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Abstract

Title of Dissertation:

Evaluation of allergy effector cell function: suppression of basophils in chronic helminth infections

David Larson, Doctor of Philosophy, 2011

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For the past several decades, allergic diseases have been increasing in industrialized countries. While the reasons for this increase are most likely multifold, both epidemiological and animal studies indicate that helminth infection suppresses atopy. The mechanisms by which helminth infection decrease allergic disease are currently unknown. The purpose of this thesis research was to determine whether helminth infection resulted in suppression of basophils, a well described allergy effector cell. To accomplish this, initial experiments were performed to determine whether

basophil responsiveness was reduced in *Litomosoides sigmodontis* and *Schistosoma mansoni* rodent helminth infection models by assessing basophil intracellular IL-4 and surface CD200R levels after IgE-mediated stimulation using flow cytometry. Reduced basophil responsiveness developed in mice with chronic helminth infections. The mechanism responsible for basophil suppression was determined to be increased production of the anti-inflammatory cytokine IL-10 during infection. Basophil responsiveness returned to baseline uninfected levels after helminths were no longer present. Additional experiments measuring histamine release from blood basophils of uninfected humans and humans with intestinal helminth infections were performed after anti-IgE and ionomycin stimulation. Basophils from uninfected humans released more histamine than basophils from infected humans. Additionally, basophils from infected children released more histamine 2 weeks after anthelmintic treatment. Together, these data indicate that reduced basophil functionality develops during helminth infection and requires the presence of live helminths. Reduced basophil functionality could have broad medical implications. Not only are basophils effector cells of allergy, but studies demonstrate that basophils play prominent roles in the development of type 2 immune responses, the type of immune response that drives allergic disease. Basophils are also important cells in the protection against some helminths. Therefore, basophil suppression could impact the development and severity of a variety of diseases and could explain data from other studies in which helminth infections conferred a protective effect against allergy. Additional data describing optimized methods to assess murine mast cell activation are also presented in this thesis, which could be used in future studies to determine whether helminth infection also suppresses this allergy effector cell.

EVALUATION OF ALLERGY EFFECTOR CELL FUNCTION: SUPPRESSION OF BASOPHILS IN CHRONIC HELMINTH INFECTIONS

By:

David Larson

Dissertation submitted to the Faculty of the Emerging Infectious Diseases Program of the Uniformed Serviced University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy 2011.

Acknowledgements

My advisor - Dr. Ed Mitre:

I will always remember your keen insight, sense of humor, and positive outlook. Thank you for all of your advice and guidance. You are a fantastic mentor.

My committee members - Dr. Davies, Dr. Via, Dr. Maynard, and Dr. Prussin:

Thank you all for taking time out of your busy schedules to provide me with guidance and helpful suggestions. I know you have made my project better.

Past and present lab members - Marina, Marc, Ellen, Kristin, Yinghui, Paul, and Holly:

Thank you all for happily providing assistance when needed. I will miss working with you and hope we can keep in touch.

My family – Parents, parents-in-law, brothers and sisters-in-law:

I have an incredibly generous family. All of you freely offered up your time to help us when we needed it. Sometimes it was to fix a broken bathroom. Other times it was to watch the kids so we could work or relax. I can't thank you enough.

My wife and kids:

Having two kids while in graduate school is tough, but you're more than worth it! Love you guys. Noelle, my friend and partner, thank you for all of your patience and support. I love you deeply.

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Chapter 1: Introduction

Helminths

Nematodes

Nematodes, also known as roundworms due to their cylindrical bodies, make up the largest group of helminths that parasitize humans. A 1997 World Health Organization study estimated that nearly 4 billion people were infected with nematodes (1). Parasitic nematodes are unsegmented, bilaterally symmetric, have digestive tracts and separate sexes. Nematode life cycles consist of 4 larval stages and 1 adult stage, with the third larval stage (L3) serving as the infectious stage for most nematode species (1).

Filarial Nematodes

Humans are the definitive host for eight different filarial nematodes. *Wuchereria bancrofti*, *Brugia malayi*, and *Onchocerca volvulus* are the species of filaria responsible for most infections (2). The life cycle is similar for all filarial worms, with infectious arthropod vectors initiating infection during blood feeding. When infected insect vectors take a blood meal, L3-stage larvae enter through the bite wound and migrate to specific niches and develop into adults. Adults mate and produce L1-stage microfilaria, which can be taken up by the vector where they mature into infectious L3-stage larvae (1). Filarial worms are long-lived with adult worms capable of producing microfilariae for 5-8 years in hosts (2).

