble antigen. Binding of the antibodies with soluble antigen results in immune complex formation. Large aggregates of immune complexes lead to activation of mast cells and leukocytes through their Fc receptors, driving an inflammatory response that often results in tissue destruction. Immune complex formation can also result in complement activation. The onset of the effector response in type III hypersensitivity is

somewhere between 3 and 12 hours. Examples are lupus nephritis and hypersensitivity pneumonitis.

Type IV hypersensitivity, which is also known as delayed-type hypersensitivity, is the only type of hypersensitivity that is not mediated by antibodies, but rather by cells. Type IV hypersensitivity is mediated by T cells, including Th1, Th2 and cytotoxic T lymphocytes. The effector stage involves antigen-specific T cells responding to an allergen. The onset of the effector response ranges between 24-48 hours. Some well-known examples include nickel allergy, haptens, poison ivy, and the purified protein derivative (PPD) skin test for tuberculosis (23). For example, in the PPD skin test, tuberculin from *Mycobacterium tuberculosis* is injected intradermally. If the individual has previously been exposed to the bacterium, Th1 cells respond with an inflammatory response that appears as swelling and redness at the site of injection 48-72 hours later (83).

TYPE I HYPERSENSITIVITY

While the impact of many of these diseases is of significance, the focus of this thesis is on IgE-mediated, type I (immediate) hypersensitivity, specifically on anaphylaxis. As mentioned previously, type I hypersensitivity is divided into two phases, the sensitization and the effector phases. With respect to a type I hypersensitivity response, sensitization refers to the process in which the immune system produces antigen-specific IgE. Sensitization to an allergen can occur through a number of different routes including intranasal, transcutaneous, and oral. The route of sensitization influences the degree of sensitization as well as the inflammatory mediators involved in subsequent allergic reactions. Type I hypersensitivity reactions include allergic asthma,

food allergy, allergic conjunctivitis, urticaria, eosinophilia, penicillin allergy, angioedema, and allergic rhinitis.

Like the sensitization stage, the effector stage of type I hypersensitivity, or re-exposure to an allergen to which an individual is sensitized, can also occur through a number of different routes. For example, re-exposure to an allergen through inhalation can trigger an asthma attack, while exposure through ingestion such as with food allergy can lead to airway constriction, facial swelling and gastrointestinal involvement.

It is worth noting that the effector stage of type I hypersensitivity can also can be triggered by cross-reactive allergens. Thus, an individual does not necessarily need to be sensitized to a particular allergen to react to it. Cross-reaction occurs when proteins from one substance closely resemble those from another, enough to bind specific-IgE and activate effector cells through IgE cross-linking. Cross-reactivity often occurs with homologues of closely related species or with molecules that are widely conserved across diverse species (74; 97). An example of cross-reactivity that sometimes affects individuals with respiratory allergy is food allergens resembling certain aeroallergens (typically pollens) and causing gastrointestinal distress. Other common examples of cross-reactivity include shrimp-mite, cat-pork and bird-egg allergens (92).

Anaphylaxis

Anaphylaxis is a type I hypersensitivity reaction. It operates as a continuum of severity, with a subset of individuals who experience severe anaphylaxis being at risk of death. A defining feature of anaphylaxis is that it involves multiple body systems. Symptoms of anaphylactic reactions vary among individuals but can include hypothermia, vomiting, low blood pressure, lethargy, labored breathing, and death.

Anaphylaxis is of significant health impact as its prevalence in the United States (US) is higher than 1.6% (8). Factors thought to increase the risk of severe anaphylaxis include age over 65 years, medication as the causative agent of anaphylaxis, and comorbidities such as lung or cardiac disease (80).

A recent study of cases cited the most common causes of anaphylaxis as medications (34%), foods (31%), and insect stings (20%) (120). In general, common medication allergies encountered in the clinic include those to antibiotics, chemotherapeutics, and the initial dose of allergen immunotherapy (15; 60). An antibiotic allergy often limits treatment options available for bacterial infections, causing an individual to receive an antibiotic that is either less effective or geared towards broad-spectrum treatment (95). Similar to antibiotics, allergies to chemotherapeutic drugs often severely restrict treatment options for cancers and autoimmune diseases. Non-preferred alternatives are often poorly tolerated or less effective, which sometimes forces an individual to choose between suboptimal treatments or undergo rush desensitization to temporarily allow for treatment to take place (16). Rush desensitization is the process by which a patient is given increasing doses of a specific allergen in order to achieve a state of tolerance to that allergen. While rush desensitization can allow an individual to tolerate a therapeutic dose of medication within several hours, it can itself result in anaphylaxis and only confers short-term protection. Therefore, rush desensitization sometimes needs to be repeated multiple times throughout the course of a disease that requires long-term treatment. Rush desensitization is also used occasionally for another type of medication allergy, specifically for patients that cannot tolerate the initial dose of allergen required for allergen immunotherapy.

While medication allergies comprise the largest category of anaphylaxis cases, food allergies constitute a very close second. Food allergy is responsible for 100-200 deaths per year in the US. Estimates on the prevalence of food allergy can be challenging to pinpoint for many reasons, and tend to vary by study. It is thought, however, that the prevalence in the US exceeds 1-2%, but less than 10% of the population (12). Additionally, food allergies impose a burden on our health system as they are responsible for an estimated 30,000 emergency room visits per year (96). The top foods that cause allergy include cow's milk, eggs, fish, peanuts, shellfish, soy, tree nuts and wheat (111).

Finally, insect stings are the third largest cause of anaphylaxis in the US. There are at least 50 fatal stings a year (5). The main order of insects associated with anaphylaxis is Hymenoptera, which includes bees, vespids, and stinging ants (39). Allergy skin tests are not considered an effective tool to predict individuals who are at risk of severe anaphylaxis from an insect sting. Therefore, as with other allergens and type I hypersensitivity reactions, it is difficult to predict whether or not exposure will result in severe anaphylaxis.

Treatments for type I hypersensitivity

The primary intervention for a severe allergic reaction such as anaphylaxis is an epinephrine auto-injector, which is administered when an individual is exposed to a known allergen or begins to display symptoms of an allergic reaction (81). Epinephrine either prevents the onset of or lessens the symptoms of anaphylaxis. It is generally effective against severe outcomes and delayed administration of epinephrine is a risk factor for death due to anaphylaxis (108). While undoubtedly an important life-saving intervention, it is not a curative treatment for allergic disease, as it does not address the

immune component of disease. Epinephrine must be followed up with steroids to block the "late phase" inflammation associated with anaphylaxis, which can also be life-threatening.

The current gold standard for preventing anaphylaxis is allergen avoidance. As implied by its name, allergen avoidance requires keeping away from known substances to which an individual is allergic. Allergen avoidance is often challenging and negatively impacts quality of life. For instance, cross-contamination of foods is a pervasive fear for atopic individuals with food allergies. Furthermore, certain environmental exposures are frequently impossible to avoid.

In contrast to allergen avoidance, several therapies exist that may lessen the predisposition to anaphylaxis. Current interventions include allergen immunotherapy (AIT), omalizumab, and medications. AIT is a type of therapy by which patients are gradually desensitized to an allergen through increasingly higher doses of said allergen. In AIT, allergen is administered subcutaneously, orally, or sublingually, depending on the allergy it is intended to treat. Subcutaneous AIT, commonly known as "allergy shots", takes place over the course of a couple of months. Sublingual AIT is administered as tablets under the tongue and is occasionally helpful for individuals with asthma or food allergies. A downside of AIT is that it must be performed in a clinical setting due to the risk of anaphylaxis. Additionally, some medical practitioners consider AIT to be controversial and its ability to confer long-term protection is disputable. Rush desensitization is a type of AIT, which was mentioned previously in relation to medication allergies.

Omalizumab is a monoclonal antibody that decreases allergy by reducing available IgE in circulation. It binds to serum IgE as well as membrane-bound IgE on B cells. Since Omalizumab does not bind to IgE bound to the surface of basophils and mast cells, it is considered safe against anaphylaxis, as it does not cross-link IgE receptors.

Medications for allergies include antihistamines. An example is histamine receptor 1 antagonist, also referred to as an H1 blocker. Histamine receptor 1 is responsible for vasopermeability and vasodilation (26). Antihistamines that block H1 receptors are used to lessen the impact of histamine in an allergic response.

Epidemiology of type I hypersensitivity

Over the past several decades, rates of allergic disease have increased in developed regions around the world (3). Although the factors driving the increased rates of disease are not fully understood, there are several hypotheses to explain the phenomenon. Factors thought to contribute to allergy are generally associated with modernization. Postulated contributors to the rise in allergic disease include alterations to the microbiome, such as through antibiotics usage early in life (66), increased rate of cesarean sections (94) and reliance on formula feeding (35; 36), vaccination (117), and reduced exposure to infectious diseases (67). Whether these factors are causative or simply correlative with the rise in allergic disease is still under investigation; however, it is appreciated that the increased prevalence of allergy is likely multifactorial including not yet elucidated mechanisms.

Another factor frequently implicated in the rise of allergic diseases is elimination of early exposure to helminths (59). Several decades of research have uncovered an inverse relationship between helminths and allergy, as areas that are endemic for

helminth infections exhibit low rates of allergy. In 1989, Strachan formulated the "hygiene hypothesis" to understand the influence of early childhood exposure to microorganisms on immune dysregulation and development of allergic disease (104). The hygiene hypothesis states that lack of early childhood exposure to infectious organisms, such as parasites, increases susceptibility to allergic disease. Since its initial conception, the hygiene hypothesis has spawned studies that have further supported the immunomodulatory capacity of helminths (34). The relationship between helminths and type I hypersensitivity is discussed later in this thesis.

Role of mast cells in type I hypersensitivity

Microbiologist Paul Ehrlich first described mast cells in 1878, calling them "Mastzellen", meaning fat or well-fed cells (2). In fact, the name referred to their appearance under a microscope, specifically that they contained large granules. Eventually, their characteristic granules were found to be the major repository for the bulk of histamine in the body. Given the importance of histamine in allergic disease, mast cells are now recognized as important inflammatory cells and are key effector cells in type I hypersensitivity reactions.

Mast cells arise from the bone marrow. Their maturation is facilitated by interactions with their receptor, CD117 (c-kit), and stem cell binding factor and the cytokines interleukin (IL)-3, IL-4, IL-9 and IL-10. Importantly for their identification, mast cells are the only hematopoietic cells that express CD117 when terminally differentiated. Mature mast cells are tissue-resident with long lifespans of months to years. Their longevity is often an important consideration in designing therapeutics to

treat allergy, because a mast cell maintains the antigen-specific IgE that is bound to its surface for the course of its lifetime.

IgE is bound to the cell surface of mast cells by the high affinity receptor FcεRI. In the effector stage of allergy, FcεRI undergoes receptor cross-linking when its bound IgE encounters its cognate antigen. FcεRI cross-linking initiates signal transduction for mast activation including degranulation. Mast cell degranulation results in the release of inflammatory mediators. These mediators include histamine, proteases, cytokines, chemokines, and lipid mediators of inflammation. They act on vasculature, mucous glands, smooth muscle, connective tissue, as well as on other inflammatory cells (10).

T HELPER TYPE 2 RESPONSE

In order to understand the impact of helminths on allergic disease, it is necessary to appreciate the immune mechanisms involved. The T helper type-2 (Th2) immune response is the predominant response to invading helminths as well as to foreign allergens (123). In response to helminths, the Th2 response drives the production of anti-parasite IgE and is generally thought to confer protective immunity (22). However, as discussed later in this thesis, helminths often suppress the Th2 response to persist chronically in their hosts. In the context of allergy, an inappropriate Th2 response is initiated in response to contact with otherwise harmless allergens. Rather than anti-parasite IgE, the response to an allergen results in production of allergen-specific IgE. The production of allergen-specific IgE constitutes the sensitization phase of Type I hypersensitivity. With respect to both helminths and allergy, the classical cytokines involved in the Th2 response include IL-4, IL-5, and IL-13. IL-4 is recognized as the cytokine that promotes B cell class switching of antibody isotype to start producing IgE.

An overview of the initiation and propagation of the Th2 response is found in Figure 1 (65). The Th2 response is generally initiated at barrier surfaces. Early mediators include the damage-associated cytokines IL-25, IL-33 and thymic stromal lymphopoietin (TSLP). Although thought to originate from epithelial cells, the specific source of these cytokines is often poorly defined. In the gastrointestinal tract, tuft cells, a rare epithelial cell type, are thought to be in the initial source of IL-25 (37; 116). IL-25, IL-33, and TSLP act on tissue-resident group 2 innate lymphoid cells (ILC2s). ILC2s produce IL-5, IL-9, and IL-13, which act on goblet cells to produce mucous. Mucous is important for expulsion of gastrointestinal helminths. The generation of mucous is also typical for certain types of allergy such as chronic rhinitis and asthma. These cytokines also drive Th2 cells to produce canonical cytokines IL-4, IL-5, IL-9 and IL-13, by providing the third signal for antigen presentation.

Generation of anti-parasite IgE or allergic sensitization requires presentation of antigen by antigen-presenting cells (APCs), such as dendritic cells, macrophages or B cells. In order to avoid anergy, antigen presentation requires co-stimulation as well as cytokines for secondary and tertiary signals, respectively. APCs present peptide to Th2 helper cells via the major histocompatibility complex (MHC) class II on the cell surface. Antigen presentation along with co-stimulation through CD80/CD86 and CD28 leads to activation of Th2 helper T cells, resulting in IL-4 secretion. Basophils are thought to provide an initial source of IL-4 that amplifies the Th2 response (106).

In the context of allergy, IL-4 provides necessary signals for allergen-engaged B cells to class-switch their antibody isotype to IgE. The class-switched B cells become plasma cells that secrete antigen-specific IgE. The secreted IgE binds to the high-affinity

IgE receptor FcεRI, which is expressed on surface of allergy effector cells, basophils and mast cells. FcεRI recognizes the constant Fc portion of the IgE antibody irrespective of the variable antigen-specific F(ab')2 portion of the IgE antibody. Relative levels of antigen-specific IgE on the cell surface of basophils and mast cells generally correlate with relative quantities in the blood (25). Individuals with elevated levels of allergen-specific IgE are sensitized to that particular allergen and considered to be "atopic".

Similar to the sensitization stage, there are a variety of routes for allergen exposure during the effector stage of allergy that can trigger an allergic response. The effector stage of allergy in the type I hypersensitivity response distinguishes itself from other types of immune responses by being uniquely mediated by IgE bound basophils and mast cells. Aggregation and cross-linking of many IgE molecules bound to FcεRI mediates allergic responses. In atopic individuals, these effector cells typically express up to 250,000 copies of the high affinity receptor for IgE, FcεRI, on their cell surfaces. Nevertheless, IgE signaling is highly sensitive, and sometimes requires the involvement of only 200-500 allergen-specific IgE receptors bound to cognate antigen to trigger degranulation and signaling though the receptor (121).

IgE receptor signaling results in the release of inflammatory mediators such as histamine. Release of pre-formed inflammatory mediators by mast cells is termed degranulation or compound exocytosis (28). Other mediators released by degranulation of effector cells include chemokines, lipids, and cytokines. Release of these inflammatory mediators drives the clinical symptoms of an allergic response.

The need to aggregate many IgE molecules to promote cross-linking is one of the few known properties that define a "good" allergen. Indeed, while humans ingest a

diverse array of foods, there are only a handful of allergens responsible for the bulk of food allergy cases (101). A good allergen is typically a long molecule with repeating domains. Allergens are usually glycoproteins of low molecular weight. They are often enzymatically active and stable against heat, proteases and acidic environments. It is thought that their high stability allows them to retain their tertiary structure, which may contribute to the overall immunogenicity of the allergen. An example of a highly allergenic protein is shrimp tropomyosin. Shrimp tropomyosin contains a number of repeating linear epitopes, which retain their ability to bind IgE when exposed to conditions ranging in pH from 1.0-3.5 (63). Thus, it is not only resistant to acid, but its many repeating domains render it exceptional in aggregating and cross-linking IgE.

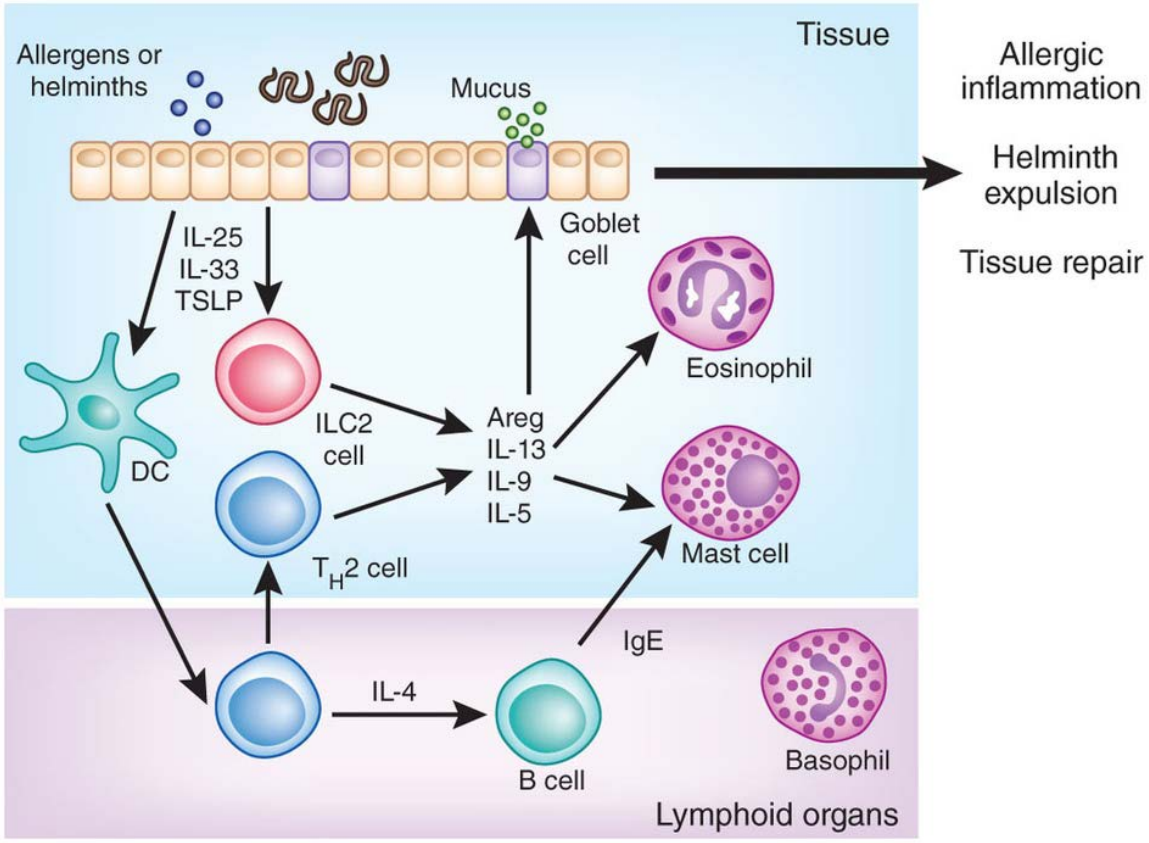


Figure 1. Initiation and propagation of type 2 response

The Th2 response is initiated by breaks in the epithelium that release the damage-associated molecular patterns (DAMPs) IL-33, IL-25 and TSLP (65). Downstream IL-4 production promotes production of classical Th2 cytokines and class switching of B cells to start producing IgE. The Th2 response results in secretion of mucous by goblet cells, which facilitates expulsion of allergens and helminths. *Figure from Licona-Limón. P.* et al. Nature Immunology 14:536.

HELMINTHS AND ALLERGY

Helminths are eukaryotic parasites that have co-evolved with humans for thousands of years (122). They are a significant cause of morbidity worldwide, particularly in developing countries where infrastructure is lacking. The relationship between helminths and allergy first became of interest nearly four decades ago when Strachan formulated the hygiene hypothesis. The hygiene hypothesis proposed that lack of early exposure to infectious agents could explain the rise in allergic diseases in the 20th century (103). To date, several epidemiologic studies have shown an inverse correlation of helminths and allergy (32). Since its initial inception, the scope of the hygiene hypothesis has expanded to postulate that early exposure to infectious agents influences the development of the immune system and dampens inflammatory diseases such as allergy and autoimmunity.

With respect to infectious diseases, helminths are of particular interest in the discussion of the hygiene hypothesis for several reasons. Helminths and allergens both elicit a Th2 response. In fact, the immunoglobulin IgE is a host defense mechanism evolutionarily linked to parasitic infections (69). An offshoot of the hygiene hypothesis speculates that perhaps allergy developed as an inappropriate immune response in the absence of parasitic infections, which were largely eradicated from certain geographical locations in the 20th century.

Although there are immunological similarities between helminth infections and allergy, there are also paradoxes that could explain the perceived protective effect of helminths. While individuals with helminth infections frequently present with allergic symptoms, especially in the early course of infection, they seem to be protective overall in response to environmental allergens (55; 90). It stands to reason that organisms that

have co-evolved with their host species for thousands of years would have evolved mechanisms to allow for their long-term survival in the host (7; 69). Indeed, a number of proteins and worm products have immunomodulatory capabilities. For example, *Brugia malayi* adult worms secrete the protein TGH-2, which is a homologue of the human immunoregulatory cytokine TGF-β (41). *Acanthocheilonema viteae, B. malayi, Onchocerca volvulus and Nippostrongylus brasiliensis* secrete cystatins, which are cysteine protease inhibitors (45). The cystatins act as immunomodulators by decreasing antigen presentation through two mechanisms. First, they inhibit cysteine proteases such as cathepsins that are required for antigen presentation, which results in diminished T cell priming (24; 71). Second, they drive IL-10 production, which decreases expression of co-stimulatory molecules on APCs and inhibits T cell proliferation (100). In addition to these molecules, there are many other examples of worm products that modulate the host immune response. Both animal models and human studies suggest that immunomodulation is an active and reversible process (73). For instance, in a human study, increased skin reactivity to bystander allergens was observed in communities that underwent long-term deworming (29). The need for active suppression to confer immunomodulation is a possible explanation for the observed immunological differences between helminths and allergens.

Nevertheless, the prospect that helminths protect against allergic disease is controversial. Some argue that the need for helminth infections in proper immune function has not been demonstrated (13). At a minimum, if helminths do indeed protect against allergic diseases, the mechanisms by which they do so remain unclear.

MURINE STUDIES OF ALLERGY AND HELMINTHS

Helminth infection has been shown to be protective against the development of allergy in a number of in vivo mouse models, including airway hyper-responsiveness (27; 70) and food allergy (6). However, only four studies have evaluated the effects of infection on pre-existing allergy, and the findings of these studies have been mixed. While it is unclear exactly why the discrepancy between the outcomes exists, these findings provoke several considerations, as thoroughly discussed in several reviews (20; 30).

The discrepancy amongst the studies could be due to differences in the lifecycles of the parasites used, differences in the durations of the parasite infections, or differences in the immune responses they elicit, as well as differences in the allergic models used. Of the two studies that demonstrated protection against allergic challenge following sensitization, one used *Strongyloides venezuelensis* as the model helminth (84), while the other used *Heligmosomoides polygyrus* (118). Both studies examined the impact of infection on allergic models of airway hyper-responsiveness, one in Wister rats and the other in BALB/c and C57BL/6 mice. In contrast, both of the studies that reported no protection against allergic reaction following sensitization used *Nippostrongylus braseliensis* as their model helminth. One of the two studies that showed no protection against allergy was performed in Hooded Lister rats and used allergic models of local and systemic anaphylaxis (51). The second of the two studies, performed in BALB/c and C57BL/6 mice, examined the effect of infection on airway hyper-responsiveness and local anaphylaxis (119).

The immune response to the parasite used for helminth infection could be important in determining whether or not there is protection against allergic challenge

(64). For instance, the two aforementioned studies failed to show protection to allergy development using *N. braseliensis*, which is an acute model of infection. *N. brasiliensis* drives a Th2 response that expels worms from the rat or mouse host by two weeks post-infection. In contrast, *H. polygyrus* establishes chronic infections in mice that last for months. Additionally, the lifecycle of these parasites has to be considered, as they migrate and reside in different parts of the body.

Differences in models of allergy could also account for disparities in ability to treat allergy. For instance, the routes of sensitization or challenge could engage distinct immune mediators. Furthermore, varying the amounts of allergen used for either sensitization or challenge also influences whether or not protection is observed, due to differential cytokine secretion. Degree of sensitization influenced by host genetics and allergen dose, could also be an important factor potentially related to protective ability (79; 87).

Fully characterizing the pathways of allergic disease as well as the mechanisms of immunologic protection are necessary in translating helminth and helminthic products in therapies. Therapeutics derived from worms and their products have the potential to overcome major limitations of current treatments for allergy. For instance, worm-based therapeutics could circumvent the need for allergen-specific therapies, and could provide broad treatment against many if not all allergens to which an individual is sensitized.

In the current study, we explored the effect of helminth infection on type I hypersensitivity. We used *Litomosoides sigmodontis*, a rodent model of chronic filariasis in conjunction with a murine model of acute anaphylaxis. As discussed in further detail in the results section, we chose the acute anaphylaxis model for our study of type I

hypersensitivity as it provided a robust allergic response by which to measure the impact of helminth infection.

LYMPHATIC FILARIASIS

For this study, we used a rodent model of filariasis that we maintain in our laboratory. Lymphatic Filariasis (LF) is caused by filarial helminth infection and is a leading cause of morbidity worldwide. The global health burden imposed by LF is severe, such that individuals with LF often endure disability, economic hardships and social stigmatization. In 2000, a Global Programme to Eliminate Lymphatic Filariasis was launched. By 2012, significant progress had been made, with cases of LF decreasing 59%, from 3.55% to 1.47% (93). However, there is still significant work that remains, as there are nearly 67.88 million cases of LF. Of those cases, 36.45 million are microfilaria carriers, 19.43 million are hydrocele cases, and 16.68 million are lymphedema cases (93).

The most common helminthic worms associated with human disease are *Wuchereria bancrofti, Brugia malayi,* and *B. timori,* which are transmitted by mosquito vectors. In the course of human infection, infective-stage L3 larvae enter the bite wound as the mosquito takes a blood meal. L3 larvae mature to adults that persist in the lymphatic system and give rise to microfilariae. Microfilariae migrate to the blood stream and circulate in the peripheral blood. Mosquitoes ingest microfilariae from the peripheral blood during subsequent blood meals to perpetuate the lifecycle. The mosquito vectors for filarial species include *Anopholes*, *Culex*, and *Aedes*.