Wuchereria bancrofti and *Brugia malayi* belong to the group of filarial worms that can cause lymphatic filariasis. The estimated global prevalence of lymphatic

filariasis is 120 million with *Wuchereria bancrofti* making up 90% of those cases (3). Mosquitoes are the vector responsible for transmitting *W. bancrofti* and adult worms develop to maturity in afferent lymphatic vessels, most commonly in the lower extremities or male genitalia (1).

Onchocerca volvulus causes the disease known as onchocerciasis or river blindness and is in the group of filarial worms that cause subcutaneous filariasis. The prevalence of onchocerciasis is nearly 40 million, with more than 30 million of those cases residing in Africa. Blackflies in the genus *Simulium* transmit infection and adult worms live in subcutaneous tissue often producing nodules known as onchocercomas. When microfilariae migrate through the eyes and other subcutaneous tissues, little pathology results. However, chronic inflammation develops around dead microfilariae that can lead to eye lesions and eventual sclerosing keratitis (3). Much of the inflammation that develops is believed to be induced by the bacterial endosymbiont, *Wolbachia*, released from dead microfilariae (1).

Five other filarial worms parasitize humans. *Brugia timori* causes lymphatic filariasis and is found in Indonesia. *Loa loa*, also known as African eye worm, and *Mansonella streptocerca* are the other two species of worms that belong to the subcutaneous filarial group (1, 3). *Mansonella ozzardi* adult worms can be found in subcutaneous tissues, yet they belong to the serous filarial group of worms along with *Mansonella perstans* since adult worms often reside in serous or body cavities (3). *Mansonella ozzardi* and *Mansonella perstans* are distributed throughout South and Central America, with *M. perstans* also being found in parts of Africa (1, 3).

Animal models of filariasis have been used to better understand how filarial infection alters the immune system and to develop anti-filaria vaccines. *Litomosoides sigmodontis* is one of the few models of filariasis capable of producing patent infections in rodents and is in the same family as the filariae that parasitize humans, Onchocercidae. While the natural host for *Litomosoides sigmodontis* is the cotton rat, *Sigmodon hispidus*, the complete life-cycle can also develop in immunocompetent BALB/c mice (4). Infectious L3 stage larvae travel through the lymphatics to the pleural, and sometimes peritoneal, cavity and develop into adults (Figure 1). Microfilariae are produced by adult worms and released into the circulation where the mite vector, *Onithonyssus bacoti,* can pick them up upon blood feeding (4). Adult worms of the human filarid *Mansonella perstans* resides in coelomic cavities of humans and also has circulating microfilaria (1), making *Litomosoides sigmodontis* an especially fitting model for that human filarial species.

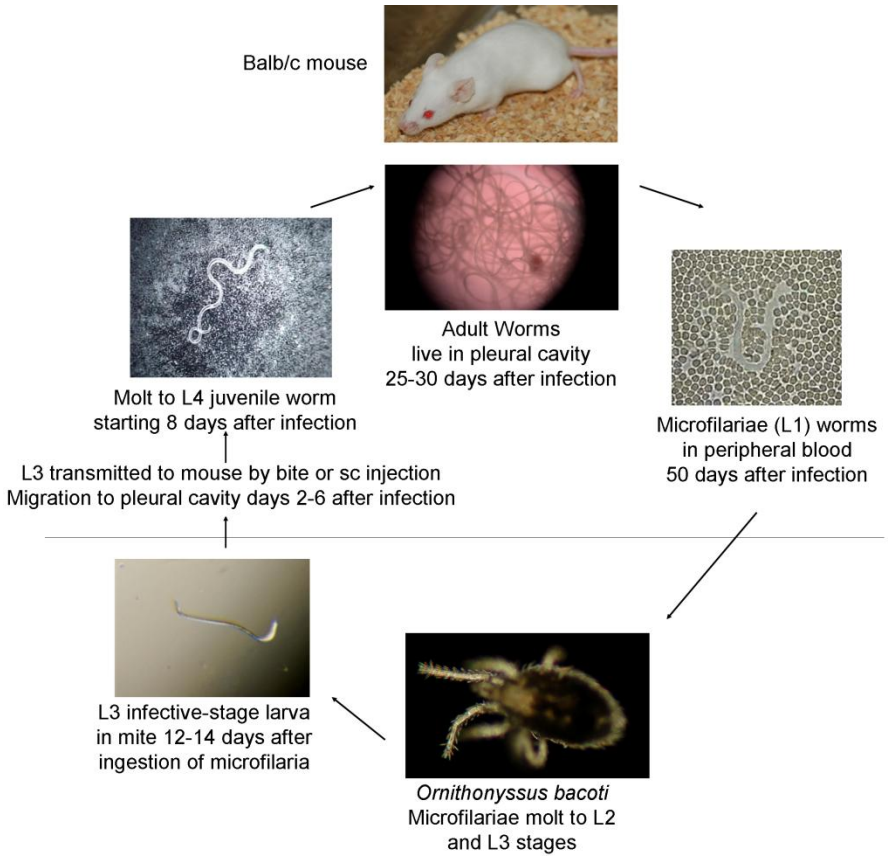
Intestinal Nematodes

Intestinal nematodes are distributed throughout tropical regions around the globe, where an estimated 1 billion people are infected. The most prevalent species are *Ascaris lumbricoides*, hookworm (*Necator Americanus* and *Ancylostoma duodenalis*), and

Figure 1. Litomosoides sigmodontis *rodent filarial life cycle.*

Infectious mites deliver L3-stage larvae to BALB/c mice when taking a blood meal. Larvae migrate to the pleural cavity, mature into adults and mate. Microfilariae (L1-stage) are released by female adult worms, which enter the circulation where they can be picked up by additional mites. Microfilariae develop into infectious stage larvae in mites.

Figure 1. Litomosoides sigmodontis *rodent filarial life cycle.*



Trichuris trichiura (5). Infection with *Ascaris lumbricoides* and *Trichuris trichiura* occurs when fertilized eggs in contaminated soil are swallowed. *A. lumbricoides* has a complicated migration through the host before ending up in the small intestine where it finishes maturation, mates and produces eggs. Adult *T. trichiura* worms reside in the large intestine with their thin heads imbedded in the mucosa and their thicker tails hanging in the lumen (1). Hookworms infect humans when infectious third-stage larvae penetrate the skin, and attach to the mucosa of the small intestine where they develop into adults (1).

Schistosomes

Schistosoma species are trematodes belonging to the phylum Platyhelminthes. More than 200 million people throughout the world have Schistosomiasis (6). The 4 species responsible for most human cases of Schistosomiasis are *Schistosoma haematobium*, *Schistosoma japonicum*, *Schistosoma mekongi*, and *Schistosoma mansoni*. Another species, *Schistosoma intercalatum*, also infects humans but to a lesser degree (1).

For *S. mansoni*, infection occurs when individuals wade in water contaminated with cercariae (6). Cercarie penetrate intact skin, becoming schistosomula, which migrate for roughly 48 hours before invading a blood vessel. Blood carries schistosomula to the lungs first and then to the liver where they mature to adult worms. Male and female adult *S. mansoni* worms form pairs and migrate to the inferior mesenteric veins and begin to lay eggs that travel to the liver and are eventually shed in

the feces. When eggs contact fresh water they hatch, releasing miracidia that swim and infect *Biomphalaria* snail intermediate hosts. Miracidia develop into cercariae in the snail and are eventually released into the surrounding water where they await future hosts (1).

Mice are used extensively for the study of schistosomiasis in humans (7). Murine infections with *S. mansoni* model human infections very well. *S. mansoni* is able to fully mature in mice yielding patent infections. While the density of infection might be greater in mice compared to humans, they develop the same pathology with deposition of eggs in the liver leading to hepatic granulomas and down-modulation of the immune system in chronic infections (7).