In response to filarial worm infection, the host immune system induces a Th2 immune response characterized by elevated Th2 cytokines including IL-4, IL-5, and IL-13 (46). Induction of Th2 immunity promotes class switching of B cells and results in

high levels of IgE. Eosinophilia is also typical during early stages of helminth infection. During chronic infection, the immune response changes to a regulatory phenotype resulting in elevated levels of the immunoregulatory cytokines IL-10 and TGF-β and an increased number of B- and T-regulatory cells, as well as alternatively activated macrophages (46; 61; 68). While helminths induce an initial pro-inflammatory immune response, they possess immunoregulatory capabilities, which allow them to reside in a host for extended periods of time.

LITOMOSOIDES SIGMODONTIS/ BALB/C MODEL

L. sigmodontis is a filarial helminth that naturally infects the cotton rat, *Sigmodon hispidus*. It is recognized as a model for filariasis, and it induces systemic immune responses that model human-specific helminths (1; 4; 89). *L. sigmodontis* is permissive in BALB/c mice and has been extensively characterized in this species (49). The mite vector *Ornithonyssus bacoti* is used to propagate the lifecycle in the laboratory. To infect mice in the laboratory, L3 larvae are either subcutaneously injected into mice or introduced by *O. bacoti* inoculation. Although mite-inoculation more closely resembles a natural infection, subcutaneous injection is often preferred because the dose of infective L3s that are administered can be controlled. During infection in mice, L3s migrate to the pleural cavity and molt into L4 juvenile worms at 8 days post infection. Adult worms (L5s) are observed in the pleural cavity 25 days post infection. Microfilariae (MF) are found in the blood 50 days post infection. Worm clearance occurs approximately 14 weeks post infection by granulocyte encapsulation. Overall, the system models human helminth infection and the use of the BALB/c strain allows for evaluation of immunological responses. The benefit of using *L. sigmodontis* as a model for filariasis

over other parasites is that it is the only infective species that can complete its entire lifecycle in a rodent.

In the laboratory mouse, *L. sigmodontis* induces an initial mixed Th1/Th2 immune response. This mixed response is followed by a predominant Th2 response during acute infection, which is characterized by production of IL-4, IL-5, and IL-13. Acute infection lasts during weeks 0-6 post-infection and also results in eosinophilia and elevated levels of parasite-specific and total IgE. As *L. sigmodontis* infection becomes chronic around ten weeks of infection, the host environment skews away from Th2 and becomes more predominantly immunosuppressive. The immunoregulatory cytokines IL-10 and TGF- β are both elevated in chronic infection (43).

METHODS TO STUDY ALLERGY EFFECTOR CELLS

Mast cells and basophils are the effector cells of type I hypersensitivity. Mast cells are granulocytes of myeloid origin that contain pre-formed mediators such as histamine and heparin. They are tissue residents. Basophils are also granulocytes that contain pre-formed mediators. Basophils circulate in the blood and represent approximately 0.5-1% of white blood cells.

Studying the effector cells of allergy can be challenging considering their granularity and hyper-responsiveness to stimuli. When working with mast cells and basophils, precautions often need to be taken in order to prevent unintentional activation, including use of calcium- and magnesium-free media, as well as slow speeds during centrifugation. Nevertheless, there are several tools available to study the effector cells that are identified in the literature.

In the laboratory, there are a few cell lines that can be used, including P815 and RBL-2H3. The RBL-2H3 is a basophil line, but is commonly used to model mast cells and histamine release (86). They are useful for studying signaling pathways. One of the drawbacks of these cell lines is that the cells typically contain less granular contents than their primary cell counterparts. Furthermore, they require an external source of IgE to become "sensitized". As an alternative to cell lines, bone marrow-derived mast cells (BMMCs) can be generated through cytokine differentiation in vitro with IL-3 (19). Cell lines and primary cells can be "sensitized" through passive transfer of antibodies, which will readily bind to FceRI. A common antigen-specific system is dinitrophenol (DNP) and anti-DNP. Anti-DNP can be passively transferred onto cells or into mice to "load" effector cells, and an effector cell response can then be initiated through reaction with DNP. While the DNP system provides a rapid, antigen-specific model to study the effector response of allergy and is useful in cell lines, it is potentially missing important bystander contributors when used in the animal model that result from active sensitization such as circulating antibodies and induction of other immune cells. In passive sensitization, antibodies are transferred to the mouse through injection (usually intravenous) and mice are sensitized and ready for challenge within 3-4 hours (58).

In addition to passive models of sensitization, active sensitization exists with regard to animal models as well. With active sensitization, mice are typically sensitized with an adjuvant such as alum or cholera toxin to stimulate Ag-specific IgE production by B cells. Active sensitization is more time consuming and it typically takes several weeks to sensitize a mouse to an allergen. Common outcome metrics used with murine models of anaphylaxis include clinical symptom scoring, core body temperature,

abundance of histamine and mMCP-1 in the blood within 1-2 hours following challenge, and presence of systemic Th2 cytokines in “late phase” allergy. In some other allergy models, allergic challenge results in a subset of animals undergoing anaphylaxis. However, the percentage of animals that demonstrate a drop in core body temperature in models of other types of allergy is typically low. An example of other allergy models that measure anaphylaxis signs in mice are models of food allergy (14).

The abundance of effector cells in each system generally dictates the outcome measurements that can be used. Thus, some outcomes that are very useful for studying human mast cells have very limited utility for small animal models. Outcome metrics for studying mast cells include measuring released contents granules and measuring markers of activation on the cell surface. Typically, cells are stimulated with an activating agent such as specific antigen, anti-CD3/anti-CD28 or PMA/ionomycin. With respect to secreted components, three different groupings of mediators exists (58). The first group of mediators includes pre-formed compounds found in granules (9; 75). This list includes histamine, β-hexosaminidase, proteases, serotonin, carboxypeptidases and various enzymes. The second group includes phospholipid metabolites that are generated from activation of phospholipase (PLA_2) activation, which includes platelet activating factor and eicosanoids (11). The final grouping consists of cytokines and chemokines that are enhanced in response to activation (38)

Within the first group of mediators, the β-hexosaminidase assay is typically the least expensive to measure. Experiments can be performed with a moderate number of cells, as the β-hexosaminidase assay typically requires 3-4 x 10^4 cells per well to detect the color change that occurs following addition of the substrate for β-hexosaminidase

(82). The assay uses the 96-well plate format. Substrate is added to cells and color change occurs in the presence of β-hexosaminidase. Results are measured 30 minutes following addition of the substrate. Purification of mast cells from a typical mouse results in 1-2 x 10^5 cells, so the β-hexosaminidase requires either pooling of mice or use of cell lines or human samples when multiple conditions need to be evaluated. To perform the β-hexosaminidase assay, mast cells can either be purified through a Percoll gradient, or whole peritoneal cells can be used. Likewise, histamine can be measured in a similar fashion, but rather than detection through substrate, it is detected by competitive ELISA. Histamine evaluation can be more sensitive in that fewer cells are needed (62). In terms of peritoneal cells, mast cells are the only source of histamine, so it is unnecessary to purify mast cells for this assay, but sometimes it is preferred. Releasability of histamine from mast cells can be reported as the amount of histamine released divided by the total amount available, which is determined by repeated freeze-thaw lysis of cells. One limitation is that it can be difficult to finely correlate histamine with degranulation.

Measuring mediators from the second group is more complicated than measuring generated PLA_2 following mast cell activation. PLA_2 releases arachidonic acid from arachidonyl-containing phosphatidylcholine. Early experiments to measure eicosanoids used radioactive-labeled arachidonic acid to measure the amount of label released following mast cell activation (102). Although an array of eicosanoids could be measured by this technique, a drawback is that their detection requires high-pressure liquid chromatography (HPLC) and use of radioactivity. Currently, there are several

ELISA kits available to measure eicosanoids to overcome the limitation of needing HPLC expertise, but they only detect a single eicosanoid and can be cost prohibitive (58).

Cytokines constitute the third grouping of mediators. Cytokines that result from mast cell activation can be measured at the mRNA or protein level. Of course, mRNA does not always correlate with protein expression, but it is often more feasible to measure an array of transcripts than to measure multiple proteins. For cytokine evaluation, cytokine-encoding mRNA transcripts can be measured by RT-PCR or semi-quantitative real time PCR. Cytokine protein levels can be measured by ELISA. Measuring cytokines from activated mast cells is generally less desirable than measuring mediators from granule contents, as cytokines require 4-8 hours post-stimulation to be detected (58).

In addition to measuring mediators from mast cells, another technique to measure mast cell activation is evaluating expression of activation markers. Flow cytometry is a useful tool to detect protein expression given the relative scarcity of mast cells compared to other cell types such as lymphocytes, but can also be difficult due to their hyper-responsiveness to cell handling and centrifugation. Common surface markers used for the identification of mast cells include proto-oncogene kit (known as CD117 or c-kit), FcεRIα and IgE (IgE is deposited on the mast cell surface as opposed to being produced by mast cells). A double stain for CD117 and ether IgE or FcεRIα is useful, because these markers alone are not exclusive to mast cells (76). CD117 is present on various hematopoietic progenitors such as melanocytes, intestinal cells, some stem cells, and early lymphoid progenitors (76). FcεRIα and IgE are found on eosinophils, basophils and epidermal Langerhans cells. Co-expression of CD117 with either IgE or FcεRIα, however, is unique to mast cells. In terms of activation markers, CD200R can be used as

a marker to study mast cell activation (62). CD200R is a type I membrane glycoprotein that contains two Ig-like domains. It is a known activation marker on mast cells. It is also possible to measure CD63 expression by flow cytometry. CD63 is a tetraspanin, also known as LAMP3, and is found on the membranes of granules of mast cells and basophils. CD63 is required for granule exocytosis and degranulation of mast cells and basophils (57).

In a clinical setting, specific IgE antibody positivity is used to diagnose allergy, as circulating antibody roughly correlates with what is observed on the surface of effector cells. More accurate for diagnosis, however, is the basophil activation test (BAT). The BAT is a flow-cytometry based assay that measures basophil responsiveness to stimulation and is often used as a diagnostic tool for food allergy (98). In the test, basophils are stimulated ex vivo and CD203c and/or CD63 expression is measured as a marker of activation (72).

In our study, we employed several of the aforementioned techniques. For studies of mast cells, we used ELISA to measure in vivo release of murine mast cell protease 1 (mMCP1), which is a marker of mast cell degranulation. Additionally, we measured histamine levels in mast cells by ELISA. We also used flow cytometry and measure CD200R and CD63 expression on mast cells. With respect to our model of sensitization, we used the ovalbumin admixed with alum to actively sensitize mice. We utilized the BALB/c strain of mice, which are Th2-skewed and conveniently permissive for chronic *L. sigmodontis* infection, which served as our model of helminth infection. Sensitization with ovalbumin admixed with alum as done in our model results in robust production of OVA-specific IgE and OVA-specific IgG. Ovalbumin is often used as a model antigen

for studying a range of immune responses, such as to cancers and pathogens, because there is a wide repertoire of molecular tools available to express it and detect it. However, in the context of allergy, it is actually a common human allergen that is naturally abundant in chicken egg white (17). Individuals with egg allergies often have OVA-specific IgE detectable in their sera.

PURPOSE OF RESEARCH AND PROPOSED SPECIFIC AIMS

Anaphylaxis is a life-threatening condition that can result from IgE-mediated allergy. It is becoming increasingly prevalent in western countries and results in approximately 500-1,500 deaths a year in the US. Current interventions are insufficient to treat I hypersensitivity and to prevent anaphylactic reactions. Evidence that helminth infections influence the development of allergy is supported by epidemiological, clinical, and animal studies. However, the underlying mechanisms behind helminth modulation of allergy remain largely uncharacterized, particularly in the context of inducing and alleviating IgE-mediated allergy. While numerous animal studies have reported that helminths are able to prevent allergic sensitization, few have evaluated the effects of infection on pre-existing allergy. The purpose of this research was to investigate the therapeutic potential of helminths on systemic anaphylaxis in sensitized mice. Our laboratory previously showed the chronic helminth infection suppresses basophils in an IL-10 dependent manner. Thus, we were interested in the impact of helminth infection on effector cells, especially on mast cells, which have been understudied in the context of type I hypersensitivity. **We hypothesized that chronic infection would protect against the clinical signs of IgE-mediated anaphylaxis in previously sensitized mice through suppression of allergy effector cells.** Insight into the impact of helminth infection on

the ability to modulate pre-existing allergy is important for development of novel therapeutics. To test our hypothesis, we proposed the following aims:

Specific aim 1: Establish and characterize a model for type I hypersensitivity.

Sub-aim 1.1: Establish OVA/Alum sensitization model of systemic anaphylaxis.

Sub-aim 1.2: Determine the mechanism of effector response in anaphylaxis model.

Sub-aim 1.3: Determine the relative contribution of the effector cells of type I hypersensitivity in acute anaphylaxis model.

Specific aim 2: Determine if chronic helminth infection protects against pre-existing type-1 (immediate) hypersensitivity.

Sub-aim 2.1: Evaluate whether infection protects against anaphylaxis at 12 weeks post-infection (chronic).

Sub-aim 2.2: Evaluate whether infection protects against anaphylaxis at 7 weeks (chronic) time point.

Sub-aim 2.3: Evaluate whether infection protects against anaphylaxis at 2 weeks post-infection (acute) time point.

Specific aim 3: Evaluate potential mechanisms of *L.s.* protection against anaphylaxis at 12 weeks post-infection.

Sub-aim 3.1: Determine if IL-10 is required for protection against anaphylaxis at 12 weeks post-infection.

Sub-aim 3.2: Determine if infection alters cytokine response to allergen.

Sub-aim 3.3: Determine if changes in mast cells occur at 12 weeks post-infection.

CHAPTER 2: Materials and Methods

ANIMALS

Female BALB/c and IL-10 knockout mice (C.129P2(B6)-Il10tm1Cgn/J) mice between the ages of 5-7 weeks were obtained from Charles River (Frederick, Maryland). For FcεRI knockout studies, female FcεRI knockout mice (C.129P2(B6)-Fcer1gtm1Rav N12) and BALB/c controls that were 6-8 weeks of age were ordered from Taconic (Rensselaer, NY). For basophil-depletion studies, female c-Kitw-sh (*Kit*$^{W\text{-}sh}$/HNihrJaeBsmJ) and C57BL/6J control mice of 6 weeks of age were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed at the Uniformed Services University of the Health Sciences (USUHS) Center for Laboratory Animal Medicine and all experiments were performed in accordance with the USUHS Institutional Animal Care and Use Committee.

MODEL OF SUB-LETHAL ANAPHYLAXIS

Mice were sensitized with 50 μg of ovalbumin (OVA) adsorbed to 2 mg aluminum hydroxide (alum) (Sigma-Aldrich) on days 0, 7 and 14. Sensitized mice were rested for two weeks and then either challenged with OVA or infected with L3s for later challenge with OVA. Mice were challenged by intraperitoneal injection with either 200 or 400 μg of OVA admixed with alum. For basophil-depletion studies, mice were injected through the intraperitoneal route with 50 μg of Ba103 or rat IgG2b isotype control antibody (BD Biosciences, San Jose, CA) 24 hours prior to challenge. The Ba103 clone recognizes CD200R3, found on basophils and mast cells, and was described previously (85). Although Ba103 binds to CD200R3 on both basophils and mast cells,

previous studies showed that it selectively depletes basophils while leaving skin and peritoneal mast cells unchanged in number (85; 106). The core temperature of each animal was measured using a rectal thermometer with lubricant every 5 minutes for either 30 minutes or one hour. After 1 hour, mice were euthanized by CO_2 asphyxiation followed by cervical dislocation. Pre-challenge serum was collected by tail-vein bleeds. Mice were warmed prior to blood collection under a heat lamp to promote vasodilation. For terminal bleeds, cardiac puncture was performed using a 25 Gauge needle. Blood samples were collected in heparin tubes and centrifuged for 1 min at 14,000 x g. Serum was obtained and stored at -20°C.

INFECTION OF MICE WITH *LITOMOSOIDES SIGMODONTIS*

Litomosoides sigmodontis was used to infect mice. L3 infective stage larvae were obtained from Mongolian jirds (*Meriones unguicultans*) that were shipped overnight from TRS Laboratory Inc., Athens, GA. L3 larvae were collected by pleural lavage with RPMI-1640 pre-warmed to 37°C. Healthy L3s were identified as active using light microscopy. L3s were collected in less than 200 µl of media for injection into mice. BALB/c mice were infected by subcutaneous injection with 40 L3-stage larvae. Mock-infected mice were administered subcutaneous injections of 200 µl of RPMI-1640.

MAST CELL PURIFICATION

Peritoneal cells were collected as described previously (52). Briefly, the ventral side of the mouse peritoneum was washed with 70% ethanol. First, an incision was made and the furbearing and subcutaneous skin were dissected away. Approximately 5 mL of 1 X HBSS was then injected into the peritoneum and the abdomen was physically agitated for one minute. A small incision was made in the layer of skin and a transfer

pipette was inserted into the abdomen to further mix the HBSS with the peritoneal cells. The cell suspension was removed by pipette and collected in a 15 mL tube on ice. An additional 5 mL of 1 x HBSS was used to further wash the peritoneal space. The removed solution was then centrifuged at 400 x g for 10 minutes and the supernatant separated off by gentle pipetting. The cells were re-suspended in 1 mL of 0.8% ammonium chloride (NH_4Cl) and incubated at room temperature for 5 minutes to lyse any red blood cells. The lysate was neutralized with 14 mL HBSS. Cells were then pelleted by centrifugation at 400 x g for 5 minutes. Cells were re-suspended in 70% isotonic Percoll (Sigma), which was overlaid with 2 mL MC media, or supplemented DMEM (L-glutamine, FBS, gentamicin, and HEPES). The suspension was pelleted for 15 minutes at 580 x g. After centrifugation, the MC media and Isotonic Percoll layers were carefully aspirated to leave an intact mast cell pellet. The mast cells were then suspended in MC media pre-warmed to 37°C. Mast cell count and viability were scored using trypan-blue exclusion and an automated cell counter (Countess, Invitrogen).

ENZYME-LINKED IMMUNOSORBENT ASSAY

Murine mast cell protease (mMCP1) ELISA Kits were obtained from Thermo Fisher Scientific (#88-7503-22). Mouse anti-OVA IgE and Anti-OVA IgG1 ELISA kits were obtained from Cayman Chemical (#500840 and #500830). Histamine ELISA kits were obtained from Beckman Coulter (#IM2015). All ELISAs were performed according to the manufacturer's instructions. Absorbance measurements were taken using a Synergy HTX Multi-Mode Microplate reader. Samples were diluted with dilution buffer until samples were within the linear range of the standard curve. Absorbance was measured using a BioTek Synergy HTX Multi-Mode Microplate

Reader. Absorbance measurements were also performed according to manufacturer's protocols at 405 nm for the histamine ELISA, or 450 nm for other ELISAs. Standard curves were generated by four-parameter or sigmoidal fits. Standard curves were generated and unknowns were interpolated using GraphPad Prism version 7. Data were graphed and statistical analysis performed using GraphPad Prism version 7. Relevant groups were compared using the Student's t-test; $p < 0.05$ was considered significant.

CYTOKINE ASSAY

Spleens were dissected from mice and placed into cold RPMI-1640 immediately. A single-cell suspension of splenocytes was prepared the same day at a concentration of $2x10^6$ cells/ml. Cells were plated at 1 ml of cells per well in 24-well plates. Splenocytes were stimulated with media, OVA (10 or 1 μg/ml), PMA/Ionomycin (50 ng/ml PMA, 0.5 μg/ml ionomycin) or anti-CD3/anti-CD28 (5 μg/ml anti-CD3, 2 μg/ml anti-CD28). The anti-CD3 (5 ug/ml in PBS) was added to the plate a day prior to stimulation and incubated overnight at 4°C. The plate was then washed once with PBS and the cells and anti-CD28 antibody were added the following day. The plates were incubated at 37°C for 3 days and then centrifuged at 500 x g for 10 minutes. Supernatants were collected and stored at -80°C in cryovials until subsequent experiments were performed. TGF-β and IL-10 in thawed supernatant samples were measured subsequently using commercially available ELISA kits. Th1/Th2 6-plex Immunoassay kits were used to measure IL-4, IL-5, IL-6, IL-12p70, TNFα, and IFN-γ levels (Invitrogen, Carlsbad, CA). Data were graphed and statistical analysis performed using GraphPad Prism version 7. Relevant groups were compared using the Student's t-test; $p < 0.05$ was considered significant.

HISTOLOGY

Peritoneal mast cells were isolated from BALB/c mice that had either been infected with *L.s.* for 12 weeks or were administered a mock treatment to serve as age-matched controls. Purified mast cells from each individual mouse were washed in cold 1% BSA-PBS and diluted in 200 µl of cold BSA-PBS. Slides were cleaned with 70% ethanol and Kimwipes and inserted into Cytofunnels. Filters were equilibrated by spinning each Cytofunnel for 2 minutes at 500 x g with 100 µl of 1% PBS-BSA in a Cytospin 4 (Thermo Fisher Scientific). Following equilibration, 100 µl of single-cell suspension was added to each Cytofunnel. Cytofunnels were spun at 500 x g for 5 minutes to adhere the cells in the samples to slides. Slides were carefully removed from Cytofunnels and dried in a desiccation chamber overnight. Slides were sent to Histoserv where the cells were fixed and stained with Toluidine blue using standard protocols (Germantown, MD).

ELECTRON MICROSCOPY

Peritoneal cells from mice that were either infected with *L.s.* for 12 weeks or had been administered a mock treatment (10 per group) were pooled prior to isolation of mast cells by Percoll purification. Mast cells were fixed with 2% formaldehyde and 2% glutaraldehyde in 0.1M cacodylate buffer for 1 hour at room temperature. Mast cells were then pelleted by centrifugation at 400 x g for 10 minutes and cell pellets were sent to the BIC at USUHS for analysis by electron microscopy.

FLOW CYTOMETRY

Peritoneal cells were isolated from each mouse by lavage with 10 mL of 1 X HBSS and the samples were collected on ice. The samples were centrifuged at 400 x g for 10 minutes to pellet the cells. Pellets were re-suspended with 1 mL of 0. 8% NH_4Cl

and samples were incubated for 5 minutes, then neutralized with 14 mL of HBSS and counted using a Countess (Invitrogen). Samples were then centrifuged at 400 x g for 5 minutes. Each cell pellet was re-suspended with 1 mL of HBSS for counting. Cells were then re-suspended to a concentration of 1.0 x 10^6 mL per tube in MC media (supplemented DMEM). Each sample was then divided into 2 fractions and each fraction was added to one well of a 6-well plate. The first fraction was stimulated with media and the second fraction was stimulated with media + 1 μg/ml of ionomycin for 1 hour at 37°C. The cells were washed 2 x with cold 1 x PBS at 400 x g for 5 minutes. Cells were fixed using 500 μl of fixative solution from a whole blood lysing reagent kit (Beckman Coulter, CA) for 3 minutes. Cells were then washed with 5 mL cold 1 x PBS. Cells were re-suspended in 500 μl of 1% BSA/PBS and incubated for 1 hour at 4°C. Approximately 500,000 cells were stained per tube in 100 μl of 1%BSA/PBS for 30 minutes, shielded from light. For analysis of mast cells, samples were stained with anti-IgE-FITC clone RME-1 (BioLegend), anti-CD117-PE clone 2B8 (BioLegend), anti-CD63-PE-Cy7 clone NVG-2 (eBioscience) and anti-CD200R-APC clone OX110 (eBioscience). Cells were washed twice with cold 1X PBS and re-suspended in a final volume of 250 μl per sample.

All antibodies were titrated to determine optimal staining conditions prior to the experiments using splenocytes from BALB/c mice. Both anti-IgE-FITC and anti-CD117-PE were titrated individually. The best concentrations for anti-IgE-FITC and anti-CD117-PE were both found to be 1 μg/ml. The activation markers anti-CD63-PE-Cy7 and anti-CD200R-APC were each titrated on cells stained with1 μg/ml anti-IgE-FITC and anti-CD117-PE. Working concentrations for subsequent experiments were 0.015 μg/ml for anti-CD63-PE-Cy7 and 0.25 μg/ml for anti-CD200R-APC. OneComp eBeads

(Invitrogen) were used to calculate compensations for each run. The fluorescence-minus-one technique was used for gating of markers of cell singlets. Data were collected with a BD LSR-II flow cytometer (BD Biosciences). Analysis of data was performed using FlowJo software (TreeStar).

PROTEOMICS OF MAST CELLS

Mast cells from mice infected with *Litomosoides sigmodontis* or administered a mock treatment for 12 weeks were purified by the Percoll purification protocol. Mast cells were enumerated using the Countess. Purified cells were pelleted at 400 x g for 10 minutes and supernatant was removed. Cell pellets contained 100,000-200,000 cells and were frozen at -80 °C. Samples were shipped overnight on dry ice to the Mass Spectrometry and Proteomics Resource Laboratory (MSPRL) at Harvard University in Cambridge, Massachusetts. Cells were lysed and proteins were extracted, digested, and labeled with Tandem Mass Tags (TMT) at MSPRL. Preliminary analysis reports were generated by MSPRL.

Abundance ratios of proteins were calculated based on normalized abundance values provided by MSPRL. MSPRL normalized values by dividing the abundance of a given protein by the abundance of that same protein in the first mock-treated (Mock) sample that was analyzed. We then calculated abundance ratios by dividing the average abundance of a protein in *L.s.*-infected (Infected) animals by the average of the abundance of that same protein in Mock animals. We considered ratios from 0.8-1.2 to be unchanged between Infected and Mock samples. We considered ratios below 0.8 to be down regulated in *L.s.*-infected compared to mock samples. We considered ratios above 1.2 were to be up regulated in *L.s.*-infected compared to mock samples.

Subsequent analysis of proteins that were differentially regulated was performed using STRING version 10.5 and PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System version 13.0. STRING is a database of known and predicted protein-protein interactions. Proteins are both direct (physical) and indirect (functional). The STRING database includes 9,643,763 proteins from 2,031 different organisms. PANTHER classifies proteins according to Family and subfamily, Molecular function, Biological process, or Pathway. The PANTHER database contains 77,172 functionally distinct protein subfamilies that represent 15,517 protein families.

MASS SPECTROMETRY ANALYSIS OF LIPIDS

Sphingolipid analysis was performed. Mast cells were purified using isotonic Percoll to render crude cell lysate. The samples were analyzed using an Agilent 1200 Series HPLC coupled to an ABSciex QTRAP 4000 MS. In general, 1 ml of methanol was added to 50-200 x g of cell lysate in a screw-top glass tube, after which 0.5 ml $CHCl_3$ containing internal standards (Avanti LM6002) was added. After brief bath sonication, the sample was heated overnight at 48°C. Phospholipids were hydrolyzed by the addition of 150 µl 1M KOH (methanol) and incubated at 37°C for 2 hours. After neutralization with acetic acid (12 µl glacial), 800 µl was transferred to a second tube for drying and LCB analysis using reverse-phase HPLC. The remaining sample was used for the preparation of complex sphingolipids by the addition of $CHCl_3$ and washing with water until the organic phase was clear. This portion was dried and analyzed using normal phase HPLC. Each sample is dissolved in 0.4 ml of mobile-phase solvent, sonicated to disperse, then centrifuged to clarify, and transferred to an HPLC vial for

analysis. Typically, 40 µl of each sample was injected for each analysis. MS analysis is in multiple reaction-monitoring mode.

Mobile reverse phase solvents were A) methanol–H_2O–formic acid (74:25:1, v/v/v with 10 mM Ammonium Formate) and B) methanol–formic acid (99:1,v/v with 10 mM Ammonium Formate). Mobile normal phase solvents were A) acetonitrile:methanol:acetic acid (97:2:1) (v:v:v) containing 5 mM ammonium acetate, and B) methanol:acetic acid (99:1) (v:v) containing 5 mM ammonium acetate.

For LCB analysis a reverse phase C18 column (100mm x 4.5uM) was pre-equilibrated with A:B (80:20,v/v) for 2.8 minutes at 1 ml/minute before injection. After injection, the flow was continued at 80:20 for 0.2 minutes then ramped to 100% B at 9 minute and held at 100% B for 36 minute before returning to 80:20 at 50 minutes.

For complex sphingolipid analysis, a normal phase column (2.1 x 50mm LC-NH_2 column) was pre-equilibrated for 0.5 minutes at 1.5ml/minute with 100% A. The mobile-phase NA was continued for 0.5 min followed by a linear gradient to 10% B in 0.2 minutes and held for 0.5 minutes, then increase18% B over 0.4 minutes and held at this composition for 0.6 minutes, ending with a linear gradient to 100% B over 0.4 minutes and that was held for 5 minutes.

Data were graphed and statistical analysis performed using GraphPad Prism version 7. Relevant groups were compared using the Student's t-test; $p < 0.05$ was considered significant.

CHAPTER 3: Results

SPECIFIC AIM 1: ESTABLISH AND CHARACTERIZE A MODEL OF TYPE I HYPERSENSITIVITY.

Sub-aim 1.1: Establish OVA/Alum sensitization model of systemic anaphylaxis.

Establishment of sub-lethal anaphylaxis model

In order to test the effect of helminth infection on pre-existing allergy, a number of different allergy models were explored, including two food allergy models and a sub-lethal anaphylaxis model. Ultimately, the systemic anaphylaxis model was found to be the most reliable in terms of reproducibility and consistency (data not shown). For the sub-lethal anaphylaxis model, mice were sensitized to either OVA or PBS admixed with alum by weekly intraperitoneal injections for 3 weeks (Figure 2A). Mice were challenged by intraperitoneal injections of 200 μg of OVA 2 weeks following the last date of sensitization (Figure 2A). Core body temperature was measured using a rectal thermometer once every 5 minutes for 1 hour. Mice that had been sensitized with PBS and challenged with OVA did not exhibit any significant difference in core body temperature over the course of an hour (n=4) (Figure 2B). This result was in contrast to significant drops in core body temperature observed with OVA-sensitized mice (n=3). All three OVA-sensitized mice responded to challenge by exhibiting a decrease in core body temperature. Maximal drop in core body temperature was reached by 30 minutes following challenge. OVA-sensitized mice showed signs of recovery to baseline temperatures around one hour following challenge. Sensitized mice exhibited clinical

signs that were consistent with anaphylaxis following challenge with OVA, such as labored breathing and lethargy.

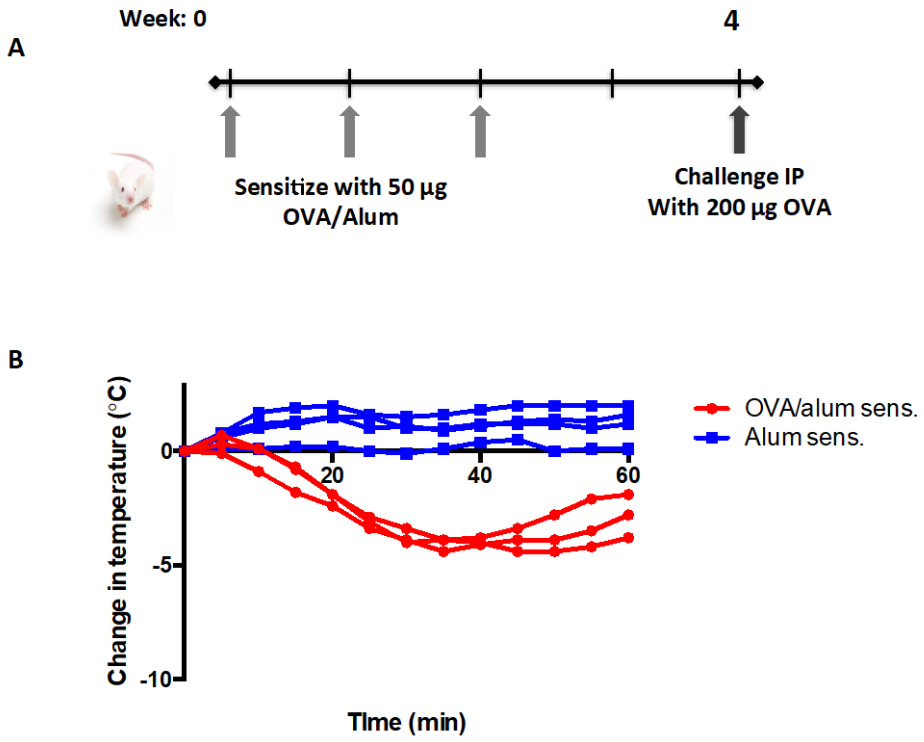


Figure 2. OVA/alum-sensitized mice undergo antigen-specific anaphylaxis in response to allergic challenge.

A) Diagram of OVA/alum sensitization regimen. BALB/c mice were sensitized once a week for 3 weeks with 50 µg of OVA in PBS admixed with alum. Mice were rested for two weeks and challenged with 200 µg of OVA by intraperitoneal injection. B) OVA/alum-sensitized mice exhibited a rapid drop in core body temperature following allergic challenge (n=3). Alum-sensitized mice maintained their core body temperature for the hour following allergic challenge (n=4).

Sub-aim 1.2: Determine the mechanism of effector response in anaphylaxis model.

Characterization of sub-lethal anaphylaxis model

In order to determine whether the sub-lethal anaphylaxis model was in fact a model for type I hypersensitivity, challenge studies were performed using transgenic mice. FcεRI knockout mice, which lack the high-affinity receptor for IgE, were used as a surrogate for IgE knockout mice. FcεRI knockout mice were compared to WT BALB/c mice in their ability to respond to OVA-challenge. Briefly, mice from each group were either sensitized with OVA/alum or PBS/alum. All groups of mice were challenged by intraperitoneal injection of 200 μg OVA after 2 weeks of rest. FcεRI knockout and BALB/c mice that were PBS-sensitized maintained their baseline core body temperature for one hour following challenge (Figure 3). In contrast, OVA-sensitized BALB/c mice responded to challenge with a rapid drop in core body temperature (Figure 3). Maximal drop in core body temperature for this group was -6.22°C and occurred at 50 minutes. Mice from this group showed signs of recovery around one hour post-challenge. OVA-sensitized FcεRI knockout mice exhibited only a mild drop in core body temperature of -1.06°C by 25 minutes and returned to baseline shortly thereafter (Figure 3). Their core body temperature over the course of an hour more closely reflected that of the PBS-sensitized mice that were either deficient in FcεRI or were of wild-type background. The serum concentration of mMCP-1 from PBS-sensitized BALB/c mice averaged 16.0 ng/ml, while serum concentration of mMCP-1 from PBS-sensitized FcεRI knockout mice averaged 63.8ng/ml (Figure 4. A). Serum from OVA-sensitized BALB/c mice was much greater than both PBS-sensitized groups at 374.34 ng/ml (Figure 4A). Serum mMCP-1 from OVA-sensitized FcεRI knockout mice appeared to be more similar to the

concentration from PBS-sensitized FcεRI knockout mice at 67.3 ng/ml (Figure 4A). Compared to OVA-sensitized FcεRI knockout mice, the average level of mMCP-1 in the serum following challenge was 5.6-fold greater.

In addition to post-challenge outcomes, pre-challenge serum levels of OVA-IgE were measured by ELISA for all groups. The average concentration of OVA-sensitized BALB/c mice was 2.3 μg/ml, while the average value of OVA-sensitized FcεRI knockout mice was approximately half of that value at 1.2 μg/ml (Figure 4B). In contrast, the average values of PBS-sensitized BALB/c and FcεRI knockout mice were merely 0.12 and 0 μg/ml respectively (Figure 4B). Although OVA-IgE levels were elevated in both OVA-sensitized groups, the amount of OVA-IgE in BALB/c mice was significantly higher than that of FcεRI knockout mice ($p=0.0084$).

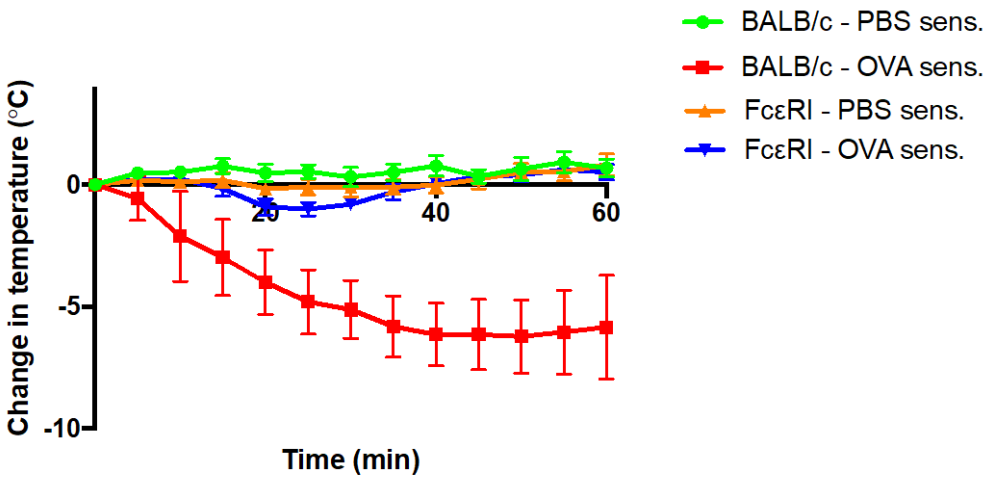


Figure 3. FcεRI is required for anaphylaxis following challenge.

A) Core body temperature was measured by rectal thermometer for 1 hour in BALB/c or FcεRI following challenge with OVA. BALB/c mice that were OVA-sensitized exhibited the most robust decrease in core body temperature, dropping 6.22 °C by 50 minutes. In contrast, FcεRI knockout mice that were OVA-sensitized only exhibited a decrease of 1.06 °C in core body temperature by 25 minutes and resolved to baseline. For all groups, n=5 mice. Results were significant by 2-way ANOVA ($p<0.0001$).

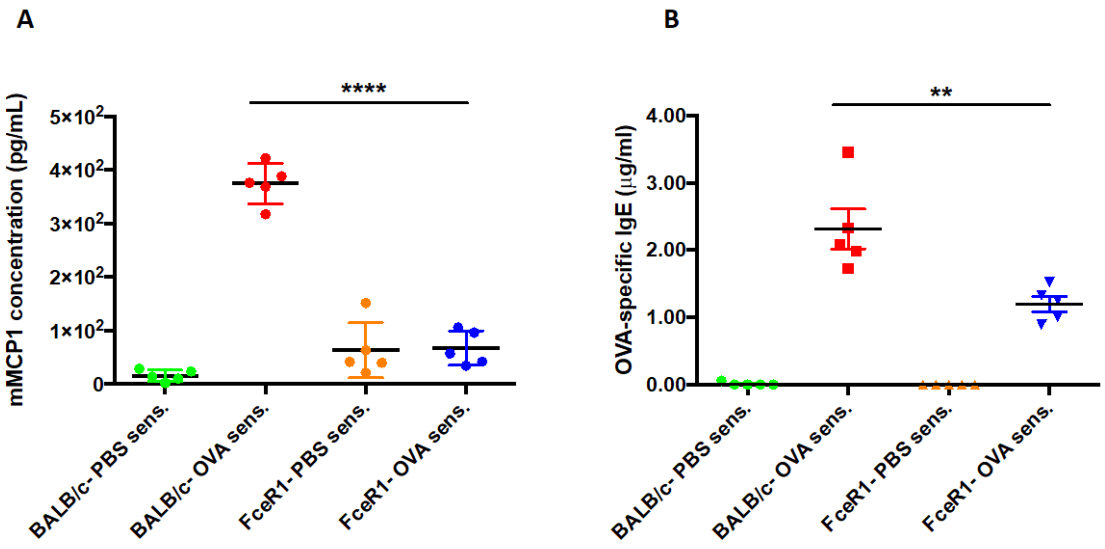


Figure 4. FcεRI is required for mMCP-1 release following challenge.

A) Serum levels of mMCP-1 were measured 1 hour following challenge with OVA. FcεRI knockout mice that were OVA-sensitized released significantly lower mMCP-1 in response to challenge compared to BABL/c mice that were OVA-sensitized. PBS/alum-sensitized BABL/c and FcεRI knockout mice served as negative controls. B) OVA-sensitized BALB/c mice demonstrated significantly higher levels of OVA-IgE than OVA-sensitized FcεRI knockout mice. Both groups of sensitized mice exhibited elevated levels of OVA-IgE compared to PBS/sensitized controls. For both experiments, n=5 per group. Relevant groups were compared by Student's t-test; p-values of <0.05 were considered significant.

Sub-aim 1.3: Determine the relative contribution of the effector cells of type I hypersensitivity in acute anaphylaxis model.

Basophil-depletion of MC-deficient and wild type mice

The respective contributions of mast cells and basophils to anaphylaxis in our model was determined through basophil-depletion of mast cell deficient and wild-type (C57BL/6J) backgrounds of mice. Mast cell-deficient mice, known as c-Kitw-sh, cKitw-sh, or sash mice, contain a mutation in the gene *Kit*. Basophils were depleted 24 hours prior to allergic challenge with the Ba103 clone, which binds to CD200R. Ba103 binds to CD200R3 on both basophils and mast cells, but selectively depletes basophils while leaving skin and peritoneal mast cells unchanged in number (85; 106). Ba103 was administered to as a single dose of 50 μg by intraperitoneal injection. An isotype control was used to account for potential off-target effects by administration of exogenous isotype-identical antibody. The strains of mice used for the experiment included the c-kit-mutant mice, which are mast cell-deficient mice of the C57BL/6J background. C57BL/6J mice were used as controls. Thus, four groups in total were evaluated: 1) wild-type mice that were isotype-control treated depleted, 2) wild-type mice that were basophil-depleted with clone Ba103, 3) mast cell-deficient mice that were isotype-control treated, and 4) mast cell-deficient mice that were basophil-depleted with clone Ba103. The experimental groups are represented in Figure 5.

Upon allergic challenge with OVA, all groups of mice exhibited a rapid drop in core body temperature. Wild-type mice that were isotype control-treated (leaving basophils and mast cells intact) exhibited the most robust drop in core body temperature,

reaching an average change in temperature of -4.12°C by 30 minutes. Wild-type mice that were Ba103 basophil-depleted (mast cells present, but no basophils) exhibited the next most robust drop in core body temperature. The average temperature decrease for this group was an average drop of -2.1°C by 30 minutes, which was significantly less than the core temperature decrease in wild-type controls that were isotype-control treated. sash mice that were isotype-control treated displayed the third least robust drop in core body temperature, with an average drop of -1.34°C by 30 minutes. Last, depletion of basophils by Ba103 treatment of sash mice had the least impact on average core body temperature drop of -0.88°C at 30 minutes post-challenge, although this difference was not significantly different from that of sash mice that were isotype-control treated.

Both histamine and mMCP-1levels were measured in mice from the above experimental groups following challenge, as shown in Figure 7. Histamine levels in the blood correlated with drops in core body temperature. Histamine levels were highest in C57BL/6J that were either isotype-treated or basophil-depleted. However, we were not able to collect samples from 4 of 5 mice in the isotype-treated group, due to their severe anaphylaxis. Thus, we were unable to discern the contribution of basophils to histamine release following challenge on the wild-type background. The average amounts of histamine released by sash mice that were either isotype- or basophil-depleted was very low. The difference in histamine released by the two groups was not statistically significant. Although not present in basophils, mMCP-1 was measured due to technical limitations in obtaining sufficient quantities of serum to measure histamine for each sample. Preliminary studies demonstrated that mMCP-1 testing was more sensitive than measurement of histamine concentrations, likely due in part to the very short half-life of

histamine (data not shown). Wild-type mice that were isotype-treated or basophil-depleted contained the greatest amount of mMCP-1 in their blood 30 minutes following challenge, and were not significantly different from one another. Likewise, levels of mMCP-1 in sash mice that were either Ba103-depleted or isotype control-treated were not significantly different and were below the limit of detection for the assay.

Additionally, pre-challenge levels of OVA-specific IgE were compared amongst groups approximately 22 hours after administration of either isotype control or Ba103 antibody (Figure 6). Serum levels of OVA-IgE from all of the mice were present in amounts sufficient to mount an anaphylactic response. The average amount of OVA-IgE from wild-type mice that were Ba103-depleted was slightly higher than that of the isotype-treated controls ($p=0.0057$) (Figure 6). In contrast, there was no significant difference in OVA-IgE levels between sash mice that were either basophil-depleted or isotype-control treated.

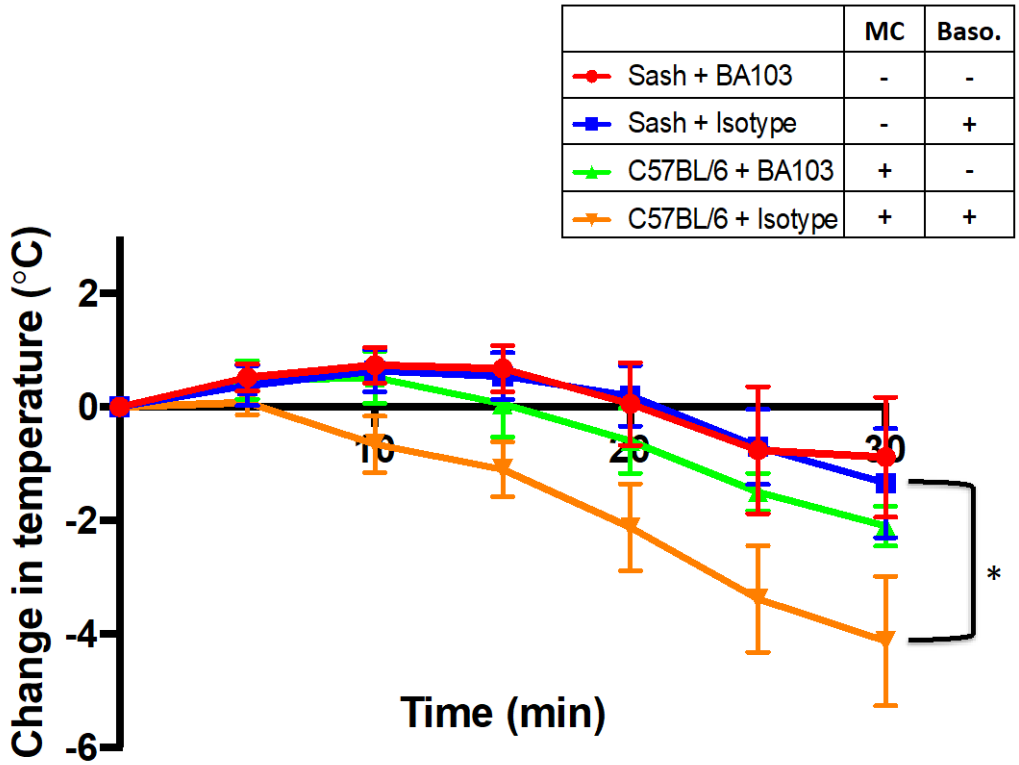


Figure 5. Anaphylaxis is primarily dependent on mast cells in OVA/alum model of sensitization.

Four groups of OVA-sensitized mice were challenged with 400 μg OVA: 1) Sash mice (mast cell-deficient) that received Ba103 to deplete basophils, 2) Sash mice that received isotype-control antibody, 3) C57BL/6 mice that received Ba103, and 4) C57BL/6 mice that received isotype-control antibody. Fifty μg of Ba103 or isotype control were administered 24 hours prior to challenge. C57BL/6 mice that were isotype-control treated served as a positive control for maximal anaphylaxis and exhibited the most dramatic decrease in core body temperature. C57BL/6 mice that were Ba103 basophil-depleted exhibited significantly less of a drop in core body temperature. However, sash mice that were isotype-treated exhibited even less of a drop in core body temperature, indicating that mast cells contribute more to anaphylaxis than basophils. While sash mice that were Ba103-depleted exhibited slight less of a decrease in core body temperature than sash mice that were isotype-control treated, the difference was not significant. Comparison of curves were made by 2-way ANOVA, with significance determined as $p<0.05$. For each group, n=5 mice.

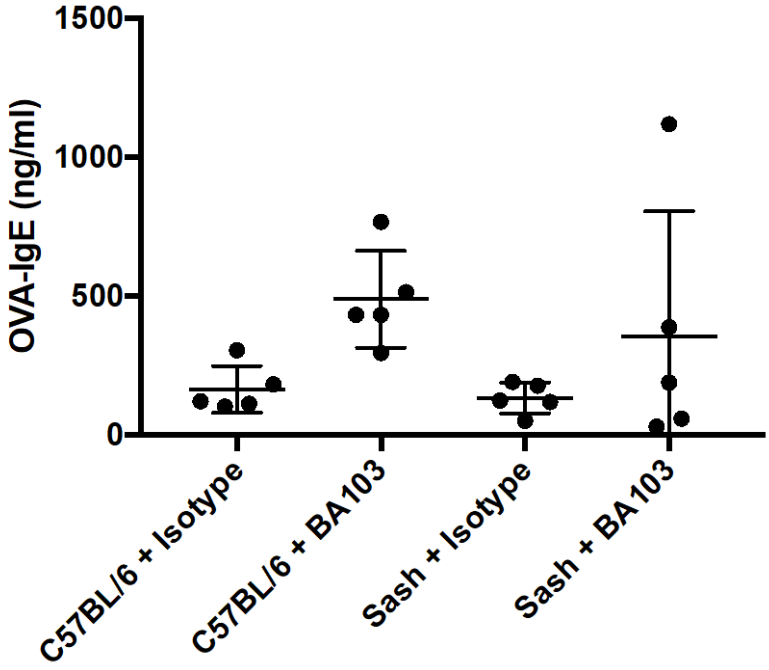


Figure 6. OVA-IgE is present in the serum of OVA-sensitized C57BL/6 and Sash mice

OVA-IgE in the serum was compared amongst the indicated groups 22 hours following administration of Ba103 antibody or isotype control. The average amount of OVA-IgE from wild-type mice that were Ba103 basophil-depleted was significantly higher than OVA-IgE in the isotype-treated controls ($p=0.0057$). In contrast, there was no significant difference in OVA-IgE levels between sash mice that were either basophil-depleted or isotype-control treated.

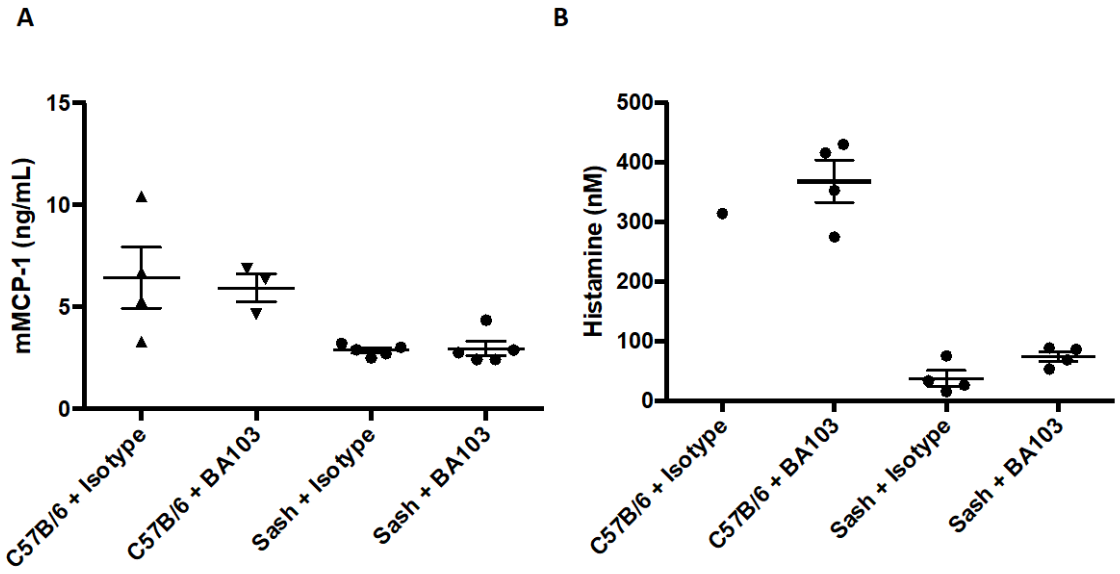


Figure 7. Mast-cell deficient mice release less histamine and mMCP-1 following allergic challenge

Levels of mMCP-1 (A) and histamine (B) were measured in serum 30 minutes following allergic challenge. As expected, mMCP-1 was barely detectable in sash mice that were either isotype-control treated or Ba103 basophil-depleted. Histamine levels were also barely detectable in these groups. On the other hand, isotype-control treated or Ba103 basophil-depleted C57B/6 mice exhibited higher levels of mMCP-1 and histamine. Results represent n=5 mice per group.

SPECIFIC AIM 2: DETERMINE IF CHRONIC HELMINTH INFECTION PROTECTS AGAINST PRE-EXISTING TYPE-I (IMMEDIATE) HYPERSENSITIVITY.

Sub-aim 2.1: Evaluate whether infection protects against anaphylaxis at 12 weeks post-infection (chronic infection).

Preliminary experiments

To directly address the question of whether helminths are protective against anaphylaxis, mice were sensitized by weekly intraperitoneal injection of either OVA/alum or PBS/alum once a week for 3 weeks (Weeks 0-2). At Week 4, mice were *L.s.*- or mock-infected. Ten weeks post-infection, immunological and clinical parameters were measured before and after intraperitoneal challenge with OVA. In sensitized mice, chronic *L.s.* infection resulted in serum levels of OVA-specific IgE that were an average of 50% lower than those observed for mock-infected mice (6,296 vs. 13,056 pg/mL, p-value= 0.1494) (data not shown). Following challenge, serum levels of mMCP-1 were significantly lower in sensitized mice that were *L.s.*-infected as compared to mock infected (86,276.81 vs. 276,635.2 pg/ml, p-value= 0.0385) (data not shown). With respect to clinical signs, *L.s.*-infected mice that had been previously sensitized exhibited an average drop in core body temperature of -4.3°C after 1 hour (data not shown). Although less than the average -6.3°C drop observed in sensitized and mock-infected mice, the difference was not statistically significant.