Type 2 Immune responses

Type 2 immune responses develop during helminth infection and allergic disease and are characterized by increased production of cytokines such as IL-4, IL-5, and IL-13 from CD4+ T cells. When T_H2 cells produce IL-4 or IL-13 while co-stimulating B cells through the interaction of CD40L and CD40, it can lead to class switching and induce B cells to make IgE (8). IgE produced against environmental antigens sensitize basophils and mast cells by binding to the high-affinity IgE receptor, FcεRI, expressed on their surfaces (8, 9). When multivalent antigen contacts IgE on the surface of a sensitized cell, the receptor becomes cross-linked leading to activation of the cell. Basophils and mast cells contain preformed molecules in cytoplasmic granules such as histamine and

proteases, which can be released rapidly after receptor cross-linking causing classical allergy symptoms (10).

Basophils and Mast Cells

Basophil Basics

Basophils are leukocytes that complete their development in the bone marrow and then enter the circulation where they primarily reside, unless recruited to sites of inflammation in peripheral tissues (10). While basophils are probably best known for their participation in the effector phase of immediate hypersensitivity reactions, some studies have suggested that they also play a critical role in the development of type 2 immune responses.

Basophil Involvement in Initiation of Type 2 Immune Responses

Initial studies indicated that basophils might be responsible for the initiation of type 2 immune responses. For instance, the dust mite allergen Der p1 was able activate basophils in vitro even though the basophils had not been sensitized to it (11). This nonspecific activation was also observed after stimulating basophils with products derived from helminths, organisms known to induce type 2 immune responses in susceptible hosts during infection. For example, stimulation of purified basophils with *Schistosoma mansoni* egg antigen and *Echinococcus multilocularis* metacestode extract resulted in IL-4 and histamine release in vitro (12, 13).

These in vitro studies hinted that basophils could initiate type 2 immune responses and were further supported by studies suggesting that basophils can migrate to lymph nodes and act as antigen presenting cells, with the ability to induce naïve CD4+ T cells to differentiate into T_H2 cells. For example, depleting basophils in these studies resulted in a drastically reduced type 2 immune response. Also, transfer of what was believed to be only basophils pulsed with antigen induced the differentiation of T_H2 cells in vivo (9, 14, 15). According to these studies, basophils are able to accomplish this because they are capable of expressing MHCII and CD80. Thus, when basophils internalize antigen, process it, and present the peptides on their MHCII molecules while producing IL-4, they can provide all necessary signals for effective T_H2 differentiation (8, 9, 14, 15). However, those studies depleted basophils using anti-FcεRI monoclonal antibodies that are not entirely basophil-specific. Subsequent studies have shown that anti-FcεRI antibody can also deplete subsets of dendritic cells (16, 17). It was also discovered that culture conditions used to generate basophils for transfer experiments produced the same subset of dendritic cells that express FcεRI (16). Therefore, while some studies may demonstrate that basophils serve to initiate type 2 immune responses, the results may be spurious due to incidental depletion or transfer of dendritic cell subsets. These experiments highlighted the need for more appropriate antibodies to specifically deplete basophils or basophil knockout mice.

Most recently, anti-CD200R3 (Ba103) Ab, which targets basophils specifically for depletion, demonstrated that basophils were dispensable for the initiation of type 2 immune responses (16, 18). In a study using house dust mite allergen, basophils did not act as antigen presenting cells and were not necessary for the induction of type 2 immune

responses, but inflammatory dendritic cells were (16). Basophils also were not necessary for the type 2 immune responses to develop in a *L. sigmodontis* infection model (18). Studies using basophil knockout mice corroborate the findings with Ba103 antibody and seem to rule out basophils as initiators of type 2 immune responses (17).

Basophil Involvement in Amplification of Type 2 Immune Responses

While basophils may not be involved in starting type 2 immune responses, it does appear that they can amplify them. In studies where basophils were depleted using the more basophil-specific Ba103 Ab, a reduction in the type 2 immune response was observed (16, 18, 19). This reduction in IgE production and type 2 cytokines is most likely due to the loss of basophil IL-4 production. However, a loss of basophil CD40L could also be playing a role in the reduced IgE production observed in Ba103 depletion studies since IL-4 and CD40L can drive class switching in B cells and IgE production (8).

Basophil Involvement in Immunity to Helminths

Finally, in addition to their participation in the amplification and effector phase of type 2 immune responses, basophils also appear to have a role in the protection against some helminth species. For example, depletion of basophils resulted in decreased clearance of the intestinal worm Trichuris muris in experimental mice (14). Also, using a basophil deficient mouse strain, which lack 90% of their basophils, demonstrated that basophils play a key role in protection against secondary infection with *Nippostrongylus brasiliensis* (20).