More than thirty studies have reported that helminth protection protects against allergic sensitization (30). We attempted to confirm this finding using our model. Again, we observed about a 1.5°C difference in core body temperature drop between *L.s.*- and

mock-infected mice (data not shown). Given the inherent variation of our outcome metric (temperature drop), we decided to perform a power to study to determine the minimum number of mice required to confirm whether or not the observed 1.5°C difference in temperature was statistically significant. The power calculation indicated a need for 12 mice per group for experiments using core body temperature as an outcome. In order to ensure enough mice at study endpoints, groups consisted of 15 mice per group in subsequent studies. Because chronicity of infection appeared to influence trends toward protection in other preliminary experiments, the duration of infection was extended to 12 weeks.

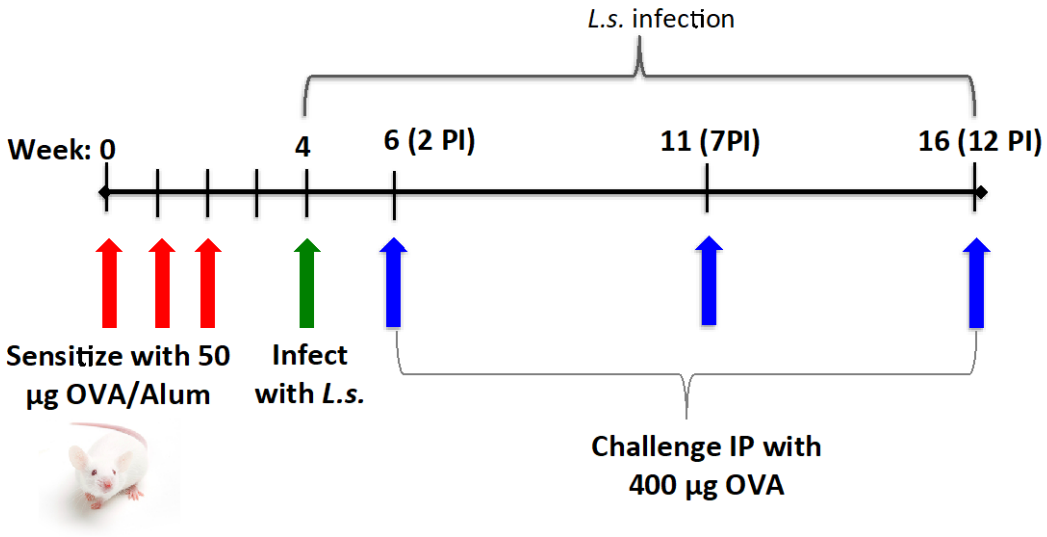


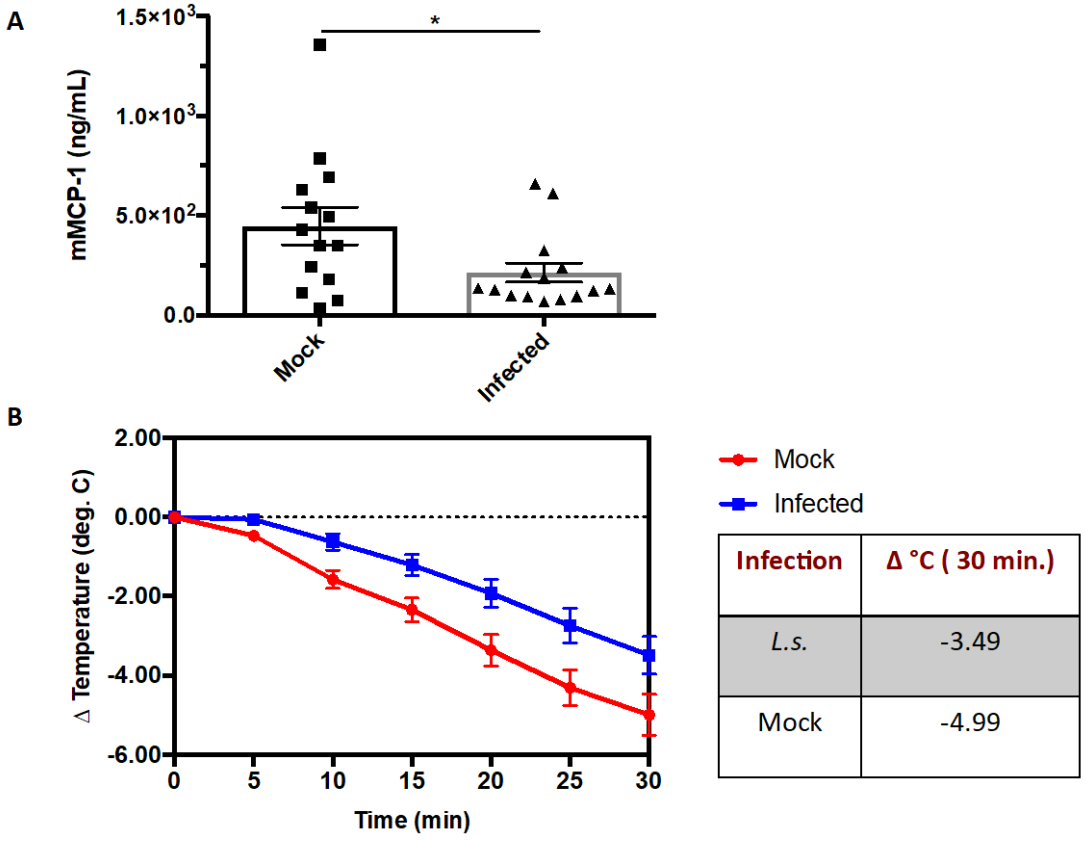
Figure 8. Timeline for evaluating the therapeutic effect of helminths on allergy

For experiments to determine the effect of helminth infection on pre-existing allergy, BALB/c mice were sensitized once a week for 3 weeks with 50 µg of OVA/alum. At week 4, mice were infected with infective-stage *L.s.* or administered a mock infection. In three separate experiments, cohorts of mice were challenged at 2, 7 or 12 weeks post-infection (n=15 mice per group, per time point). Mice were challenged by intraperitoneal injection of 400 µg of OVA and clinical and immunological parameters were measured.

Challenge at 12 weeks post-infection

Mice were challenged by intraperitoneal injection with OVA during the twelfth week of infection (n= 15 per group, Figure 8). mMCP-1 was measured in the serum 30 minutes following challenge (Figure 9A). Serum levels of mMCP-1 were significantly lower in sensitized mice that were *L.s.*-infected as compared to mock-infected mice ($p= 0.0316$). Additionally, *L.s.*-infected mice that had been previously sensitized exhibited an average drop in core body temperature of -3.5°C after 30 minutes, which was significantly less than the -5.0°C decrease observed in OVA-sensitized controls, as measured by 2-way ANOVA ($p<0.05$) (Figure 9B).

Pre-challenge serum measurements revealed that OVA-IgE levels were not significantly affected by infection with *L.s.* at 12 weeks post-infection (Figure 10A). However, OVA-IgG1 levels were significantly reduced in mice that were chronically infected with *L.s.* as compared to mock-infected controls (Figure 10B). IgG1 is the murine equivalent of human IgG4, which is found in circulation and can function as a blocking antibody to prevent binding of antigen to the surface of allergy effector cells. We tested IgG1 levels in our model because allergens and certain helminths are typically good inducers of human IgG4 (114). While we found OVA-IgG1 levels to be decreased in the setting of chronic infection, we would expect total IgG1 levels to be elevated. We will consider testing total levels of IgG1 in the future.



Infection	Δ °C (30 min.)
L.s.	-3.49
Mock	-4.99

Figure 9. *L. sigmodontis* protects against anaphylaxis at 12 weeks post-infection

BALB/c were either administered a mock infection (Mock) or infected with *L.s.* (Infected). At 12 weeks post-infection, mice were challenged with 400 µg OVA by intraperitoneal injection (n=15 mice per group). A) Average mMCP-1 in the serum was significantly lower in Infected mice compared to Mock controls at 30 minutes post-challenge as measured by ELISA ($p=0.0316$). B) Core body temperature was measured in Mock and Infected mice for 30 minutes following challenge. Changes in temperature were significantly different between the two groups as determined by statistical analysis using 2-way ANOVA ($p<0.05$).

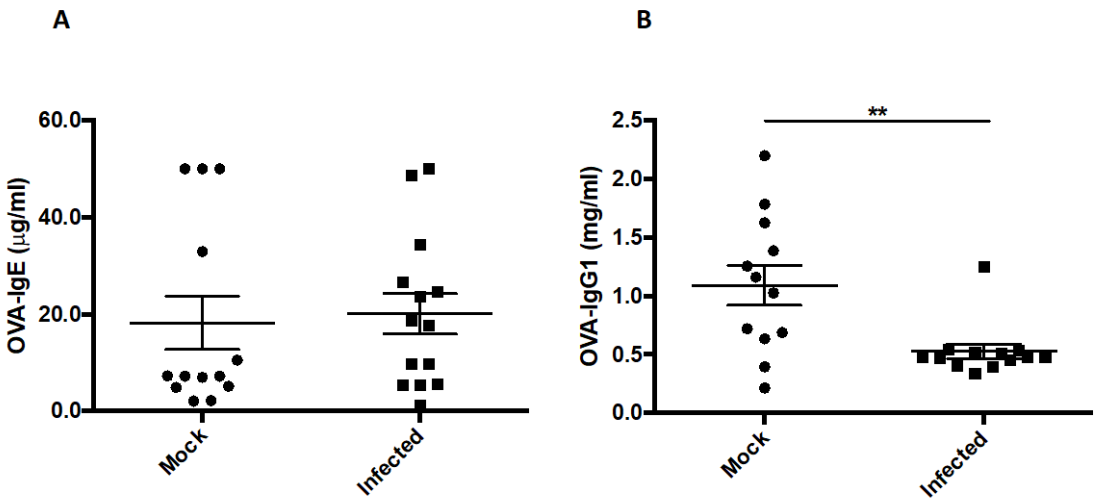


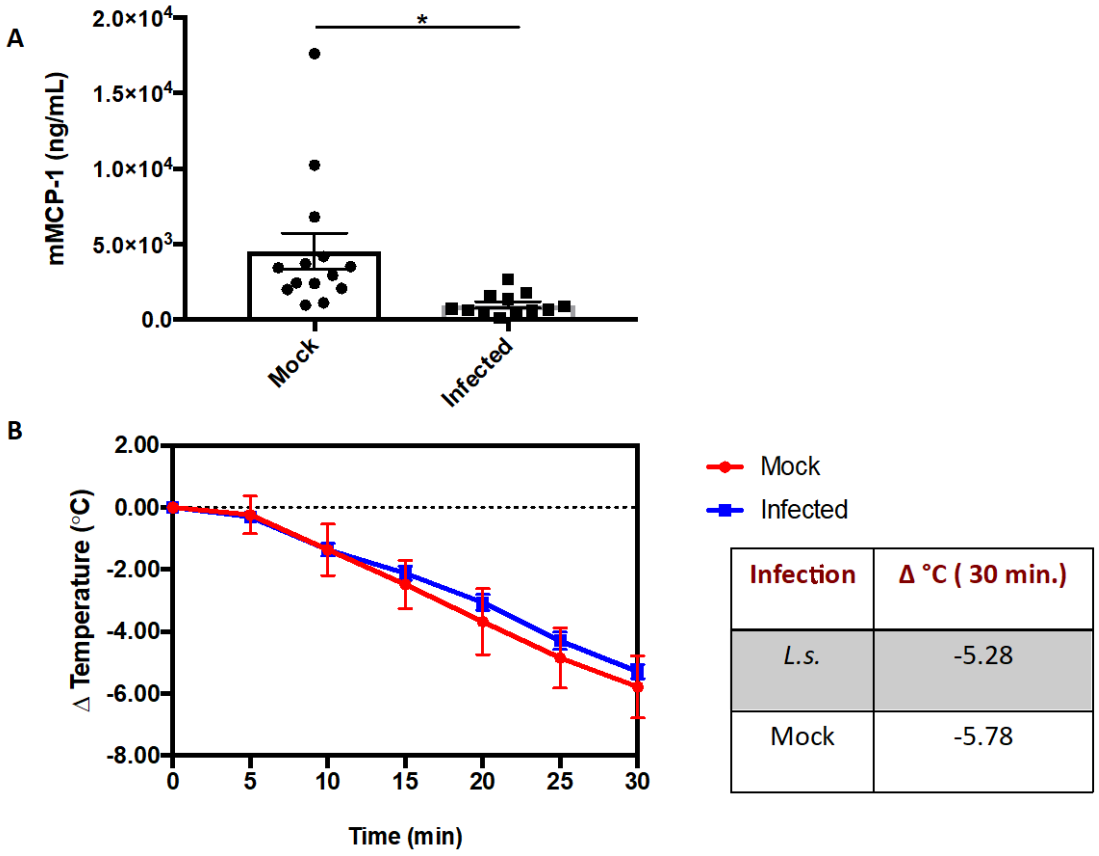
Figure 10. Chronic infection with *L. sigmodontis* does not affect OVA-IgE levels, but decreases OVA-IgG1 at 12 weeks post-infection

A) OVA-IgE levels in pre-challenge sera of mice were not different between mice that were OVA-sensitized and either administered a mock infection (Mock) or infected with *L.s.* (Infected). B) OVA-IgG1 levels were significantly decreased in Infected as compared to Mock mice (p=0.0066). For both experiments, n=15 mice per group.

Sub-aim 2.2: Evaluate whether infection protects against anaphylaxis at 7 weeks (chronic) time point.

Challenge at 7 weeks post-infection

In order to determine whether chronic helminth infection could protect against clinical signs of anaphylaxis at an earlier time point, mice were sensitized and *L.s.* or mock infected (n=15 per group). A timeline of the experiment is shown in Figure 8. Infection was established and allowed to progress until 7 weeks post-inoculation. Mice were challenged with 400 μg of OVA by intraperitoneal injection and core body temperature was measured every 5 minutes for 30 minutes. Change in core body temperature was plotted for each group and the difference between the two groups was analyzed by 2-way ANOVA. A p-value of less than 0.05 was considered significant. There was no significant difference between two groups at the 7-week time point (Figure 11B). The concentration of mMCP-1 was measured in the serum 30 minutes following challenge and was found to be significantly lower in *L.s.*-infected as compared to mock-infected mice (p=0.012) (Figure 11A). The concentrations of OVA-IgE and OVA-IgG1 antibodies were measured in pre-challenge serum at the 7-week time point. The concentration of OVA-IgE was significantly elevated in the setting of chronic *L.s.* infection (p=0.0156) (Figure 12A). In contrast, the concentration of OVA-IgG1 was significantly lower in the setting of chronic *L.s.* infection as compared to mock-infected controls (p=0.0359) (Figure 12B).



Infection	Δ °C (30 min.)
L.s.	-5.28
Mock	-5.78

Figure 11. *L. sigmodontis* impairs mast cell degranulation, but does not protect against hypothermia at 7 weeks post-infection

BALB/c were either administered a mock infection (Mock) or infected with *L.s.* (Infected). At 7 weeks post-infection, mice were challenged with 400 µg OVA by intraperitoneal injection (n=15 mice per group). A) Serum levels of mMCP-1 were compared by ELISA between Mock and Infected mice 30 minutes following challenge. Average mMCP-1 was significantly lower in Infected as compared to Mock mice ($p=0.012$). B) Core body temperature was measured in Mock and Infected mice every 5 minutes for 30 minutes following challenge. There was no significant difference in change in core body temperature as analyzed by 2-way ANOVA using a p-value of <0.05 to determine significance.

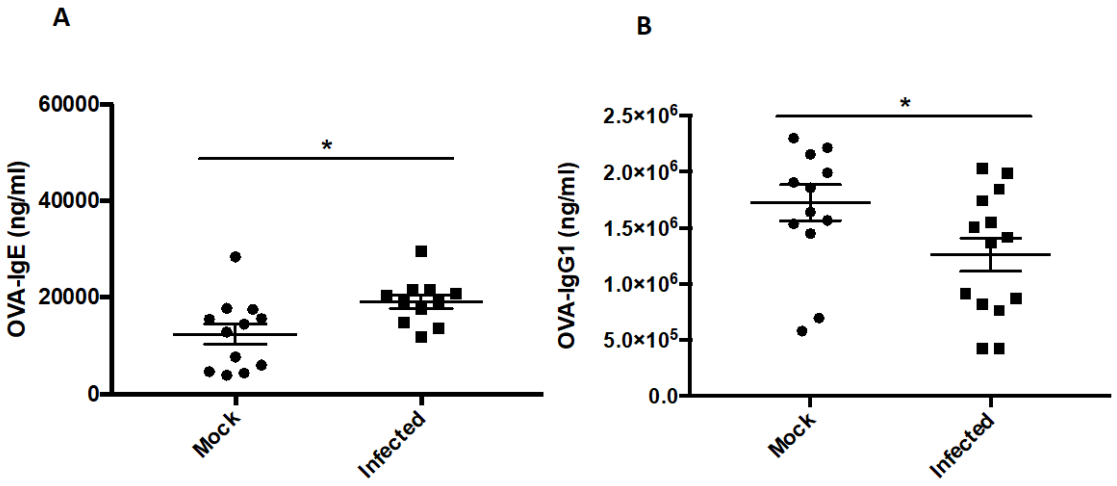


Figure 12. *L. sigmodontis* increases OVA-IgE, but decreases OVA-IgG1 at 7 weeks post-infection

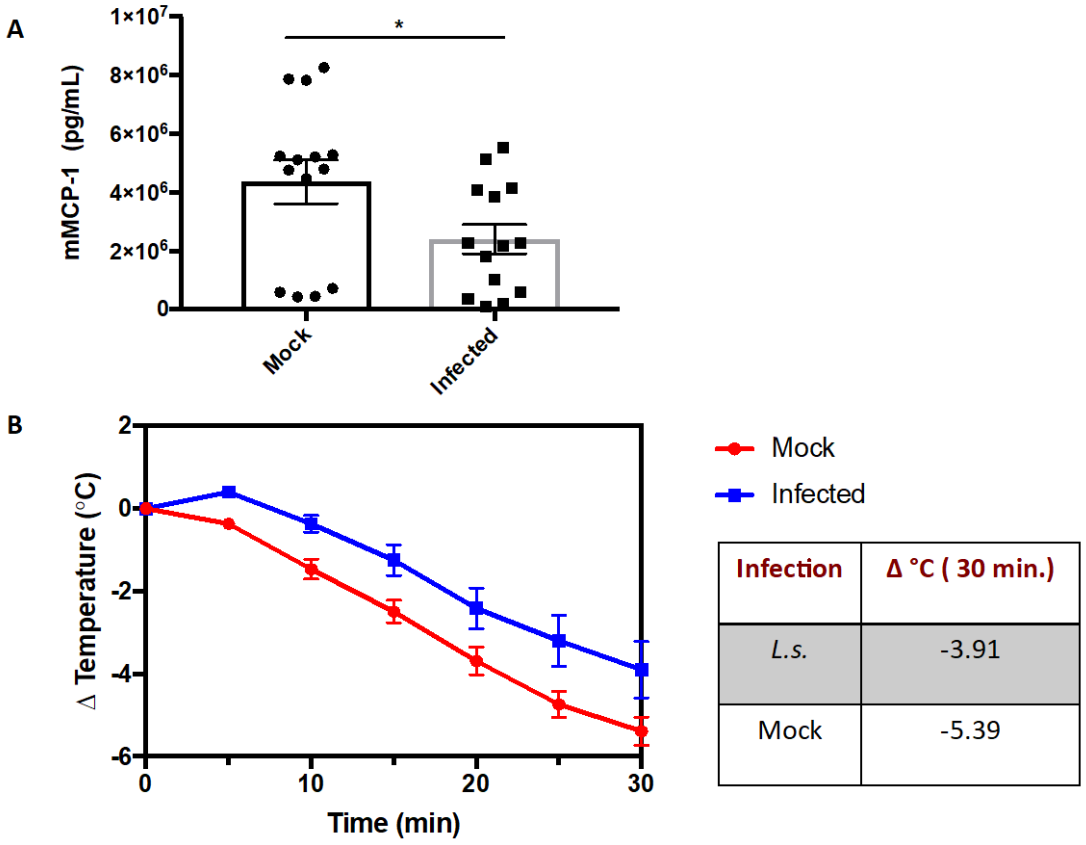
Pre-challenge sera were collected from mice that were OVA-sensitized and either administered a mock infection (Mock) or infected with *L.s.* (Infected) and compared by ELISA (n=15 mice per group). A) Average OVA-IgE was significantly increased in Infected as compared to Mock mice (p=0.0156). B) Average IgG1 was significantly decreased in Infected as compared to Mock mice (p=0.359).

Sub-aim 2.3: Evaluate whether infection protects against anaphylaxis at 2 weeks post-infection (acute) time point.

Challenge at 2 weeks post-infection

OVA-sensitized BALB/c mice that were either mock-or *L.s.*-infected were challenged with OVA at 2 weeks post-infection (n=15 per group), as outlined in Figure 8. OVA-sensitized mice that were administered a mock infection responded to challenge by dropping an average of 5.39 °C by 30 minutes (Figure 13B). In contrast, OVA-sensitized mice that were infected with *L.s.* responded to challenge by dropping an average of 3.9°C by 30 minutes; a statistically significant difference (Figure 13B). Levels of mMCP-1 were measured in the serum 30 minutes following challenge. The concentration of mMCP-1 in the sera of mock-infected averaged 4.35 x 10^6 pg/ml, whereas, the concentration of mMCP-1 in the sera of *L.s.*-infected mice was significantly lower at 2.39 x 10^6 pg/ml (Figure 13A). The difference was statistically significant by Student's t-test (p=0.0387).

Pre-challenge serum levels of OVA-IgE were measured in both Mock and Infected groups. There was no significant difference in OVA-IgE levels between the two groups (Figure 14). Although not significant, the average level of OVA-IgE trended higher in infected mice than in mock controls.



Infection	Δ °C (30 min.)
L.s.	-3.91
Mock	-5.39

Figure 13. *L. sigmodontis* protects against anaphylaxis at 2 weeks post-infection

OVA-sensitized BALB/c were either administered a mock infection (Mock) or infected with *L.s.* (Infected). At 2 weeks post-infection, mice were challenged with 400 µg OVA by intraperitoneal injection (n=15 mice per group). A) Serum was collected from mice 30 minutes following challenge. Mast cell degranulation was impaired in the setting of acute helminth infection at 2 weeks post-infection, as mMCP-1 levels were significantly lower in Infected mice ($p=0.0387$). B) Acute helminth infection protected against hypothermia at 2 weeks post-infection with *L.s.* (2-way ANOVA, $p<0.05$).

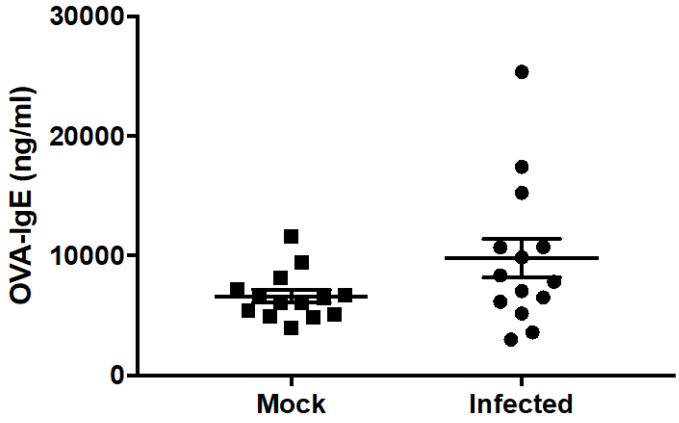


Figure 14. *L. sigmodontis* does not significantly alter OVA-IgE at 2 weeks post-infection

Pre-challenge sera were collected from mice that were OVA-sensitized and either administered a mock infection (Mock) or infected with *L.s.* (Infected) and compared by ELISA (n=15 mice per group). Average OVA-IgE levels were not significantly altered in Infected as compared to Mock mice; however, the average concentration in Infected mice trended slightly higher than in Mock.

SPECIFIC AIM 3: EVALUATE POTENTIAL MECHANISMS OF *L.S.* PROTECTION AGAINST ANAPHYLAXIS AT 12 WEEKS POST-INFECTION.

Sub-aim 3.1 Determine if IL-10 is the mechanism for protection against anaphylaxis at 12 weeks post-infection.

The role of IL-10 at 12 weeks

IL-10 knockout mice were sensitized with OVA/alum and separated into two groups. Mice from each group were either infected with *L.s.* (n=8) or administered a mock injection with media (n=7). At 12 weeks post infection, mice were challenged and core body temperature was measured by rectal thermometer every 5 minutes for 30 minutes. IL-10 knockout mice that were infected with *L.s.* were significantly protected against hypothermia by 2-way ANOVA ($p < 0.05$) compared to IL-10 knockout mice that were administered a mock treatment (Figure 15A). Furthermore, mast cell degranulation in IL-10 knockout mice, as measured by release of mMCP-1, was significantly impaired in *L.s.*-infected mice compared to mock-treated ($p=0.042$) (Figure 15B).

OVA-specific IgE was measured in pre-challenge serum from IL-10 knockout mice that were first sensitized with OVA/alum and then infected with *L.s.* or administered a mock infection. There was no significant difference in OVA-IgE between the two groups (Figure 16A). OVA-IgG1 was also measured in pre-challenge serum from the same two experimental conditions. Mice that were *L.s.*-infected had significantly lower levels of OVA-IgG1 than levels in mice that were administered a mock treatment (Figure 16B).