Mast Cell Basics

Mast cells are produced in the bone marrow and circulate in small numbers before migrating to tissue sites throughout the body where they become fully mature. The most common sites where mast cells are found are skin, airways, intestine and in close proximity to blood vessels (8, 21). Residing in these tissues provides mast cells with a strategic location since they are exposed to the external environment, providing evidence that they play a role as sentinel cells in host defense (21).

Mast Cell Involvement in Type 2 Immune Responses

Like basophils, mast cells are probably best known for their participation in the effector phase of allergic responses. The release of mediators, such as histamine and leukotriene C_4 (LTC_4), is responsible for many of the symptoms of allergic disease by increasing bronchoconstriction and vascular permeability (21). In addition to the role mast cells play in the effector phase of type 2 immune responses, murine mast cells also have the ability to amplify them by producing IL-4 and expressing CD40L (8), although they produce far less IL-4 on a per cell basis than basophils.

Mast Cell Involvement in Immunity to Helminths

Considering mast cells are often found in sites that ensure interaction with pathogens and the fact that they can quickly release preformed mediators, it is not surprising that mast cells have been found to play a role in the immunity against some helminth species. Expulsion of the rodent intestinal helminths, *Trichuris muris* and *Nippostrongylus brasiliensis*, corresponded with the release of specific mast cell proteases (21). Mice that lacked a mast cell-specific protease had delayed clearance of the intestinal dwelling helminth *Trichinella spiralis* (22). Interestingly, mast cells were

recently demonstrated to play a critical role in susceptibility to the filarial nematode, *Litomosoides sigmodontis*. In this study, mast cell recruitment to the skin and degranulation during L3 challenge lead to increased vascular permeability, larval entry, and higher worm burdens 10 days later. The recruitment and degranulation of mast cells in this experiment seemed to be dependent on dendritic cell-produced chemokine CC17 (23). However, whether mast cells play a role in immunity to *L. sigmodontis* once *L. sigmodontis*-specific IgE is present is unclear.

Allergic Disease

Allergy Prevalence

Allergies can develop to normally harmless environmental antigens. Some examples of environmental allergens are pollen, pet dander, dust mites, and even certain foods (8). Industrialized countries have seen a dramatic increase in allergic diseases over the last several decades. This fact was highlighted by an epidemiological study that analyzed cross-sectional surveys of children living in Aberdeen, United Kingdom from 1964 – 1999. The results of the surveys indicated that children had a steady rise in reported cases of asthma, eczema, and hay fever over that period of time (24). Increases in allergic diseases have also been observed in other industrialized countries. For example, in the United States when information on food allergy among children aged 0 – 17 years was collected by the National Health Interview Survey (NHIS), it revealed an 18% increase in prevalence from 1997 – 2007. Hospitals have seen increased workloads due to food allergies as outpatient visits and hospitalizations related to food allergies

were statistically higher during that same time frame (25). Additionally, atopic dermatitis prevalence is also now 2 – 3 times higher in industrialized countries than it was 30 years ago. Up to 30 % of children and 10 % of adults are affected, with children often going on to develop other atopic diseases (26). It was estimated that 1 in 5 children from industrialized countries were afflicted with an allergic disease in 1998 and this amount has increased over the last 10 years, with increases in asthma being the most dramatic (27). Interestingly, one study showed that asthma prevalence was higher in children after immigrating from a developing country to a developed country (24).

Potential Reasons for Increased Atopy

Several hypotheses have been put forward to explain the rather precipitous rise in allergic diseases. One factor demonstrated to be positively associated with atopy is economic status. As family income goes up, so does the incidence of atopic dermatitis. However, the specific socio-economic factor responsible for increases in allergy is not clear from these studies (27).

The food consumed today in industrialized countries has changed considerably from when allergy prevalence was much lower, with less fresh products making up a part of the daily diet. As an example, the consumption of fresh green vegetables has decreased in the United Kingdom from 392 g to 240 g per day per person over a 50 year period (24). Also, for many years now, food in industrialized countries have undergone extensive processing and prolonged storage, which has been concurrent with declines in the mineral content of fruits, vegetables, and meats (24). Whether these reductions in mineral content are a result of changed agricultural practices or because there has been

increased storage and transportation of foods is unclear. It has been proposed that these changes in diet are responsible for decreased consumption of antioxidant-rich vitamins and nutrients. The decreased intake of antioxidants result in increased damage to the lungs stemming from decreased antioxidant-mediated defenses. Damage to the lungs then leads to increased permeability of the lung mucosa, allowing allergens more penetration and inappropriate allergen exposure to the immune system (24). The few observational studies that have been undertaken seem to support the role nutrients play in allergy development. Skin test positivity was inversely correlated with vitamin E intake and blood levels of vitamin E, β-cryptoxanthin, and α-carotene. However, interventional studies where nutrients such as vitamin C, vitamin E, or selenium were given to individuals with disease, no improvement in symptoms were observed (24).

Epidemiological studies have demonstrated that while allergic diseases have been increasing in industrialized countries, vitamin D levels have been decreasing (28). Plasma vitamin D levels can be increased by eating certain foods or by exposure to sunlight. Therefore, differences in vitamin D levels may be from either a more sedentary lifestyle with less outdoor exercise, or eating different foods. Theories as to how vitamin D might be able to suppress atopy stem from its immunomodulatory properties. It is believed that vitamin D can contribute to the development of Foxp3+ regulatory T cells and aid production of anti-inflammatory cytokines such as IL-10 (28). At this time, while the associations are compelling, there is very little proof that decreased vitamin D levels are to blame for the increase in allergic diseases. For example, it is not currently known whether improving plasma vitamin D levels will also improve allergic diseases as intervention studies have not been initiated (28).

One other potential explanation for the increase in allergic diseases is that individuals in industrialized countries get fewer infections. This theory is often referred to as the hygiene hypothesis and was first formalized by David Strachan in 1989 after observing that the prevalence of hay fever and asthma decreased as number of siblings increased (27, 29). According to the hypothesis, atopy is thought to be a result of public health measures such as water decontamination, sterilization of milk, vaccinations, and antibiotic use in developed countries which have resulted in a reduction of infections. Living conditions where increased exposure to infectious diseases can occur are thought to be protective for the development of allergy (27). For example, children who attend day care centers have many more infections, yet are at a reduced risk for the development of atopic dermatitis and asthma. Atopic diseases are also prevented by early life exposure to farms and cowsheds (27). Some of the strongest data for the hygiene hypothesis comes from studies evaluating whether helminths protect against allergic disease.

Allergy and Helminth Infection

The idea that helminth infection could protect against allergic disease at first appears counterintuitive. After all, it would seem that the increased cytokine production from T_H2 cells during a helminth infection would also help drive allergic responses to allergens. While the type 2 immune responses that develop to both helminths and allergens are largely identical, anti-inflammatory responses develop in chronic immune responses to helminths, but do not in responses to allergens (30). Numerous epidemiological studies in children indicate a positive association between helminth infection and protection against atopy. For example, in a *Schistosoma mansoni*

hyperendemic area of Brazil, only 4.8 % of infected individuals were skin test positive to allergens compared to 24.3% of uninfected individuals, an association that remained after adjusting for age and gender. Despite having more total IgE, infected individuals had less allergen-specific IgE in their plasma compared to the uninfected group (31). Another study in an *Ascaris lumbricoides* and *Trichuris trichuria*-endemic Venezuelan slum measured skin test responses to allergens in children at the beginning of the study and then 22 weeks later. A group of children who declined anthelmintic treatment had an increase in the prevalence of infection, which was associated with decreases in allergen specific IgE and skin test responses to allergens. Interestingly, in the group of children who underwent monthly anthelmintic therapy, which removed intestinal helminth infection, allergen-specific IgE increased as did skin test positivity to allergens (32).

The idea that helminth infection suppresses allergic disease is further strengthened by animal studies. Mice that were uninfected or had *Schistosoma mansoni* infections were then sensitized to Penicillin V. When mice in these groups were challenged with Pen V, body temperature was measured along with serum levels of the mast cell products mMCP-1 and histamine. Twenty percent of uninfected mice died from anaphylaxis after challenge, and the average levels of mMCP-1 and histamine in serum was also very high (33). However, none of the infected mice died after challenge, only had a negligible change in body temperature, and had much lower levels of mMCP-1 and histamine in the serum demonstrating that *Schistosoma mansoni* infection can provide a strong protective effect against allergy (33).