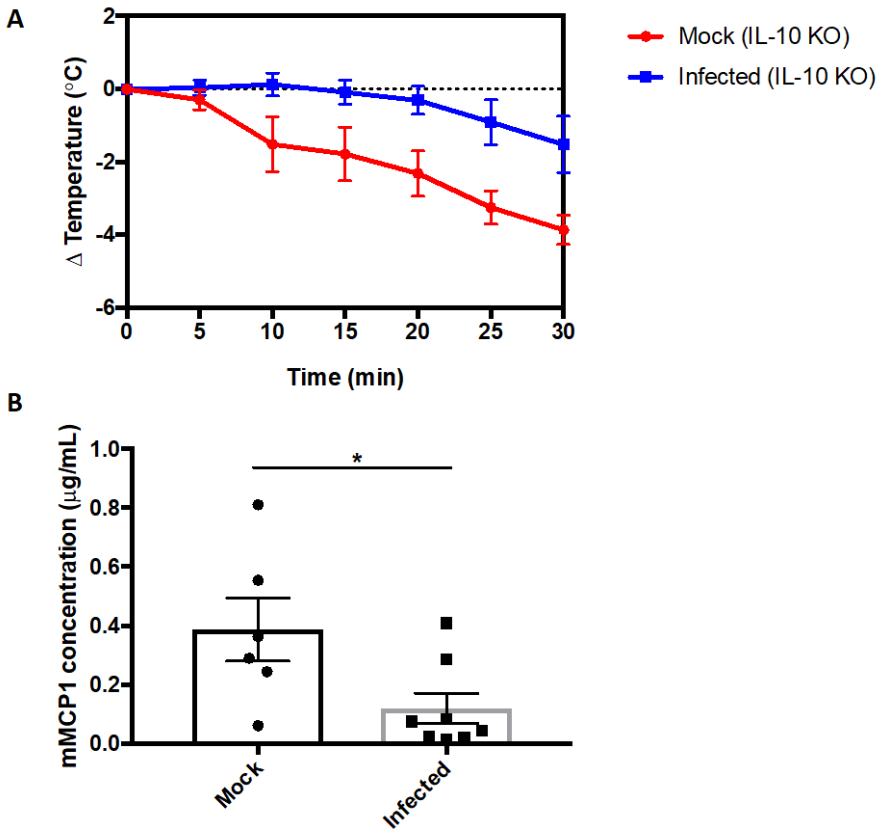


Figure 15. IL-10 is not required for protection against anaphylaxis at 12 weeks post-infection with *L. sigmodontis*

A) Core body temperature was measured by rectal probe for 30 minutes following intraperitoneal challenge with 400 μg OVA in IL-10 knockout mice. Change in core body temperature was significantly different between *L.s.*-infected (Infected) and mock-infected (Mock) mice by 2-way ANOVA ($p<0.05$). B) Levels of murine mast cell protease 1 (mMCP-1) in the serum of mice 30 minutes following challenge were measured by ELISA and were found to be significantly lower in IL-10 knockout mice that were infected with *L.s.* compared to mock-treated controls ($p=0.042$). Comparison of mMCP-1 levels was conducted by Student's t-test.

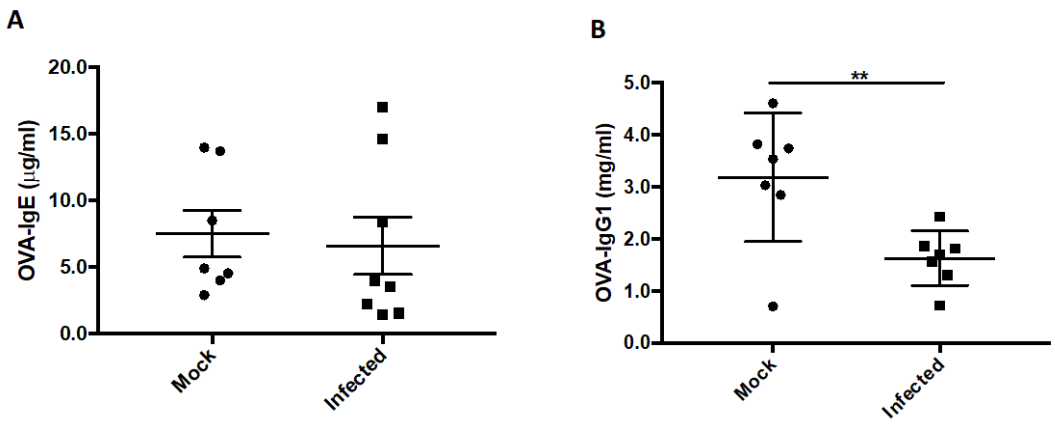


Figure 16. OVA-IgE levels in the serum were not significantly different in IL-10 knockout mice infected with *L. sigmodontis*, but OVA-IgG1 levels were decreased compared to mock controls

A) OVA-IgE levels were measured in pre-challenge serum 24 hours prior to allergic challenge of mice. OVA-IgE levels were not significantly different between mock- (Mock) and *L.s.*-infected (Infected) mice. B) OVA-IgG1 was measured in the sera of pre-challenge mice. OVA-IgG1 levels were significantly decreased in mice that were infected with *L.s.* for 12 weeks (Infected) compared to mice that were administered a mock infection ($p<0.05$). Statistical analysis was performed by Student's t-test.

Sub-aim 3.2: Determine if infection alters cytokine response to allergen.

Evaluation of cytokines at 12 weeks post-infection

BALB/c mice were sensitized with OVA/alum according to the previously described sensitization regimen. At week 4, mice were infected with *L.s.* or administered a mock treatment with media (n=6 per group). Infection progressed until 16 weeks (12 weeks post-infection) at which point mice were euthanized. Ex vivo stimulation of splenocytes was performed using the following stimuli and controls: media, anti-CD3/anti-CD28, and 10 μg of OVA. Anti-CD3/anti-CD-28 stimulation was used as a positive control. Cytokines were measured in the supernatants of cell cultures 72 hours following stimulation. Using a BioPlex assay, Th1/Th2 skewing was measured using a panel of antibodies to quantify the following cytokines: interleukin (IL) 4, IL-5, IL-6, tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), and IL-12p70. In addition, commercially available ELISA kits were used to measure the regulatory cytokines IL-10 and TGF-β.

There was a significant increase in TNF-α in OVA-stimulated samples as compared to controls at 72 hours (Figure 17). However, there was no significant difference in TNF-α levels between Mock and Infected groups at this time point (Figure 19). There was no significant difference in IFN-γ levels between media- and OVA-stimulated groups (Figure 17). Unsurprisingly, there was also no significant difference in IFN-γ levels between mock- and *L.s.*-infected mice (Figure 19). Additionally, there was no significant difference in IL-6 or IL-12p70 levels secreted from splenocytes that underwent OVA-stimulation as compared to media-stimulation (Figure 18).

In terms of classical Th2 cytokines, IL-4 and IL-5 were both significantly elevated above media controls in response to stimulation with OVA (Figure 17). While there was no significant difference in IL-4 levels between mock- and *L.s.*-infected mice at 72 hours, there was a significant difference in IL-5 (Figure 19). In fact, IL-5 was significantly decreased in mice that were chronically infected as compared to mock-treated controls ($p=0.0095$).

With respect to the immunoregulatory cytokines, there was no significant difference in IL-10 between samples that were stimulated with OVA (10 μg/mL) as compared to media-stimulated controls (data not shown). Because the baseline IL-10 from media-stimulated splenocytes from mock-infected mice was significantly higher than that from *L.s.*-infected mice ($p=0.0396$), we compared the amount of IL-10 secreted by *L.s.*- or mock-infected mice. However, there was no significant difference between the two groups.

Both active and total TGF-β levels in supernatant were assayed following stimulation of splenocytes. Active TGF-β was elevated in OVA-stimulated splenocytes compared to media-stimulated controls ($p=0.0141$) (Figure 18). However, there was no significant difference in active TGF-β levels in the supernatants of stimulated splenocytes from *L.s.*- and mock-treated mice (Figure 20). Total TGF-β was measured according to manufacturer's protocol. Basal difference (media stimulation) in total TGF-β was slightly higher in *L.s.*-infected mice compared to mock-infected animals, but not statistically significant (data not shown).

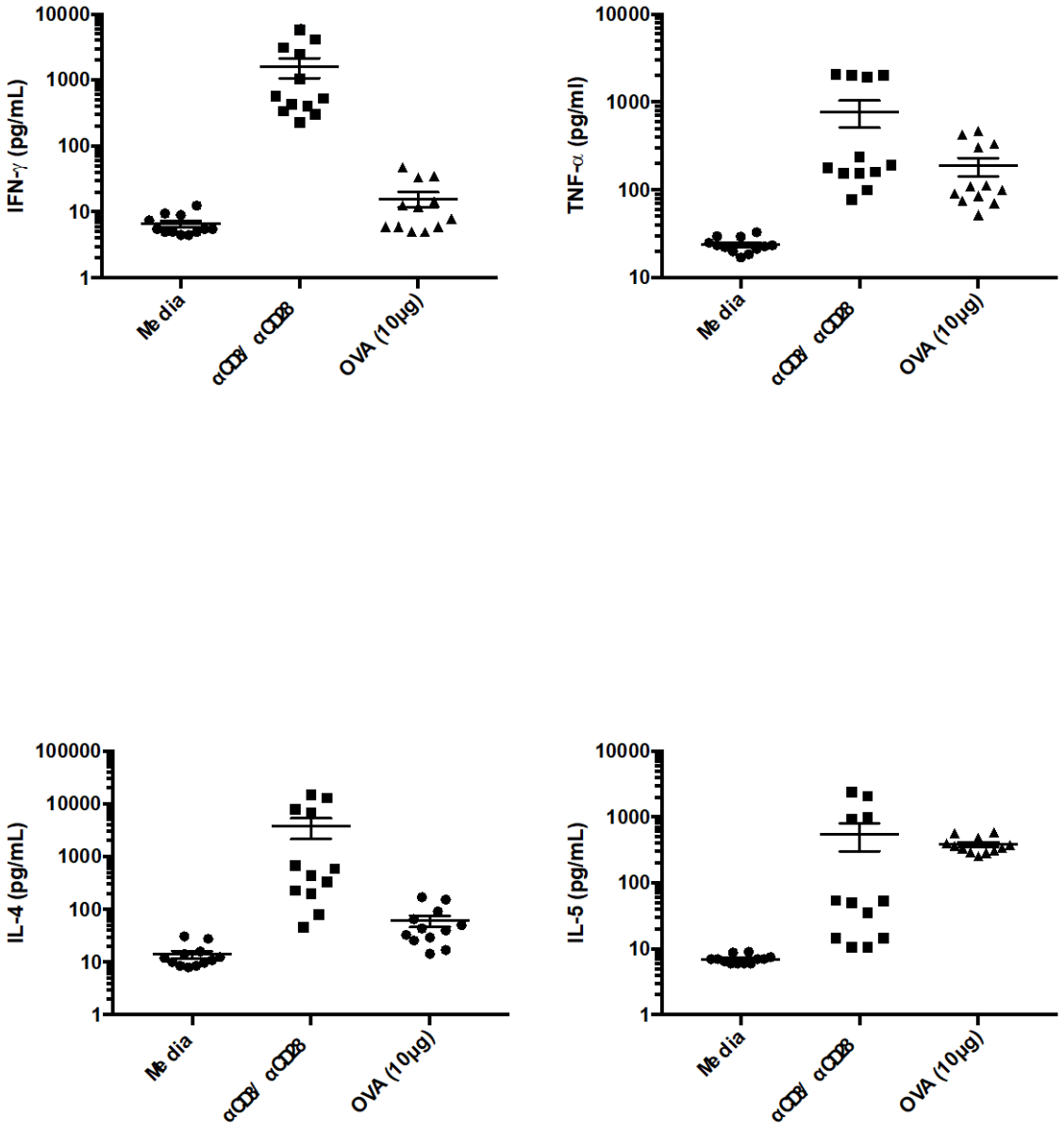


Figure 17. Stimulation of splenocytes from sensitized mice results in cytokine secretion.

Splenocytes were isolated from mice that were chronically infected with *L.s.* for 12 weeks or were mock infected. Cells were cultured and stimulated for 72 hours with media, αCD3/ αCD28 or OVA (10 µg/ml). Data for *L.s.*- and mock-infected mice were graphed together. IFN- γ (top left), TNF-α (top right), IL-4 (bottom left) and IL-5 (bottom right) were all significantly elevated above media stimulation in response to stimulation with αCD3/ αCD28 or OVA. Statistical analysis was performed by Student's t-test ($p<0.05$) on untransformed data.

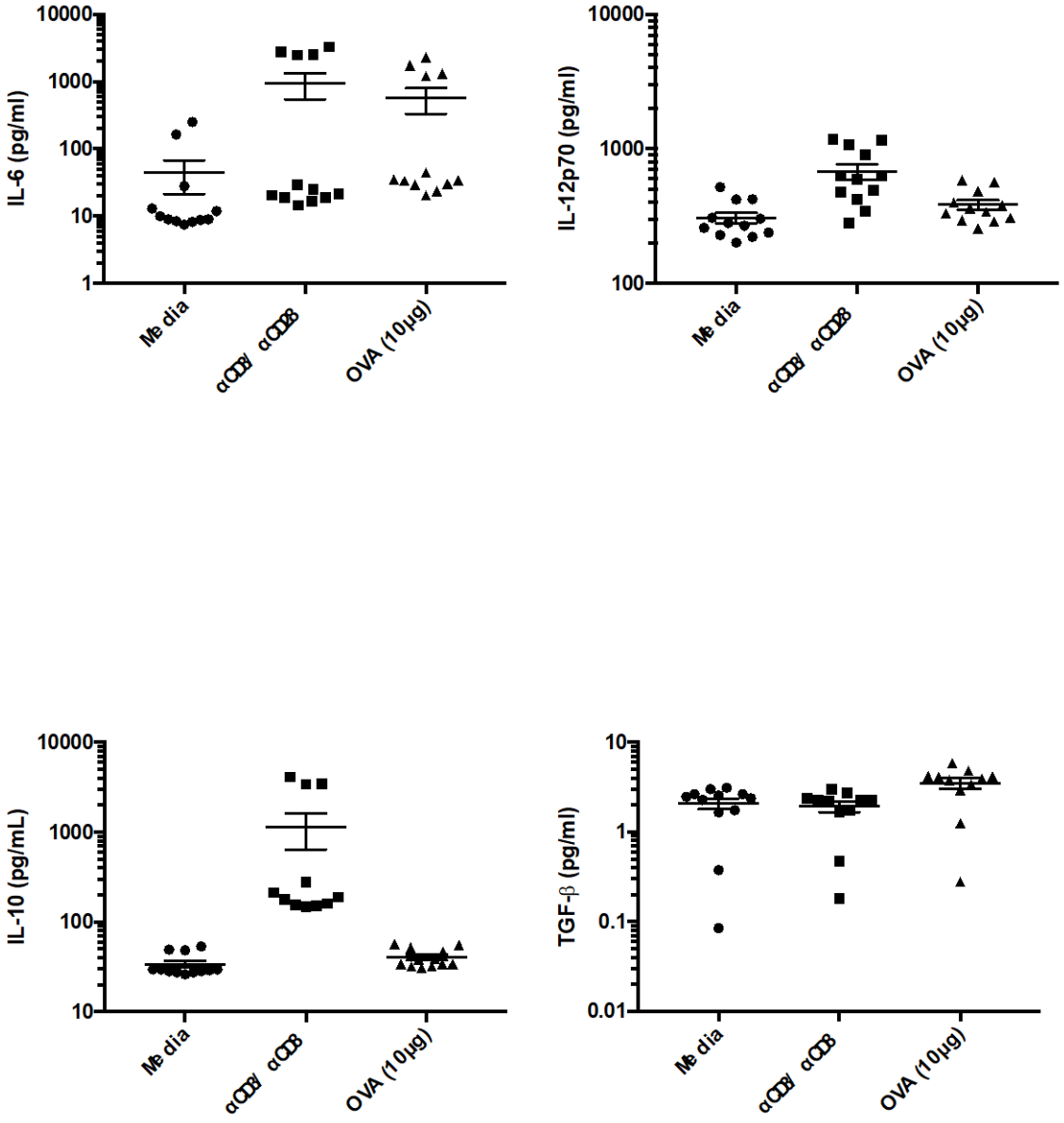


Figure 18. Stimulation of splenocytes from sensitized mice results in cytokine secretion.

Splenocytes were isolated from mice that were chronically infected with *L.s.* for 12 weeks or were mock infected (n=12). Cells were cultured and stimulated for 72 hours with media, αCD3/ αCD28 or OVA (10 μg/ml). Data for *L.s.*- and mock-treated mice were graphed together. Splenocytes responded to stimulation with αCD3/ αCD28 IL-6 (top left), IL12p70 (top right), and IL-10 (bottom left), but not TGF-β (bottom right). IL-6, IL-12p70, IL-10, and TGF-β were secreted in response to OVA stimulation. Statistical analysis was performed by Student's t-test ($p<0.05$) on untransformed data.

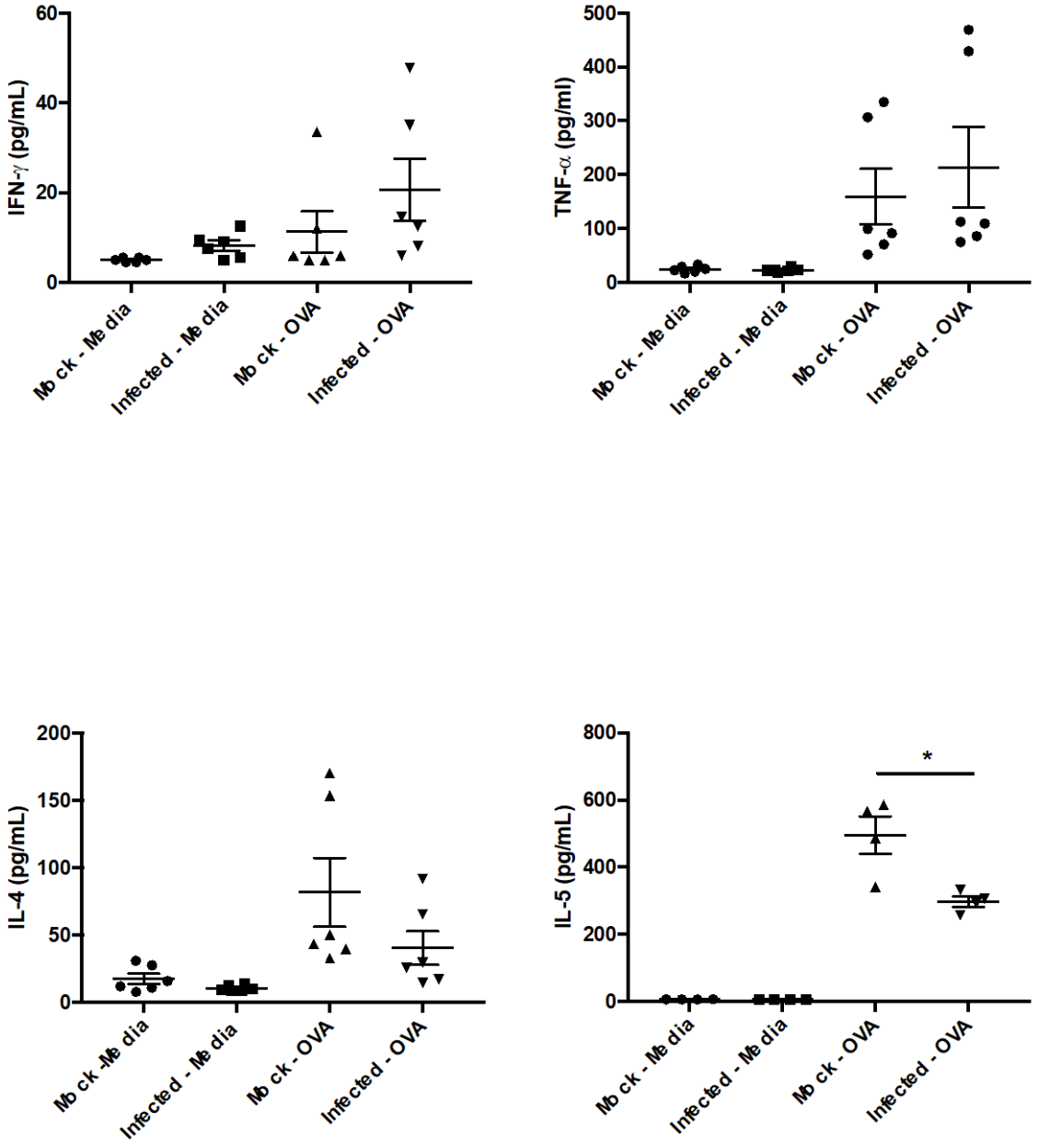


Figure 19. Cytokine profile of *L.s.*- and mock-infected mice at 12 weeks.

Splenocytes were isolated from mice that were chronically infected with *L.s.* for 12 weeks or were mock-treated(n= 6 per group). There was no significant difference in IFN- γ (top left), TNF-α (top right), and IL-4 (bottom left) secretion in response to stimulation with OVA (10 μg) between Mock and Infected mice. In contrast, IL-5 levels were lower following stimulation with OVA in Infected mice compared to Mock controls. Statistical analysis was performed by Student's t-test (p<0.05).

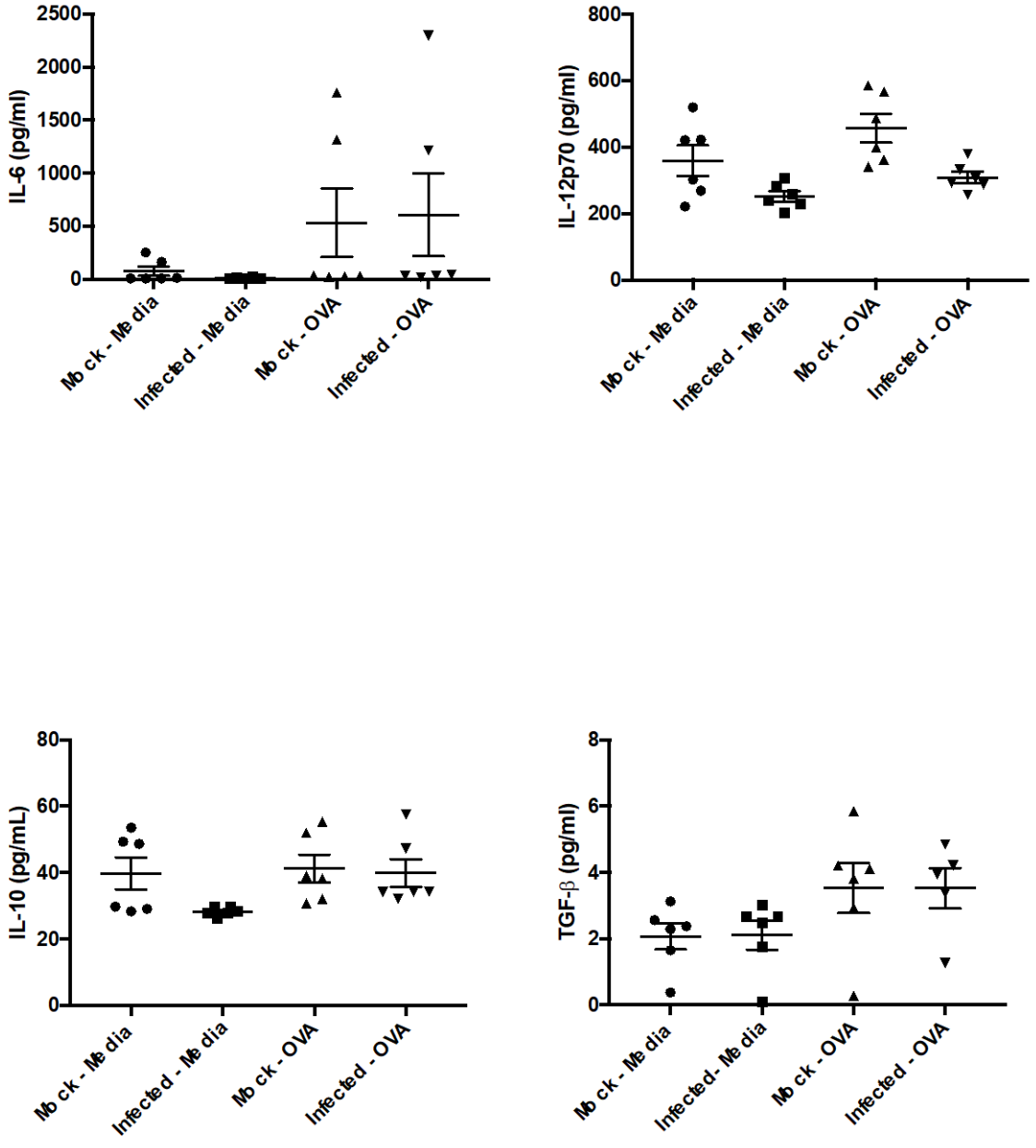


Figure 20. Cytokine profile of *L.s.*- and mock- infected mice at 12 weeks.

Splenocytes were isolated from mice that were chronically infected with *L.s.* for 12 weeks or were mock-treated(n= 6 per group). There was no significant difference in IL-6 (top left), IL-12p70 (top right), IL-10 (bottom left), and TGF-β (bottom right) from Mock and Infected mice stimulated with OVA. Statistical analysis was performed by Student's t-test ($p<0.05$).

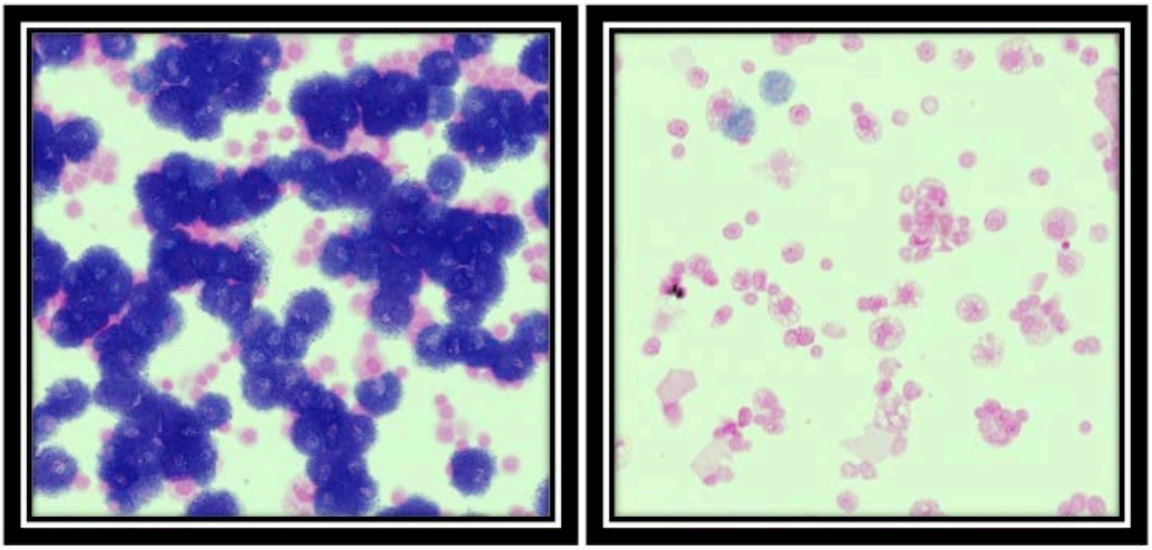
Sub-aim 3.3: Determine if changes in mast cells occur at 12 weeks post-infection.

Cell counts, histology and electron microscopy

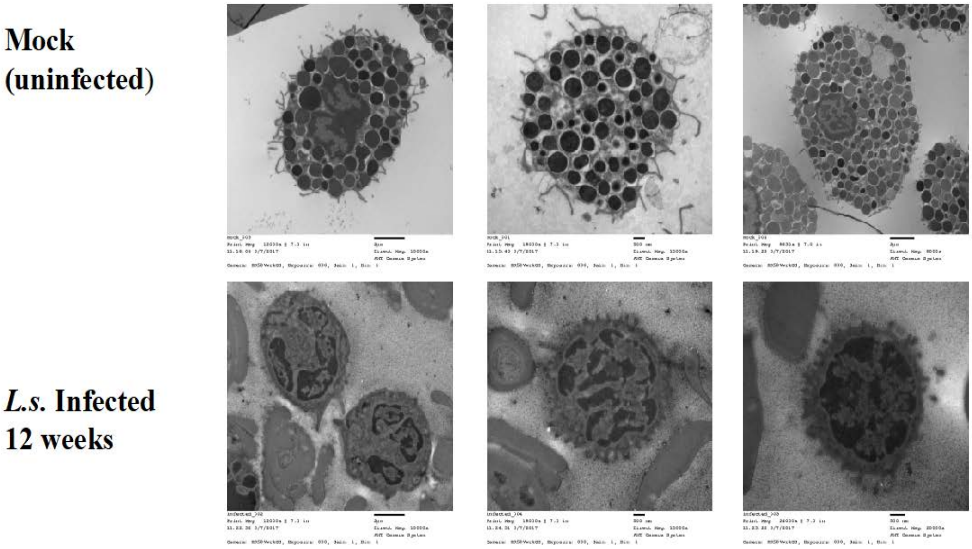
Mast cells were purified from *L.s.*- and mock-treated mice by Percoll purification and enumerated using an automated cell counter (n=10 mice per group). No significant difference in the number of mast cells between the two groups was observed (Figure 22). There was no significant difference in viability between the groups as determined by trypan blue exclusion (data not shown).

Peritoneal mast cells were isolated from mice that were chronically infected with *L.s.* for 12 weeks (Infected) or mock controls (Mock) (n=5 per group). Mast cells were affixed to slides and stained with Toluidine blue, which stains granules. The appearance of mast cells from Infected mice was dramatically different than that of Mock (Figure 21). In the Mock group, mast cells that stained bluish purple were loaded with granules. A subset of mast cells in this group appeared to have pink nuclei. In contrast, mast cells in the Infected group appeared predominantly pink and were smaller in size than the average cell found in the Mock samples. Furthermore, they were devoid of granules and some of the cells even displayed prominent vacuoles. Single slides were prepared from each of five mice per group, Infected or Mock. Images displayed in the figure are representative of the entire group.

Representative images of mast cells were obtained by electron microscopy. Mast cells from Mock animals were densely packed with granules; whereas, mast cells from Infected mice were devoid of granules and showed signs of activation (Figure 21).



Mock Infected



Mock (uninfected)

L.s. Infected 12 weeks

Figure 21. Chronic *L. sigmodontis* infection alters the appearance of peritoneal mast cells

Top) Purified mast cells from mice that were chronically infected with *L.s.* for 12 weeks or mock infected were stained with Toluidine blue (40x magnification). The above pictures serve as an overall representative of the respective groups. Bottom) Representative mast cells from both experimental groups were identified by electron microscopy (20x magnification).

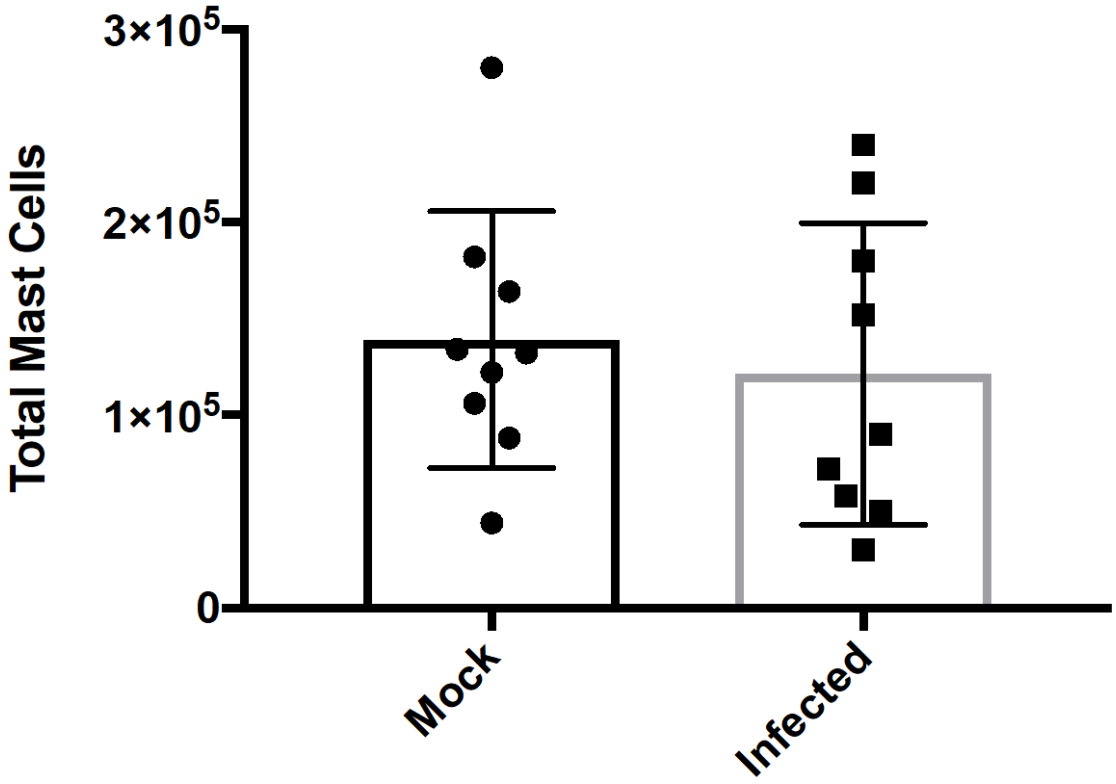


Figure 22. *L. sigmodontis* infection does not alter the number of mast cells in the peritoneum at 12 weeks

Mast cells were purified by Percoll isolation and enumerated using the automated cell counter the Countess. No significant difference was observed between Mock and Infected groups. Statistical analysis was performed by Student's t-test ($p<0.05$).

ELISAs of mast cell lysates

ELISAs were performed on the contents of mast cells purified from mice that were either *L.s.*- or mock-treated for 12 weeks. Mast cells were ruptured by two freeze-thaw cycles. Total histamine levels were measured and compared between groups on a per cell basis (n= 5 per group). Mast cells from mock-infected mice had an average 9.11 μMOL histamine per 10^5 cells, while mast cells from *L.s.*-infected mice had an average of 1.77 μMOL histamine per 10^5 cells (Figure 23). Thus, mast cells from *L.s.*-infected mice contained significantly less histamine than those from mock-infected mice ($p=0.041$). Additionally, we attempted to measure baseline mMCP-1 levels but were unsuccessful (data not shown). Although mMCP-1 is pre-formed in cells, we were unable to detect it in either group despite repeated attempts. Total histamine was also measured in the serum during 12 weeks of challenge in order to determine baseline histamine levels derived from basophils. There was no significant difference in the baseline level of histamine in the serum from *L.s.*-infected compared to mock-treated mice (Figure 24).

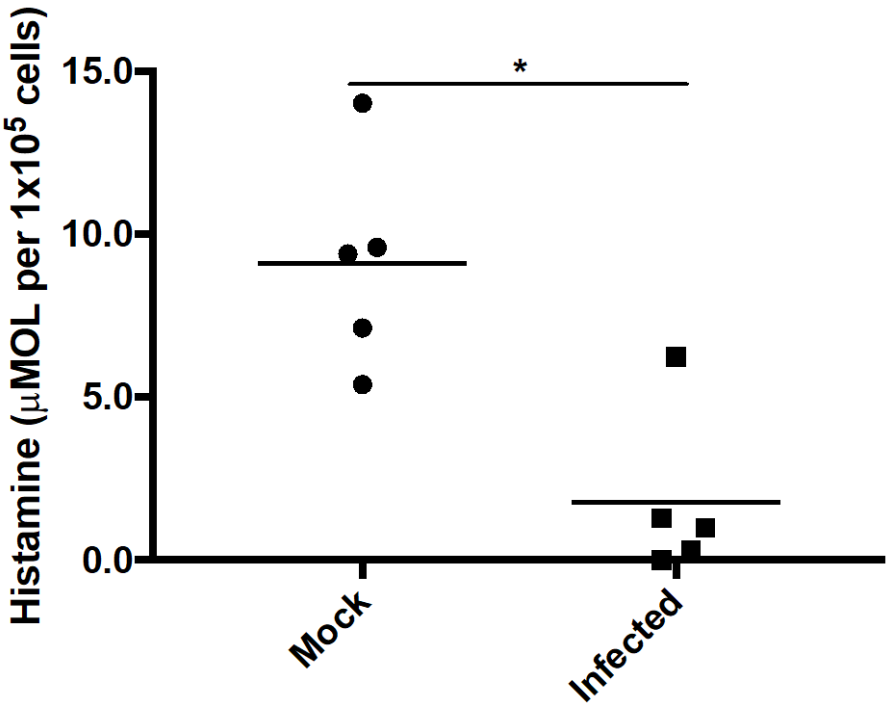


Figure 23. Mast cells from *L.s.*-infected mice contain significantly less histamine at 12 weeks post-infection than mock-infected mice

Mast cells were isolated from mice that were either administered a mock infection (Mock) or infected with *L.s.* (Infected) at 12 weeks post-infection (n=5 per group). Average histamine levels per 10^5 cells were significantly lower in mast cells from Mock compared to Infected mice, at 9.11 μMOL histamine and 1.77 μMOL histamine respectively by Student's t-test (p=0.041).

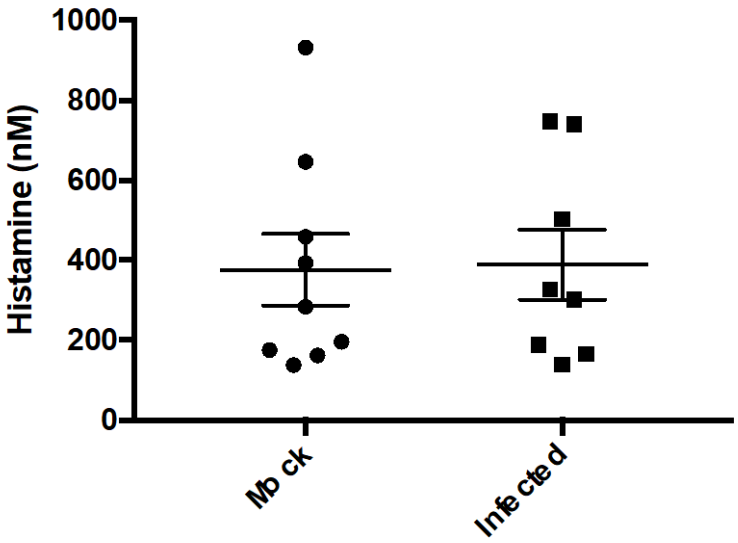


Figure 24. *L.s.* infection does not alter total histamine in the blood at 12 weeks post-infection

BALB/c mice were infected with *L.s.* (Infected) or administered a mock infection (Mock) (n=10 per group). Twelve weeks following infection, blood was collected and lysed. Histamine levels were measured by ELISA. There was no significant difference in total histamine between Mock and Infected mice. Statistical analysis was performed by Student's t-test ($p<0.05$).

Flow cytometry of mast cells

Peritoneal mast cells were analyzed by flow cytometry at 12 weeks following infection with *L.s.* or mock (n=10 per group). The gating strategy for mast cells is shown in Figure 25. An initial forward scatter and side scatter gate was established. Singlets were identified by FSC-H and FSC-A from the parent population. From the singlets, mast cells were identified from this population through co-expression of IgE and CD117 (c-Kit). The average percentage of total cells that were mast cells was significantly lower in infected mice than in mock controls (Figure 26), which we believe corresponds to a loss of CD117 expression. However, absolute numbers of mast cells from mice were not determined by flow cytometry. The average median fluorescence intensity of IgE on mast cells from infected mice was significantly higher than that of mock controls (Figure 26), which could reflect only a subset of mast cells. We plan to explore this finding as well as the loss of CD117 in the future by characterizing and comparing mast cells purified from the peritoneum of mock- and *L.s.*-infected mice.

Side Scatter (SSC), which is a measure of granularity, was significantly lower in mast cells from *L.s.*-infected than mock-infected mice ($p<0.0001$) (Figure 27). Furthermore, it was found that the average median fluorescence intensity (MFI) of the activation marker CD200R was significantly lower at baseline on mast cells from *L.s.*-infected mice compared to mock-treated mice ($p=0.0005$) (Figure 27). We also observed that average MFI of CD63 was significantly lower on mast cells from mice that were infected with *L.s.* as compared to mock-treated mice ($p=0.0002$) (Figure 27).

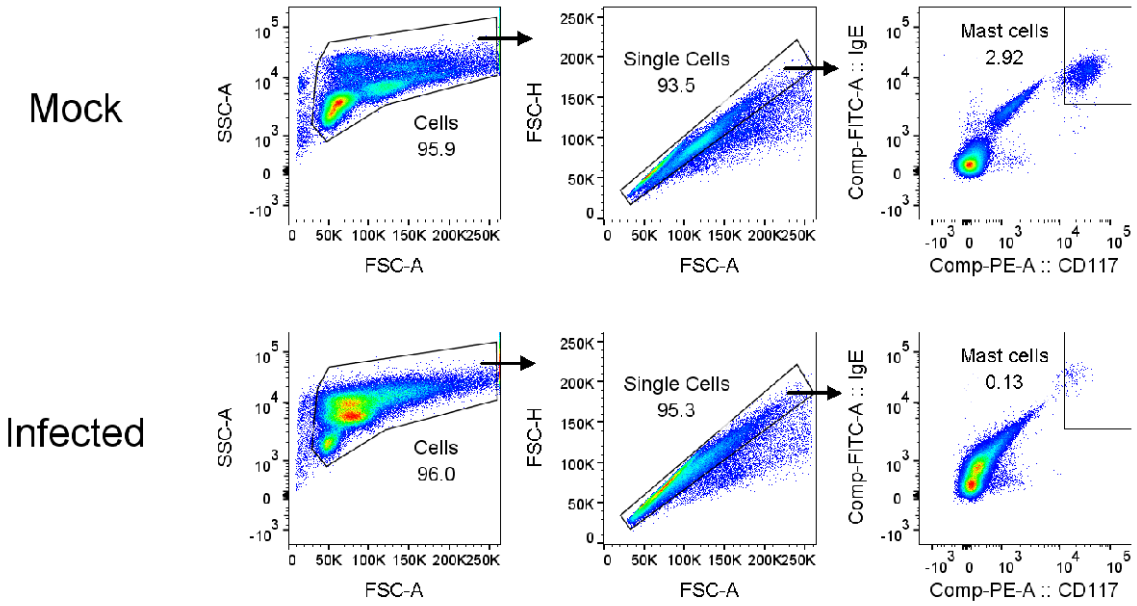


Figure 25. Mast cell gating strategy

Initial gating on forward scatter and side scatter was performed. Using that parent population, a gate was set to identify singlets. Mast cells were identified within the singlet population as $CD117^{+}$ and IgE^{+}. Representative images of a mock infected (top: Mock) and *L.s.*-infected mice (bottom: Infected) are shown.

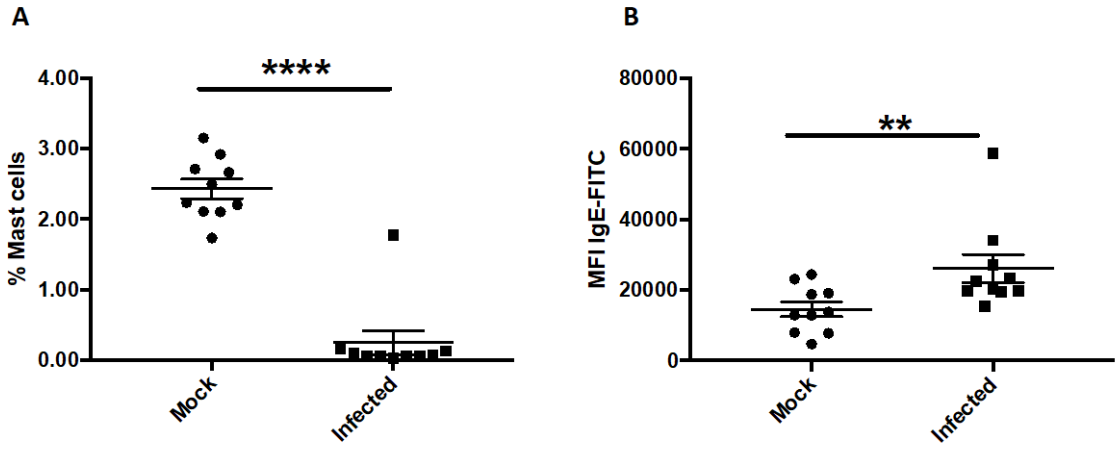


Figure 26. Mast cells from Infected comprise less of the total cell population that from Mock mice and their median expression of IgE is higher

Mast cells were identified using the mast cell gating strategy. A) Mast cells comprised a lower percentage of total peritoneal cells in infected mice compared to mock controls ($p<0.0001$). B) Median fluorescence intensity (MFI) of IgE is higher in infected mice compared to mock controls ($p=0.0068$). Statistics were performed using a 2-tailed Mann-Whitney test.

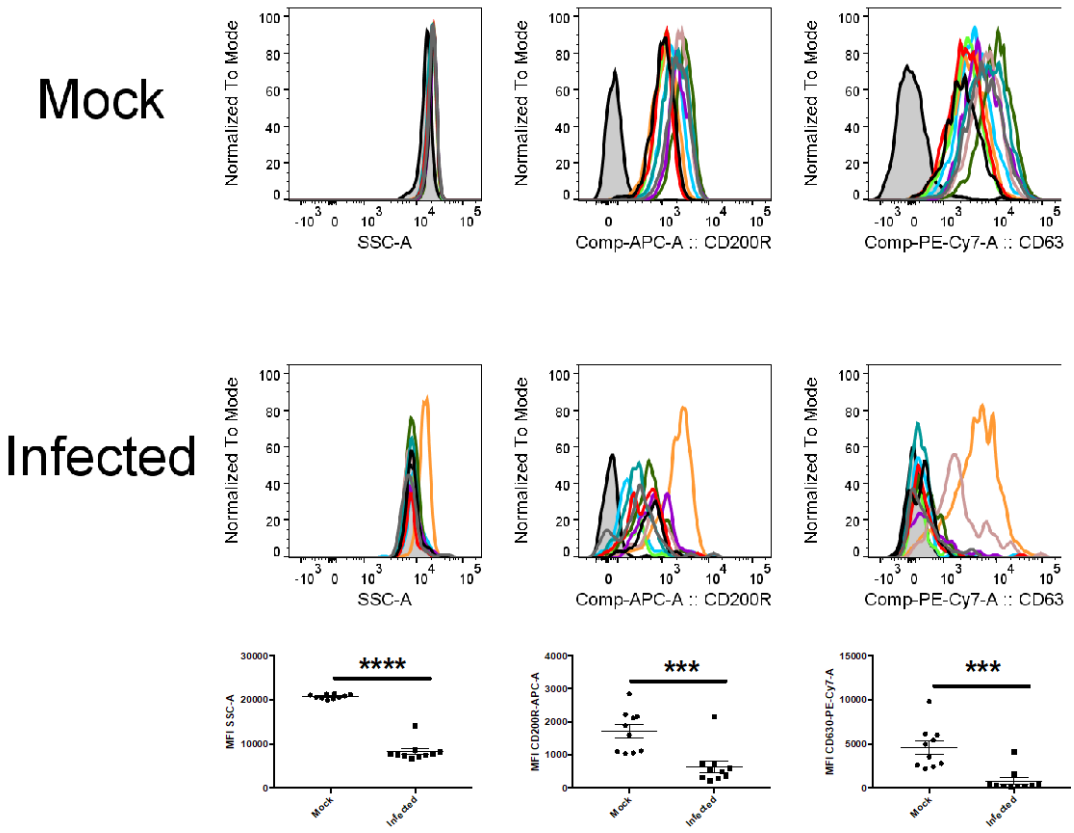


Figure 27. Baseline expression of activation markers is lower on mast cells from chronically infected mice

Mast cells were identified using the mast cell gating strategy. Histograms of side scatter (SSC-A), CD200R, and CD63 are shown for Mock and Infected mice are shown on the top and middle rows, respectively. The bottom row represents a comparison between the two groups for the designated characteristic. Average SSC-A was significantly lower in Infected compared to Mock mice. Average median fluorescence intensity (MFI) of CD-200R-APC was significantly lower in Infected compared to Mock control mice. Average MFI of CD63-PE-Cy7 was significantly lower in Infected compared to Mock mice. Statistical analyses were performed by two-tailed Mann-Whitney test.

Proteomics of mast cells

Mast cells were isolated by isotonic Percoll purification from *L.s.*- (Infected) or mock-treated (Mock) mice at 12 weeks (n=5 per group). Samples were shipped on dry ice for proteomics analysis to the Mass Spectrometry and Proteomics Resource Laboratory (MSPRL) at Harvard University, Cambridge, MA. Cells were lysed and proteins were CRA solution-digested, and labeled with Tandem Mass Tags (TMT) for relative quantitation by mass spectrometry (MS). Sample size of approximately 100,000 cells per sample limited the depth of fractionation, so analysis was limited to only highly abundant proteins. Unique proteins were identified by recovery of at least one matched peptide sequence.

Sixty-nine unique proteins were identified from the purified mast cells. All 69 identified proteins were compared against a database of common contaminants by MSPRL. Only samples that were identified as "false" contaminants (i.e. not likely to be contaminants) were included in subsequent analyses. For instance, keratin was identified as highly abundant in samples, but it was identified as a likely contaminant. Thus, it was excluded from subsequent analyses. Other "true" contaminants included common media components such as bovine serum albumin. Ten proteins were identified as "true" contaminants and were eliminated. In total, fifty-three proteins had sufficient information in their reports from MSPRL for subsequent analysis and were determined to not be likely contaminants. Identification of mast-cell specific proteins such as mast cell carboxypeptidase A added to the confidence of this analysis.

The average normalized abundance of each of the fifty-three proteins was calculated for Mock and Infected samples. The relative abundance ratio for each protein was calculated by dividing its average abundance in Infected by its average abundance in Mock samples. A ratio within 0.8-1.2 was considered to be within the normal range of variation and was not considered different between the two groups. Ratios either above or below this range were determined to be different between Infected and Mock groups. A ratio below 0.8 indicated that the protein was less abundant in Infected samples than in Mock. A ratio above 1.2 suggested that the protein was more highly abundant in Infected than in Mock. Twenty-four proteins were identified as either less or more highly abundant between Mock and Infected mice. Of these, nine were more highly abundant (up-regulated) in Infected than Mock and fifteen were less highly abundant (down-regulated). Up-regulated proteins are found in Table 1 and down-regulated proteins are listed in Table 2. Twenty-nine proteins were found to be in the normal range (0.8-1.2) and were considered neither up-regulated nor down-regulated (Table 3).

Of the proteins found to be up-regulated in Infected samples, several have known roles in cell repair, including peroxiredoxin-6, tubulin alpha 1B, alpha enolase, actin and isoform 2 of histone H2B, and a putative tropomyosin. Additionally, alpha-2-macroglobulin (A2M) and myeloperoxidase were up-regulated in Infected mast cells. A2M is a protease inhibitor and myeloperoxidase is an enzyme that produces hypochlorous acid.

Fifteen proteins were identified as down-regulated in Infected compared to Mock samples. Seven of those proteins have known functions in metabolic processes, including arginase-1, S100-A6, myeloblastin, AHNAK nucleoprotein, annexin A1, mast cell

carboxypeptidase A, and 40S ribosomal protein S9. Several of the proteins were localized to extracellular regions, such as mast cell carboxypeptidase A, leukocyte elastase inhibitor, and myeloblastin. Additionally, S100a6, also known as calcyclin, was down-regulated. S100a6 is an S100 calcium binding protein and could function as a calcium sensor and modulator. Calcium flux is required for degranulation of mast cells.

The accession numbers of the 24 differentially abundant proteins were entered into STRING. STRING generated an interaction network of physical and functional interactions that included the 20 proteins of the 24 that were identifiable in its database. The results are shown in Figure 28. The majority of the proteins clustered into 3 interaction networks, indicating that many of the proteins are involved in similar pathways. Six of the proteins, including alpha-2-macroglobulin and carboxypeptidase A, did not cluster into any network.

The accession numbers of the 24 differentially abundant proteins were also entered into PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System. PANTHER classifies proteins based on Family and subfamily, Molecular function, Biological process or Pathway.

Graphs revealed that a large portion of the differentially expressed proteins had either catalytic or binding functions (Figure 29).

Table 1. Proteins up-regulated in Infected mast cells

Accession	Description of Protein	Peptides (#)	Abundance Ratio
H3BJQ7	Peroxiredoxin-6 OS=Mus musculus GN=Prdx5 PE=2 SV=1	1	2.20
D3Z2H9	Uncharacterized protein OS=Mus musculus GN=Tpm3-rs7 PE=3 SV=1	3	1.88
Q6GQT1	Alpha-2-macroglobulin-P OS=Mus musculus GN=A2mp PE=2 SV=2	1	1.66
P43274	Histone H1.4 OS=Mus musculus GN=Hist1h1e PE=1 SV=2	6	1.66
P05213	Tubulin alpha-1B chain OS=Mus musculus GN=Tuba1b PE=1 SV=2	3	1.54
P11247	Myeloperoxidase OS=Mus musculus GN=Mpo PE=2 SV=2	1	1.47
P17182	Alpha-enolase OS=Mus musculus GN=Eno1 PE=1 SV=3	1	1.42
P62737	Actin, aortic smooth muscle OS=Mus musculus GN=Acta2 PE=1 SV=1	8	1.26
Q8CGP2-2	Isoform 2 of Histone H2B type 1-P OS=Mus musculus GN=Hist1h2bp	3	1.23

Table 2. Proteins down-regulated in Infected mast cells

Accession	Description of Protein	Peptides (#)	Abundance Ratio
Q02566	Myosin-6 OS=Mus musculus GN=Myh6 PE=1 SV=2	1	0.432
Q91XV3	Brain acid soluble protein 1 OS=Mus musculus GN=Basp1 PE=1 SV=3	2	0.491
A4QPC5	Chymase OS=Mus musculus GN=Cma1 PE=2 SV=1	3	0.555
P26350	Prothymosin alpha OS=Mus musculus GN=Ptma PE=1 SV=2	2	0.558
Q8VHP7	Leukocyte elastase inhibitor B OS=Mus musculus GN=Serpinb1b PE=1 SV=1	2	0.568
P51437	Cathelin-related antimicrobial peptide OS=Mus musculus GN=Camp PE=2 SV=1	1	0.591
Q6ZWN5	40S ribosomal protein S9 OS=Mus musculus GN=Rps9 PE=2 SV=3	1	0.598
P14069	Protein S100-A6 OS=Mus musculus GN=S100a6 PE=1 SV=3	1	0.621
P10107	Annexin A1 OS=Mus musculus GN=Anxa1 PE=1 SV=2	2	0.629
D3Z6C3	40S ribosomal protein S3a OS=Mus musculus GN=Rps3a2 PE=3 SV=1	1	0.659
B2RT41	Protein Zfc3h1 OS=Mus musculus GN=Zfc3h1 PE=2 SV=1	1	0.685
E9Q616	Protein Ahnak OS=Mus musculus GN=Ahnak PE=2 SV=1	4	0.719
P15089	Mast cell carboxypeptidase A OS=Mus musculus GN=Cpa3 PE=2 SV=1	2	0.753
Q61096	Myeloblastin OS=Mus musculus GN=Prtn3 PE=2 SV=2	1	0.759
Q61176	Arginase-1 OS=Mus musculus GN=Arg1 PE=1 SV=1	1	0.767

Table 3. Proteins unchanged between Infected and Mock mast cells

Accession	Description of Protein	Peptides (#)	Abundance Ratio
A6ZI46	Fructose-bisphosphate aldolase OS=Mus musculus GN=Aldoart1 PE=2 SV=1	2	0.802
Q93092	Transaldolase OS=Mus musculus GN=Taldo1 PE=1 SV=2	2	0.820
P62806	Histone H4 OS=Mus musculus GN=Hist1h4a PE=1 SV=2	1	0.835
P68433	Histone H3.1 OS=Mus musculus GN=Hist1h3a PE=1 SV=2	1	0.838
P43277	Histone H1.3 OS=Mus musculus GN=Hist1h1d PE=1 SV=2	7	0.860
P01942	Hemoglobin subunit alpha OS=Mus musculus GN=Hba PE=1 SV=2	1	0.860
P02089	Hemoglobin subunit beta-2 OS=Mus musculus GN=Hbb-b2 PE=1 SV=2	1	0.876
Q61599	Rho GDP-dissociation inhibitor 2 OS=Mus musculus GN=Arhgdib PE=1 SV=3	1	0.884
G5E8N5	L-lactate dehydrogenase OS=Mus musculus GN=Ldha PE=3 SV=1	1	0.920
P08249	Malate dehydrogenase, mitochondrial OS=Mus musculus GN=Mdh2 PE=1 SV=3	1	0.949
P27661	Histone H2AX OS=Mus musculus GN=H2afx PE=1 SV=2	2	0.951
Q61233	Plastin-2 OS=Mus musculus GN=Lcp1 PE=1 SV=4	1	0.953
P63101	14-3-3 protein zeta/delta OS=Mus musculus GN=Ywhaz PE=1 SV=1	2	0.966
O08692	Myeloid bactenecin (F1) OS=Mus musculus GN=Ngp PE=2 SV=1	2	0.970
F6W687	Non-histone chromosomal protein HMG-17 (Fragment) OS=Mus musculus GN=Hmgn2 PE=4 SV=1	2	1.00
P63017	Heat shock cognate 71 kDa protein OS=Mus musculus GN=Hspa8 PE=1 SV=1	2	1.01
F6VW30	14-3-3 protein theta (Fragment) OS=Mus musculus GN=Ywhaq PE=2 SV=1	1	1.03
P14094	Sodium/potassium-transporting ATPase subunit beta-1 OS=Mus musculus GN=Atp1b1 PE=1 SV=1	1	1.04
Q9ERD7	Tubulin beta-3 chain OS=Mus musculus GN=Tubb3 PE=1 SV=1	1	1.07
E9QNP0	KxDL motif-containing protein 1 OS=Mus musculus GN=Kxd1 PE=2 SV=1	1	1.10
P02088	Hemoglobin subunit beta-1 OS=Mus musculus GN=Hbb-b1 PE=1 SV=2	1	1.12
P52480	Pyruvate kinase PKM OS=Mus musculus GN=Pkm PE=1 SV=4	2	1.12
Q3UKW2	Calmodulin OS=Mus musculus GN=Calm1 PE=2 SV=1	1	1.15
Q03265	ATP synthase subunit alpha, mitochondrial OS=Mus musculus GN=Atp5a1 PE=1 SV=1	2	1.16
Q8VDD5	Myosin-9 OS=Mus musculus GN=Myh9 PE=1 SV=4	2	1.18
F8WGL3	Cofilin-1 OS=Mus musculus GN=Cfl1 PE=2 SV=1	1	1.18
P56480	ATP synthase subunit beta, mitochondrial OS=Mus musculus GN=Atp5b PE=1 SV=2	2	1.19

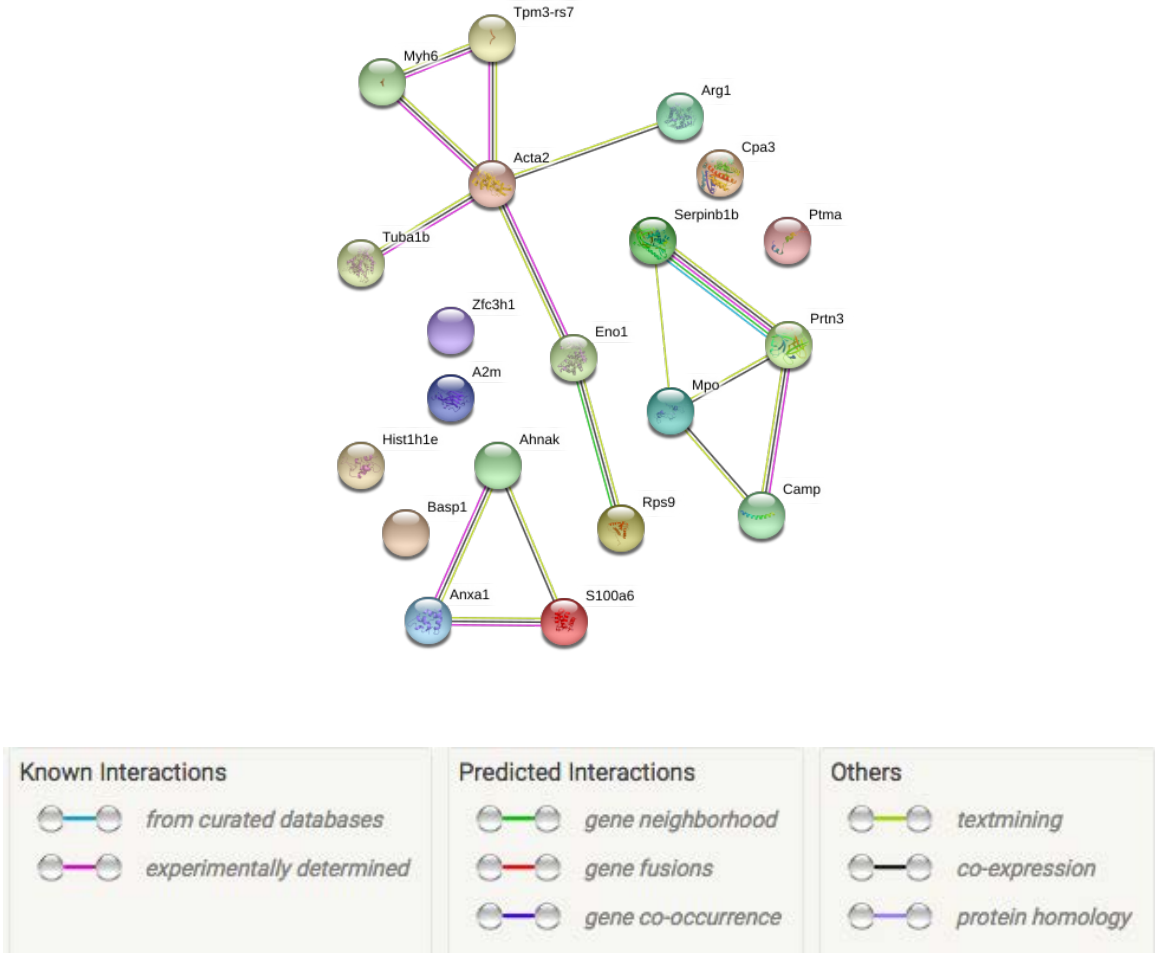


Figure 28. Network of proteins differentially regulated in mast cells from chronically infected versus mock-infected mice

Proteomics analysis was performed on expression data from peritoneal mast cells that were isolated from mice either chronically infected with *L.s.* or mock infected for 12 weeks. An interaction network of proteins differentially expressed in *L.s.*-infected mice was generated using STRING. Lines connecting nodes indicate known or predicted interactions.

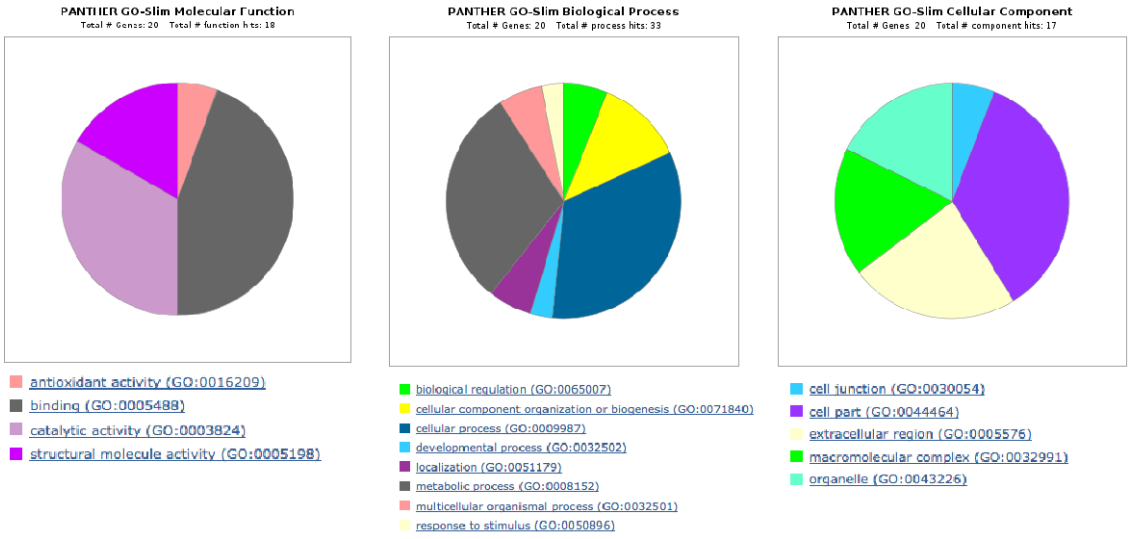


Figure 29. Proteins differentially regulated in mast cells from chronically infected mice

Proteomics was performed on expression data from peritoneal mast cells that were isolated from mice either chronically infected with *L.s.* or mock infected for 12 weeks. Pie charts were generated using PANTHER and included only differential expression data correlating with infection status. Charts represent abundance of differentially expressed proteins based on Molecular function (left), Biological process (middle) and Cellular component (right).

Lipid analysis of mast cells

Lipid extracts from purified mast cells were compared from *L.s.*- and mock-treated mice to determine whether or not chronic infection influences sphingolipid composition. Differences observed in mMCP-1 release following challenge could have resulted from differences in signaling. Thus, we hypothesized that the profile of sphingolipids from the mast cells of chronically infected mice would be significantly different than that from mock-treated mice.

Sphingolipids are composed of a sphingosine, a fatty acid, and a functional (-R) group. When the -R group is a hydrogen, the resulting sphingolipid is known as ceramide; a sphingolipid in which the -R group is a phosphocholine is a sphingomyelin; a sphingolipid in which the -R group is a sugar is a glycosphingolipid.

Lipid extracts were compared from 5 *L.s.*- and 5 mock-treated mice. No significant differences in sphingolipid content were observed between *L.s.*- and mock-treated mice. On a per cell basis, levels of sphingomyelin, lactosyl-ceramide, glucosyl-ceramide, and ceramide were no different in mast cells from Mock and Infected mice at 12 weeks following infection with *L.s*. (Figure 30A). On a per cell basis, there were no differences in Phosphatidylinositol (PI), Phosphatidylethanolamine (PE), Phosphatidylserine (PS), or in total PI/PE/PS (Figure 30B). No significant differences were found among 11 species of phosphatidylcholine when *L.s.*-infected and mock-treated mice were compared (Figure 30C). These included PC 30:0, PC 30:1, PC 30:2, PC 32:0, PC 32:1, PC 34:1, PC 34:2, PC36, PC 36:1, and PC 36:2. The total amount of phosphatidylcholine per 1 x 10^6 cells (Total PC) was also not statistically significant between *L.s.*- and mock-infected groups (Figure 30C).

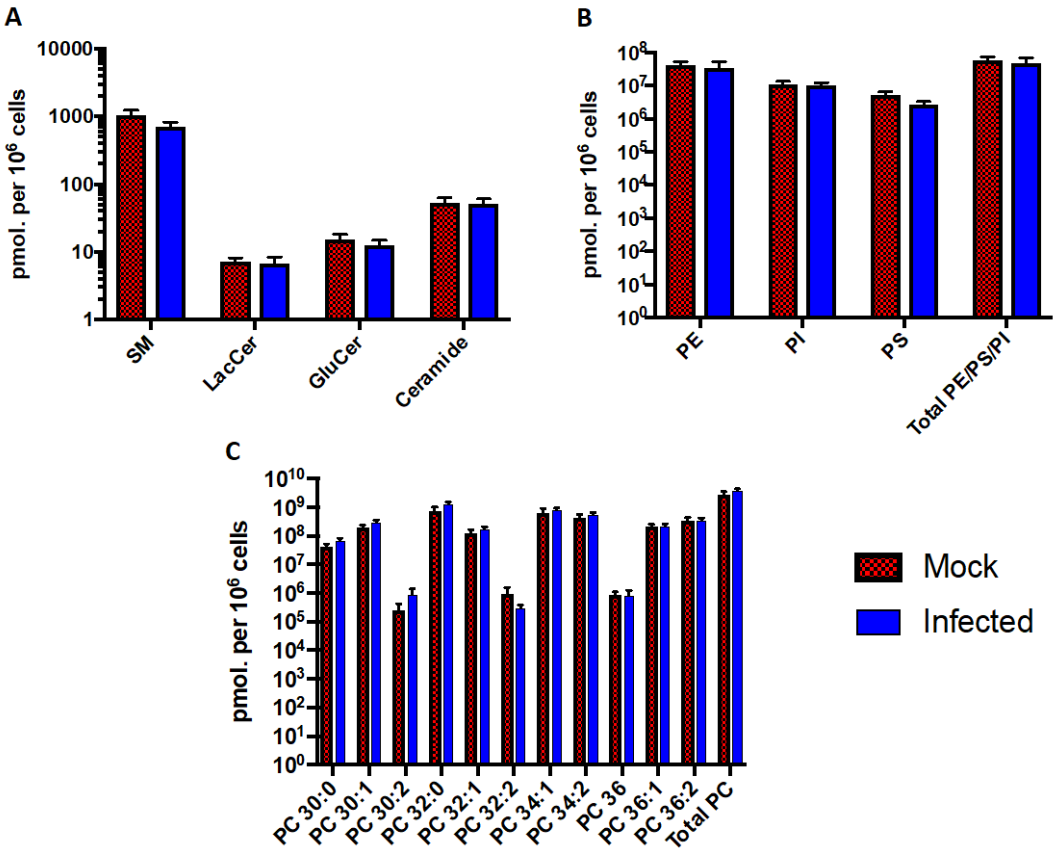


Figure 30. Chronic infection with *L. sigmodontis* did not alter sphingolipid composition of mast cells at 12 weeks post-infection

A) Levels of sphingomyelin, lactosyl-ceramide, glucosyl-ceramide, and ceramide were not different in mast cells from mock and infected mice at 12 weeks following infection with *L.s.*, B) No differences were observed between *L.s.*- (Infected) and mock-infected (Mock) mice in Phosphatidylinositol (PI), Phosphatidylethanolamine (PE), Phosphatidylserine (PS), or in total PI/PE/PS. C) No differences were observed in mast cells from *L.s*- and mock-treated mice in Phosphatidylcholine (PC) 30:0, PC 30:1, PC 30:2, PC 32:0, PC 32:1, PC 34:1, PC 34:2, PC36, PC 36:1, PC 36:2 and total PC per 1 x 10^6 cells. For all experiments, n=5 mice per group.

CHAPTER 4: Discussion

The overarching goal of this project was to determine whether helminth infection could protect against allergic challenge in mice that have been previously sensitized to an allergen. We hypothesized that chronic helminth infection would protect against the immunological manifestations and clinical signs of anaphylaxis in previously sensitized mice. Furthermore, we hypothesized that infection would protect by suppression of allergy effector cells, specifically mast cells. In order to address our hypothesis, we employed a model of sensitization whereby BALB/c mice are sensitized with OVA/alum by repeated intraperitoneal injections. We utilized this model in conjunction with the *L. sigmodontis* rodent model of filariasis, which is permissive in BALB/c mice. Since *L. sigmodontis* is able to persist in BALB/c mice for up to 14 weeks, we were also able to explore the impact of duration of infection on protection against allergy. Our study is novel in that it is one of only a few to examine the therapeutic potential of helminths on pre-existing allergy. Furthermore, it is the only study we know of to look at the impact of helminth infection on mast cells in the context of allergic disease. Our study addresses a necessary question if helminths and helminthic products are ever to be successfully translated into clinical therapies.

Characterizing the Model

We first demonstrated that allergic challenge by intraperitoneal injection of OVA in OVA/alum-sensitized mice resulted in a rapid decrease in core body temperature (Figure 2). In contrast, PBS-sensitized mice maintained their initial core body temperature over the hour immediately following challenge. The disparity between these

two responses indicated to us that responsiveness to allergic challenge is antigen-dependent in this model. It also demonstrated that this model reflects clinical manifestations of anaphylaxis, specifically hypothermia. We noted that in addition to hypothermia, OVA-sensitized animals that were challenged with OVA demonstrated other clinical signs of allergy including labored breathing, lethargy and decreased mobility. While we observed clinical signs in addition to hypothermia, we chose to only monitor core body temperature following challenge, as assessing rectal temperature interferes with clinical scoring. We rationalized that measuring core body temperature was the more quantitative, non-subject measurement of clinical impact of the two measurements that we could perform.

We next characterized our anaphylaxis model as a model of type I hypersensitivity. Experiments with transgenic animals revealed that anaphylaxis was dependent on IgE signaling, as OVA-sensitized FcεRI knockout mice exhibited less than a one-degree drop in core body temperature following intraperitoneal OVA-challenge, which resolved within 30 minutes (Figure 3). The response of OVA-sensitized FcεRI knockout mice was not significantly different from that of PBS-sensitized FcεRI knockout mice, a group that represented non-specific reaction to challenge with OVA. Furthermore, in terms of their response to allergen challenge, OVA-sensitized FcεRI knockout mice behaved more similarly to PBS-sensitized BALB/c mice than to OVA-sensitized BALB/c mice, which exhibited a robust drop in core body temperature following challenge. Following OVA-challenge, OVA-sensitized FcεRI knockout mice released mMCP-1 at a level that was not significantly different from the level of mMCP-1 released by PBS-sensitized FcεRI knockout mice (Figure 4). Although the amount of

mMCP-1 released by OVA- and PBS-sensitized FcεRI knockout mice was slightly higher than in PBS-sensitized BALB/c mice, the amount of mMCP-1 released by OVA-sensitized BALB/c mice was significantly greater. Thus, it appeared that mast cell degranulation, as measured by mMCP-1 in the serum post-challenge, relied on signaling through FcεRI. Analysis of OVA-IgE levels within the groups revealed that OVA-IgE levels were significantly lower in FcεRI knockout mice that were OVA-sensitized compared to BALB/c mice that underwent the same regimen, but much higher than negative controls (Figure 4). Nevertheless, although the average level of OVA-IgE was significantly lower than in FcεRI knockout mice, it was available in sufficient quantity to mount an anaphylactic response as determined by other experiments to optimize sensitization regimens (data not shown). Thus, anaphylaxis in our model is dependent on IgE signaling. We hypothesize that OVE-IgE levels were significantly lower in FcεRI knockout mice because of the need for signaling through the IgE pathway to provide positive feedback mechanisms for IL-4 production, which in turn drive IgE production. Results from experiments with FcεRI knockout mice indicated that our anaphylaxis model is IgE-mediated and supported its suitability as a model for type I hypersensitivity.

We further characterized our model in order to determine which effector cell type was most important in driving anaphylaxis in our model. As mast cells and basophils are the primary effector cells of type I hypersensitivity, we elucidated their relative contributions to anaphylaxis with an experiment using transgenic mice along with cell depletion. Briefly, basophils were antibody-depleted or isotype control-treated in mast cell-deficient and wild type mice. Eliminating mast cells from the anaphylaxis model exhibited the greatest impact on hypothermia of a single cell type (Figure 5). In fact,

mast cell-deficient mice exhibited only minimal drops in core body temperature in response to challenge with OVA. Depletion of basophils in mast cell-deficient or wild type mice revealed that this cell type also plays a role, albeit to a much lesser extent. Depleting basophils on the wild type background had a lesser impact on hypothermia than knocking out mast cells, however the difference was not statistically significant. Depleting basophils on the mast cell deficient background had a minor impact on hypothermia that was also not statistically significant from eliminating mast cells alone. Histamine levels amongst experimental groups following challenge correlated with hypothermia (Figure 7). However, we were not able to determine histamine levels for all of the animals from the wild-type background group. This was due to challenges with blood collection during anaphylaxis. It appeared that mice that were exhibiting severe signs of anaphylaxis often had very little blood in their hearts likely due to massive vasodilation and leakage. Levels of mMCP-1 following challenge were also measured in the serum, but could not be used to determine relative contributions of mast cells and basophils, as mMCP-1 is specific only to mast cells (Figure 7). Nevertheless, the fact that mMCP-1 was absent in the serum of mast cell-deficient mice was consistent with the identity of our transgenic mice and lent credence to the validity of our experiment. Thus, we demonstrated that mast cells were the primary effector cell type responsible for hypothermia and histamine in our model. While we found that basophils also contributed to anaphylaxis in our model, we found it to be to a slightly lesser extent. Thus, we focused our study primarily on the effect of helminth infection on mast cells.

Challenge at 12 Weeks Post-infection

We demonstrated that challenge at 12 weeks post-infection with *L.s.* resulted in clinical protection against anaphylaxis, as determined by relative changes in core body temperature between mock-treated and *L.s.*-infected groups. Mice that were chronically infected with *L.s.* were protected against hypothermia by an average of 1.5°C (n=15 per group) (Figure 9B). Furthermore, we observed profound immunological changes in terms of mast cell degranulation were observed, as *L.s.*-infected mice demonstrated significantly less mMCP-1 in the serum following challenge compared to mock-treated mice (Figure 9A). Although our study did not demonstrate complete protection against either the clinical signs or immunological markers of anaphylaxis, our results serve as proof of concept that therapeutic infection with *L.s.* could be a beneficial treatment for type I hypersensitivity reactions. These results are important because they show that helminths and helminthic products could potentially reverse pre-existing allergic disease, and thus could be translated into therapeutics to treat pre-existing allergic disease.

We hypothesized that the difference observed in mast cell degranulation between *L.s.*-infected and mock-treated mice resulted from alterations in cell signaling. Thus, we compared the lipid composition of *L.s.*- and mock-infected mice at 12 weeks post-infection, as sphingolipids have been shown to play a role in cell signaling (Figure 30). We analyzed an array of sphingolipids as a relatively unbiased approach to identify potential differences between *L.s.*- and mock-infected groups. We did not observe any differences between the experimental and control groups for any of the sphingolipids that were analyzed. However, in our experiment we only analyzed lipid extract from whole cells. A more sensitive and directed approach would be to isolate the lipid membrane components for analysis. Additionally, our approach only looked at the lipid composition

of cells, and not the localization of species throughout the cells or across the plasma membrane. Differences in localization of lipids involved in signaling could lead to differences in signaling, despite relative composition of specific lipid species remaining unchanged. This possibility could be addressed through immunohistochemistry.

Serum levels of antigen-specific IgE are thought to correlate with surface levels on effector cells (77). Thus, we evaluated OVA-IgE in the pre-challenge serum of mice. We found that serum levels of OVA-IgE were not altered by *L.s.* infection at 12 weeks post-infection (Figure 10). If OVA-IgE concentrations were the mechanism of protection at 12 weeks, we would have expected the levels to be lower in *L.s.*-infected mice than in mock-treated controls. Instead, OVA-IgE levels appeared to be unaffected by infection with *L.s*. Furthermore, although we observed decreased serum levels of OVA-IgG1 due to infection, they were not able to explain the observed protection at 12 weeks. IgG1 in BALB/c mice, which is the equivalent of human IgG4, primarily serves as a blocking antibody that would act by binding free OVA and preventing it from binding the OVA-IgE on effector cells. Thus, if OVA-IgG1 played role in protection, we would have expected levels to be elevated in the *L.s.*-infected group as compared to mock-infected controls.

To further explore potential mechanisms of protection at 12 weeks post-infection, we analyzed a number of Th1 and Th2 cytokines at 12 weeks post-infection (Figures 17-20). Levels of the inflammatory cytokine TNF-α were slightly elevated in OVA-stimulated as compared to media-stimulated mice. However, as there was no significant difference in TNF-α levels between mock- and *L.s.*-infected mice, we concluded that TNF-α did not play a role in protection at 12 weeks post-infection. The immune

responses in helminth infections and allergic disease are both Th2-skewed. Thus, there was predictably no significant difference in OVA-stimulated compared to media-stimulated groups in the levels of their secretion of the Th1 cytokines INF-γ and IL-12p70, or the inflammatory cytokine IL-6. The Th2 cytokines IL-4 and IL-5 were both elevated in OVA-stimulated groups compared to media-stimulated controls. This result was also somewhat unsurprising since alum drives a Th2-skewed immune response. While IL-4 levels trended higher in mock-treated compared to *L.s.*-infected mice, the difference was not statistically significant. On the other hand, IL-5 levels were significantly higher in the supernatant of mock-infected samples compared to *L.s.*-infected samples ($p=0.0095$). The IL-4 and IL-5 cytokine data suggest that the de novo Th2 response is slightly dampened in the setting of chronic helminth infection in our model. Thus, these trends in IL-4 and IL-5 cytokine levels were consistent with the immunoregulation observed with chronic helminth infection. In an allergic response, IL-5 is usually delayed, so we think it was unlikely that IL-5 played a role in dampening the immediate anaphylactic response of our model. However, we predict that the slightly decreased IL-5 levels we observed in the setting of chronic *L.s.* infection would dampen a late-phase hypersensitivity response by inhibiting recruitment of eosinophils. Alternatively, IL-5 could have been low in the splenocyte culture due to excess eosinophils that were already present and consuming IL-5 rapidly.

Hyperendemic exposure to helminths is thought to result in immune hyporesponsiveness through constitutive secretion of IL-10 and TGF- β (107). Chronic helminth infection has also been shown to down-modulate anti-parasite immune responses in part by production of IL-10 and TGF- β (46). Thus, we evaluated levels of

the immunoregulatory cytokines TGF-β and IL-10 from supernatants of stimulated splenocytes derived from *L.s.*- or mock-infected mice at 12 weeks post-infection to determine if they correlated with allergic responsiveness. We observed no significant differences in IL-10 between samples that were stimulated with OVA or media. However, we noticed that baseline IL-10 (media stimulated) was significantly higher from mock-treated mice than that from *L.s.*-infected mice ($p=0.0396$). Thus, we decided it was worthwhile to compare the amount of IL-10 secreted by *L.s.*- or mock-infected mice that were OVA stimulated as well; however, we did not observe a statistically significant difference. Our cytokine data did not indicate that IL-10 served as the mechanism for protection against anaphylaxis as 12 weeks post-infection as it was not differentially secreted. However, the data were consistent with the results of our studies in IL-10 knockout mice described below.

Active TGF-β was higher in the supernatants from cultures of OVA-stimulated splenocytes compared to media-stimulated controls. There was no significant difference, however, in active TGF-β levels between *L.s.*- and mock-infected groups. Basal levels of total TGF-β were slightly higher from *L.s.*-infected mice compared to mock-infected animals, but the result was not statistically significant. This result is consistent with the idea that chronic helminth infection promotes an immunoregulatory environment. In the future, we would like to look at TGF-β in the serum prior to challenge to determine whether or not it is naturally elevated in the context of chronic infection in our model. Given previous data from our laboratory, we think that it would be and it might be a move relevant indicator of whether or not TGF-β was playing a role in suppressing anaphylaxis.

Overall, our cytokine data suggest that, perhaps, chronic *L.s.* infection promoted a dampened Th2 environment. Both IL-4 and IL-5 trended lower in supernatants of splenocytes from mice that were *L.s.* infected, but only the IL-5 result was statistically significant. However, we did not pursue mechanistic studies of either cytokine. We hypothesize that dampening of either IL-4 and IL-5 individually by chronic *L.s.* infection would not fully explain the protection against anaphylaxis that we observed at 12 weeks. More likely, they were indicative of a more immunoregulatory environment being promoted by chronic *L.s.* infection.

While we did not see an increase in IL-10 derived from OVA-stimulated cells at 12 weeks post-infection, we did not measure serum IL-10 at baseline. For this reason, as well as a number of others, we decided to conduct additional experiments exploring IL-10 as a potential mechanism for allergic protection in our model. IL-10 has been shown to suppress mast cell function both in vitro and in vivo (53). In some models of allergy, IL-10 from mast cells has been shown to act on mast cells to reduce inflammation (42). IL-10 has also been shown to induce both antigen-specific as well as naturally occurring Tregs to control inflammation (44; 115). Furthermore, a study from our laboratory made the link between helminths and allergy effector cells by demonstrating that IL-10 suppresses basophil responsiveness in mice chronically infected with *L. sigmodontis.* (61). Previous experiments in our laboratory demonstrated that chronic infection with *L. sigmodontis* results in elevated levels of IL-10, so even though we did not see evidence for a role for IL-10 without cytokine data, we wanted to test our initial hypothesis that IL-10 confers protection by another experimental method.

We found, however, that IL- 10 was not required for *L.s.*-mediated protection in our model, as OVA-sensitized IL-10 knockout mice were protected against hypothermia in a similar fashion to OVA-sensitized BALB/c mice (Figure 15). We also demonstrated that, following challenge with OVA, the concentration of mMCP-1 in the serum of OVA-sensitized IL-10 knockout mice was similar to that of OVA-sensitized BALB/c. As observed in BALB/c mice, the serum concentration of OVA-IgE between *L.s.*- and mock-infected mice was not statistically significant. Additionally, the levels were similar to those observed in BALB/c mice. Although not statistically significant, the difference in OVA-IgG1 levels between *L.s.*- and mock-infected mice displayed the same trend as that observed with BALB/c mice, where OVA-IgG1 was decreased in chronically infected mice. Thus, IL-10 was not sufficient for *L. sigmodontis* infection to protect against anaphylaxis at 12 week post-infection in our model of anaphylaxis. Furthermore, IL-10 did not appear to have any effect on the immunological parameters that we evaluated including mMCP-1, OVA-IgE, and OVA-IgG1 (Figures 10-11). Although previous studies in our laboratory showed that IL-10 is elevated in the setting of chronic *L.s.* infection compared to naïve controls, we found in our current study by ELISA that IL-10 was not differentially expressed between *L.s.*- and mock-infected mice that were also previously sensitized. Thus, we demonstrated with two layers of evidence that IL-10 was not a mechanism of protection against anaphylaxis in this model.

While our results clearly indicate that IL-10 was not required for protection against anaphylaxis, we were unable to determine whether or not it played a redundant role with other regulatory cytokines such as TGF-β. To accomplish this in the future, we would first examine serum levels of TGF-β in pre-challenge serum to determine whether

or not it was elevated above the level of mock-infected controls in the setting of chronic *L.s.* infection. If we found a significant difference, we would then conduct an experiment to determine whether or not TGF-β plays a redundant role in protection with IL-10. In order to test our hypothesis, we would deplete TGF-β in IL-10 knockout mice for several weeks prior to challenge. Antibody depletion of TGF-β has been successfully performed in our laboratory previously (48). TGF-β would also be singly depleted in BALB/c mice to determine whether it confers protection against allergic challenge independent of IL-10. If we demonstrated that mice continue to be protected by 1.5°C in core body temperature in the absence of IL-10 and TGF-β, we would conclude that IL-10 and TGF-β were not the mechanisms of protection against anaphylaxis in our model. If we lost protection in the experimental group lacking both IL-10 and TGF-β, but not in the group lacking TGF-β alone, we would conclude that the two cytokines acted redundantly to confer protection. In contrast, if we did not observe protection in the mice lacking both cytokines, and did not observe protection in mice lacking TGF-β, we would conclude that TGF-β was our mechanism of protection. We would not expect to observe other outcomes besides those two possibilities since we have already demonstrated that IL-10 was not required for protection against anaphylaxis in our model. We think it is unlikely that both IL-10 and TGF-β are required for protection against anaphylaxis at 12 weeks post-infection, as cytokine levels from our ex vivo splenocytes were somewhat low, and we failed to notice even a modest effect from knocking out IL-10 alone.

Challenge at 7 Weeks Post-infection

Seven weeks of infection with *L.s.* did not protect against clinical signs of anaphylaxis in OVA-sensitized mice, as measured by changes in core body temperature following challenge (Figure 11B). Interestingly, clinical protection was not achieved despite the significant impairment of mast cell degranulation at 7 weeks of infection as measured by mMCP-1 levels in the serum following challenge (Figure 11A). However, compared to at 12 weeks post-infection, the serum values of mMCP-1 from both *L.s.*- and mock-infected mice were both significantly higher at 7 weeks. In fact, the concentration of mMCP-1 in the serum from *L.s.*-infected mice as compared to mock-infected mice was nearly 6.5-fold lower.

Based on our results, we propose two possibilities to explain our data. First, we hypothesize that mast cell degranulation and release of granule contents at 7 weeks post-infection exceeded a threshold whereby clinical differences could be measured in terms of temperature difference. For instance, an animal will exhibit a maximal drop in temperature within a given time period. If both groups meet that threshold, the temperature reading will not be different between them. Second, we cannot rule out the possibility that mechanisms in addition to mast cell suppression are at play in the observed protective effect. It is possible that inflammatory mediators were differentially expressed between 7 and 12 weeks post-infection. A future experiment could examine the expression of histamine receptors on endothelial linings as decreased expression of histamine receptors could translate to increased resistance against vasodilation. In order to elucidate the mechanistic differences at the seven and twelve week post-infection time points, we would ideally like to perform a number of additional studies that we were unable to perform at the seven week time point in this study.

Challenge at 2 Weeks Post-infection

OVA-sensitized mice that were either *L.s.*- or mock-infected were challenged at 2 weeks post-infection (n=15 per group). Analysis of core temperature by 2-way ANOVA revealed that *L.s.*-infected mice were protected against hypothermia as compared to mock-infected mice (Figure 13B). Additionally, mMCP-1 in the serum following challenge was significantly less in *L.s.*-infected mice compared to mock-infected controls (Figure 13A).

The results of this particular experiment were surprising to us, as we had hypothesized that duration of infection influenced protection against anaphylaxis. Secondarily, we had theorized that degree of sensitization also influenced the ability to protect. Nevertheless, we observed that only 2 weeks of infection with *L.s.* was sufficient to confer significant immunological and clinical protection (Figure 13B). We compared levels of mMCP-1 between groups at various time points and showed that even though levels are higher in both *L.s.*-infected and mock-infected controls at 2 weeks than at 7 weeks, clinical protection is still observed. Thus, we hypothesize that either mechanisms in addition to mast cell suppression are at play, or an alternative mechanism for clinical protection is at play at 2 weeks compared to 12 weeks.

Mast Cell Studies at 12 Weeks Post-infection

We were interested specifically in examining the effects of helminth infection on mast cells. Previous research in our laboratory demonstrated that chronic helminth infection is able to suppress the other major effector cell type of type I hypersensitivity,

the basophil, through an IL-10-dependent mechanism. Given the finding that helminth infection could suppress an effector cell of type I hypersensitivity, as well as the strong link between helminths and suppression of allergy in the literature, we naturally wondered whether or not helminth infection could also suppress mast cells. In fact, we found that the impact of helminth infection on mast cells, particularly in the context of allergic disease, was understudied. Since we observed a marked suppression of mast cell function at 12 weeks post-infection as measured by mMCP-1 release, we decided to directly compare mast cells from *L.s.*-infected versus mock-infected mice directly at this time point. We took a number of approaches to compare mast cells. We compared mast cells at 12 weeks post-infection by 1) microscopy, 2) ELISA, 3) flow cytometry, 4) proteomics, and 5) lipid analysis.

Overall, the data from our comparison of mast cells from chronically infected mice and mock-infected controls suggest that changes in mast cell functionality were central to the protective effect that we observed at 12 weeks post-infection. The results of histology were striking. Mast cells from chronically infected mice were almost entirely devoid of granules compared to mast cells from naïve mice, which were densely packed with granules. We identified representative images of mast cells from each of these experimental groups by electron microscopy. While the mast cells from *L.s.*-infected mice resembled mast cells in their activated state, mast cells from naïve mice resembled mast cells in their resting state.

Our flow cytometry results offered insights into our histological observations. We identified mast cells using by double staining for IgE and CD117 (Figure 25). First, the side scatter of mast cells from mice that were chronically infected with *L.s.* was lower

than from mock-infected mice (Figure 27). This result indicated that, at baseline, mast cells from chronically infected mice were less granular than their mock-infected mice. We hypothesize that mast cells in the mice that are chronically infected with *L.s.* are "depleted" or "exhausted" of their granule contents. In addition to our SSC observation, we also noticed that CD200R levels were expressed at lower levels on mast cells from chronically infected mice (Figure 27). CD200R is a known activation marker on mast cells (62). CD200R, a type I membrane glycoprotein, contains two Ig-like domains (124). Overexpression of CD200R has been shown to act as an inhibitory receptor on mast cells, preventing degranulation through FcεRI signaling (18). We think that in the setting of chronic mast cell activation, CD200R served as a negative feedback mechanism to prevent further degranulation of mast cells following periods of chronic activation.

We also observed by flow cytometry that CD63 expression on mast cells from chronically infected mice was either absent or very low (Figure 27). CD63 is present on the cell surface of mast cells and in their lysosomes, specifically in secretory lysosomes that contain allergic mediators of inflammation (56). CD63, also known as LAMP3, is a tetraspanin (91). CD63 is required for degranulation of mast cells, as studies with CD63-deficient mice exhibit diminished cutaneous anaphylaxis in response to challenge (57). Thus, it is reasonable to hypothesize that mast cells depleted of their granules would exhibit decreased CD63 expression. We also found that total IgE expression on mast cells from chronically infected mice was slightly elevated compared to controls (Figure 26). The total IgE result again suggested to us that alteration in IgE expression were not the mechanism for protection against anaphylaxis at 12 weeks post-infection. Mast cells are able to undergo repeated rounds of degranulation without dying (99). We

hypothesize that mast cells from *L.s.*-infected mice were less granular because they are depleted of their granule contents, potentially by repeated activation through the IgE signaling pathway.

By ELISA, we showed that total histamine from *L.s.*-infected mast cells was significantly less than total histamine from mock-infected mice, supporting our hypothesis that granule contents are depleted in chronically infected mice (Figure 23). We were not able to compare baseline mMCP-1 between groups, as we were not able to detect any from freeze-thaw ruptured cells by ELISA. We speculate that the reason we were unable to detect mMCP-1 from cells is that it either needs to be stabilized in a complex with plasma serpin to prevent its degradation, or that one of the antibodies used in the ELISA kit recognizes it only when in complex with serpin (88). In addition to baseline histamine in mast cells, we also examined baseline histamine levels in the blood, which would represent basophil-derived histamine. However, unlike with mast cells, we did not observe any differences between *L.s.*- and mock-infected mice, emphasizing the importance of mast cells in protection against allergy in this model. One limitation of this experiment was that we did not enumerate basophils to determine whether or not infected animals were masking lower per cell levels of histamine through increased cell numbers. This is a relevant consideration in evaluating this piece of data as chronic infection with helminths has been shown to drive basophilia (78). Regardless, the availability of histamine in the blood is not significantly different between mock- or *L.s.*-infected mice.

We also found differences in mast cells from mice that were infected with *L.s.* for 12 weeks compared to mock controls by MS and proteomics analysis. Of the 53 proteins

that were identified as non-contaminants in our mast cells, 24 were differentially regulated between *L.s*-infected and mock mice. Of those 24 proteins, 9 were up-regulated (Table 1) in the setting of chronic infection and 15 were down-regulated (Table 2).

In terms of proteins that were up-regulated, we found several that are involved in cell repair, including peroxiredoxin-6, actin, tubulin alpha 1B, and isoform 2 of histone H2B (DNA repair). Elevated levels of these proteins could indicate that mast cells in the state of chronic infection are undergoing repair. Peroxiredoxin-6 is implicated in phospholipid turnover. In the setting of helminth infection, chronically activated cells could indicate that there is increased membrane and phospholipid turnover. On the other hand, peroxiredoxin-6 is also involved in protection against oxidative injury. Elevated levels during infection could indicate the presence of increased oxidative products form mast cells constantly undergoing degranulation. The protease inhibitor alpha-2-Macroglobulin (A2M) was also elevated in infected mast cells. Elevated levels could be in response to increased activation and trypsin release in the setting of chronic infection. Finally, myeloperoxidase, which catalyzes the production of hypochlorous acid, was also increased in infected samples. Elevated levels could indicate that mast cells were attempting to make inflammatory products in response to repeated activation.

We also found interesting proteins within the fifteen that were down-regulated in mast cells from Infected mice compared to the Mock controls. A number of these proteins have known roles in metabolism. These data suggest that metabolism could be altered in mast cells from chronically infected mice. We found that carboxypeptidase A (Cpa3), a secretory granule protease, was down regulated in mast cells from chronically

infected mice (31). Other extracellular proteins that were down regulated include elastase inhibitor and myeloblastin. This finding contributes to our hypothesis that granule contents are depleted in the setting of chronic infection compared to mock infected mice. We also found that S100A6, which is a calcium binding protein, was lower in Infected compared to Mock. Since calcium is required for mast cell degranulation, this difference could indicate that mast cells in the setting of chronic helminth infection are in an "exhausted" state.

The results of our proteomics, though certainly interesting, are hypothesis generating and not conclusive. In order to validate our findings, we need to confirm our results by secondary methods such as Western blotting. It would then be interesting to test our hypotheses regarding the roles of some of the more promising proteins that we identified in our model.

Overall, the results of histology, flow cytometry and protein analyses demonstrate that chronic helminth infection dramatically impacts the baseline status of mast cells in this model. The results support the idea that alterations to mast cells are the central mechanism of protection against anaphylaxis in this model. We hypothesize that mast cells from chronically infected mice are depleted of their granule contents, likely through repeated activation through IgE signaling. Although efforts to pursue the mechanism have been limited to date, we hypothesize that chronic activation occurs through repeated signaling through IgE. We know that helminth infection drives polyclonal expression of IgE (50; 109). We also know that helminth-specific IgE becomes bound to the cell surface of mast cells. We hypothesize that mast cells become repeatedly activated through IgE by either circulating antigen or excretory-secretory product. Because we do

not observe any adverse effects in mice, and normal helminth infection does not result in spontaneous anaphylaxis, we conclude that degranulation of mast cells occurs gradually over time, at a very low level.

Future Directions

While several future experiments were discussed in previous sections, there are a few additional experiments that would be important to address in continuing this work. For instance, an important future experiment would be to examine the expression of OVA-IgE on mast cells in *L.s.*- as compared to mock-infected mice. Although serum levels of OVA-IgE were not significantly different between the two groups, and serum IgE generally correlates with relative abundance of cell surface expression of FcεRI and IgE on mast cells, our studies cannot conclusively exclude the possibility that OVA-IgE expression was differentially expressed in mock- vs. *L.s.*-infected mice (40). There are at least two experimental approaches that we could perform in the future to compare relative amounts of OVA-IgE. The first approach would be to acid-wash purified mast cells to liberate bound OVA-IgE. ELISA could be performed to quantitate OVA-IgE and a comparison between *L.s.*- and mock-infected mice could be made. Alternatively, flow cytometry could be performed using OVA-GFP to compare relative expression levels of antigen specific antibodies bound to the cell surface.

Our current study makes a strong case that alterations in mast cells are primarily responsible for the differences in protection observed between mock- and *L.s.*-infected mice. However, in order to conclusively demonstrate that mast cells are the mechanism of protection, we would ideally perform adoptive transfer studies. These studies are

challenging given the granularity, size, and hyper-responsiveness to stimuli of mast cells. Theoretically, however, there are a number of variations in how these studies could be performed. One way would be to purify mast cells from mock- and *L.s.*-infected mice. These cells would then be transferred into sash mice, which are deficient in mast cells. Approximately 24 hours following transfer, survival of mast cells in recipient mice would be confirmed by performing flow cytometry on a few animals. Mast cells would be identified as $CD117^+$ and IgE^+ and enumerated in recipient mice. Survival would be compared between groups to ensure that equal numbers of mast cells from the two experimental groups survived transfer. Once mast cell transfer and survival was confirmed, the experiment would be performed in order to determine whether or not transfer of mast cells from chronically infected mice resulted to dampened anaphylaxis compared to transfer of mast cells from mock-infected mice. In addition to the difficulty in adoptively transferring mast cells, a challenge of this study is the number of animals needed. At least fifteen animals per recipient group (sash mice either receiving mast cells derived from mock- or *L.s.*-infected mice) would be needed in order to determine whether potential difference in core body temperature between the groups was significant. About 2-3 additional mice per group would be needed to perform survival analysis of mast cells by flow cytometry. If we saw protection with transfer of mast cells from chronically infected mice into naïve sash mice (i.e. decreased hypothermia relative to mast cells transferred from mock-infected), we would be able to conclude that altered mast cell functionality was central to helminth-dependent protection against anaphylaxis in our model.

Another future direction would be to determine the mechanism by which mast cells are altered in the setting of chronic *L.s.* infection. Indeed, experiments with knockout mice could reveal a mechanism for the changes in granularity and activation marker expression observed by flow cytometry. Jh mice could be used to determine whether or not antibodies are required for the decreased granularity of mast cells at 12 weeks post-infection. These mice carry a deletion in the endogenous J segment of the Ig heavy chain locus, which renders them deficient in IgM and IgG. Jh mice could be infected with *L.s.* or mock and compared 12 weeks post-infection to determine whether or not activation through antibodies is required for the changes observed with mast cells. Alternatively, FceRI knockout mice could be used to determine if signaling through the high affinity IgE receptor is required for the observed phenotype in mast cells.

Our results might call into question whether or not the changes caused by chronic *L.s.* infection are due to changes in precursor cell lineage or mature mast cells. Although we believe that chronic helminth infection is affecting mature mast cells, we could rule out the possibility that is affecting precursor cells by analyzing precursor frequencies in the bone marrow to address this issue. Demonstrating that chronic infection does not alter precursor frequencies would support our current hypothesis regarding the mechanism of mature mast cell "exhaustion". Along the same vein, if the morphological differences were shown to result from changes to mature mast cells as we hypothesize, it would be interesting to determine whether or not these changes were reversible. Performing flow cytometry to analyze mast cells from mice that have cleared their chronic helminth infections could address this question. With our proposed mechanism, we would expect mast cells to return to their "resting" phenotype following worm

expulsion, as they would be able to replenish their granules and would not be constantly activated through exposure to worm antigen or excretory-secretory product.

Finally, an important future direction would be to determine if antigen-derived from *L.s.* could produce the same protective effect as live infection in this model of anaphylaxis. These experiments are challenging, as they require dosage optimization and large quantities of antigen. Ultimately, however, the goal of this type of research is to treat patients therapeutically with a worm or worm product that does not cause adverse pathology. Helminthic products such as surface proteins and secretory-excretory products have been shown to be immunomodulatory (113). We hypothesize that worm-derived antigen could be an important therapeutic compared to live infections, which cause lymphatic obstruction in humans. A study demonstrated that antigen from the free-living nematode *Caenorhabditis elegans* induces a similar immune response to parasitic filarial nematodes (105). In fact, our laboratory previously published that infection with *L. sigmodontis* protects against the onset of autoimmune diabetes in NOD mice (47). Unpublished data from our laboratory also revealed that nematode antigen could be used as a surrogate for infection in preventing the onset of diabetes in the NOD model. Thus, we think that nematode antigen could be similarly useful for protection against development of and therapeutic alleviation of allergic disease.

While over 30 studies to date have demonstrated that helminths can protect against the development of allergy when animals are infected prior to sensitization, this study is one of just a few to evaluate the effects of helminth infection after sensitization. Furthermore, it is one of the first, if not only, studies to directly examine the effect of chronic *L.s.* infection on mast cells, in particular by flow cytometry and to observe the

dramatic changes of infection on mast cell granularity. To date, studies evaluating the therapeutic potential of helminths in pre-existing allergy have demonstrated mixed results. However, our study demonstrates several of the challenges and limitations of such experiments on mast cells, including the difficulty in assaying their function in small animal models.

Significance

One of the most important findings of the current study was that helminth infection altered the baseline status of mast cells as 12 weeks post-infection. These results clearly demonstrate that helminths modulate the function of a primary effector cell of type I hypersensitivity. While we observed dramatic immunological protection at all of the time points that were evaluated, we only observed clinical protection at 2 and 12 weeks post infection, and not at 7 weeks post infection. Our results suggest that clinical protection might depend on the specific point during infection that allergic challenge occurs. We hypothesize, as others have before, that timing of infection is an important consideration in the ability of helminths to protect against allergy (21). Timing of challenge may partially explain why clinical trials to date have not been met with success.

A major question elicited by this work is exactly how a 1.5°C difference in core body temperature translates to human health. This question raises a myriad of points; among them, but not trivial, how relevant any animal system is in modeling human disease. While a 1.5°C difference may not initially seem noteworthy, we believe it is actually fairly dramatic given the robust levels of allergic sensitization and challenge

these animals endured. In the future, we would like to perform an experiment in which we infected animals that are sensitized using a single dose of OVA in order to determine if helminth infection can be absolutely protective against clinical signs in a model of low-level sensitization. It is also worth mentioning that in our studies we did not optimize worm burden in our model to determine whether or not mast cell suppression could be even further suppressed. However, we think it is somewhat unlikely, given our system, that we could suppress mast cells even further, as the chronic infection is systemic and robust. Although we were unable to show absolute protection against anaphylaxis, we believe our study serves as proof of concept that helminth infection can in fact result in desirable clinical outcomes. It serves as an important first step in demonstrating that 1) helminth infection can modulate pre-existing allergy, 2) timing of infection could be important in determining therapeutic translatability, and 3) chronic infection with *L.s.* dramatically impacts baseline status of allergy effector cells.

Our study is the first to directly evaluate the effects of helminth infection on mast cell functionality. One of the most noteworthy findings of our study was that helminth infection leads to decreased release of mMCP-1 at all analyzed time points (weeks 2, 7, and 12 post-infection). Our data from challenge at 2 weeks post-infection suggest that duration of infection was not necessarily required for clinical protection against anaphylaxis. However, we were unable to fully explain the mechanistic differences between the various time points that we analyzed. Thus, we are not certain that the mechanism of clinical protection at 2, 7 and 12 weeks is the same. Since we operated under the hypothesis that duration of infection confers clinical protection, the majority of our studies were performed at the 12-week time point, where we first saw clinical

protection in our sequence of experiments. In fact, observing clinical protection at the 2-week time point came later in our studies, and was a great surprise to us. It will undoubtedly be of primary interest in moving forward with this research.

Nevertheless, the dramatic immunological effect on mast cells suggests that infection could be useful for protection against other types of type I hypersensitivity. Anaphylaxis is perhaps the most severe type I hypersensitivities. The challenge to mast cells in this type of reaction most likely exceeds a threshold by which helminths are sufficient for protection. In a more mild example of type I hypersensitivity, however, mast cell suppression might be sufficient to confer absolute clinical protection. As mast cell suppression does not always translate into clinical protection in this model, further research needs to be done in order to understand other contributing mechanisms. These experiments were largely outlined in earlier chapters of this thesis. Given our results, it is almost certain that mechanisms downstream of mast cell degranulation contribute to the clinical protection or lack thereof observed in this model. Further studies will increase our understanding of precisely how helminths and helminthic products could protect against type I-hypersensitivity reactions and may lead to novel approaches to treat pre-existing allergy.

We think that using helminthic therapy to treat pre-existing allergy is a real possibility in the future. We speculate that there are important alterations that need to be made in order to achieve full protection, however. While it is possible that we would see enhanced protection with a higher worm burden, it might not be feasible given the adverse pathology of helminthic infections. One experiment we plan to do is to use *C.*

elegans antigen as a surrogate for infection in our anaphylaxis model. By fine-tuning the dosage of antigen, we hope to observe complete protection.

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