In another study, mice were infected with the filarial worm *Litomosoides sigmodontis* and then sensitized to OVA. When these mice were later challenged, all

allergic symptoms were eliminated. Airway reactivity and eosinophilia in the lung was markedly reduced, compared to uninfected sensitized controls. Some of the protection in this model was associated with TGF-β and regulatory T cells. However, infected animals were largely resistant to OVA sensitization, which may have also contributed to the observed protection in this study (34).

While most studies indicate that helminth infection protects against allergic disease, a few studies suggest that they do not or can even exacerbate allergies (30). The discrepancy may be related to how long individuals or animals were infected before evaluation of the allergic response took place. The most potent anti-inflammatory responses are induced by helminths that are long-lived and establish chronic infections in humans (30), which would be important if aspects of the regulatory immune response are required for suppressing allergic disease.

Determining the mechanism by which helminth infection can decrease allergic disease is a focus of current research. In chronic helminth infections, there is enhanced production of anti-inflammatory cytokines such as IL-10 and TGF-β and increased percentages of CD4+Foxp3+ regulatory T cells and alternatively activated macrophages (30).

Evidence that IL-10 could be suppressing the response to allergens in helminth infection comes from studies in humans with schistomsome infections. The infected individuals had reduced skin test positivity to allergens that was associated with increased IL-10 production from PBMCs when stimulated with worm antigen (35). Also, in the study where mice infected with *Schistosoma mansoni* were protected from anaphylaxis,

the transfer of IL-10-producing B cells from infected animals was enough to confer protection. However, only the transfer of B cells from IL-4 deficient mice was successful and not when B cells from wild type mice were transferred further complicating the mechanism of protection (33).

Developing macrophages can become alternatively activated when they encounter type 2 cytokines such as IL-4 and IL-13 during their migration to sites of active inflammation (36). Alternatively activated macrophages and regulatory T cells have similar abilities in that they can produce the suppressive cytokines IL-10 and TGF-β and also inhibit T_H2 cell proliferation by cell contact (30). Due to these abilities to inhibit immune responses, it is possible that alternatively activated macrophages or regulatory T cells are playing a role in helminth-mediated protection against allergy.

Additionally, it is possible that helminths produce and release substances that suppress the development of type 2 immune responses or even directly suppress allergy effector cells. For example, when extracts prepared from adult *Ascaris suum* worms (ASC) were combined with egg white protein and implanted in mice, type 2 cytokine and OVA-specific IgE production in mice was reduced compared to controls (37). Additionally, the numbers of eosinophils infiltrating airways of ASC treated mice were lower than mice that did not receive ASC. Using anti-IL-10 and anti-IL-4 blocking antibodies suggest that the protective effects of ASC are in some way mediated by these two cytokines (37).

Another study added excretory/secretory products, collected from adult *Nippostrongylus brasiliensis* worms (NES), to OVA during the sensitization to determine

whether NES conferred any protection to allergy. Similar to experiments with *Ascaris suum* extracts, decreased type 2 cytokines and OVA-specific IgE were observed in mice that received NES in addition to OVA during sensitization (38). These decreases in type 2 immune responses to OVA were observed in parallel with the prevention of airway eosinophilia and reduced airway hyperreactivity after OVA challenge (38).

When excretory/secretory products obtained from the filarial nematode *Acanthocheilonema viteae* (ES-62) were added to cultures of bone marrow mast cells (BMMCs), activation through FcεRI was suppressed, leading to a severe reduction in mediator release. ES-62 was also successfully used to protect mice in allergy models, reducing airway hyperreactivity and lung inflammation, by injecting ES-62 during sensitization. It is believed that ES-62 directly inhibits mast cells by binding to TLR4, leading to a reduction in the mast cell signaling molecule PKC-α, which inhibits activation signals (39).

Summary

In summary, increases in the prevalence of allergic disease have been occurring for the past several decades in industrialized countries. While these increases most likely depend on multiple factors, strong evidence suggests that one of those factors is helminth infection. Epidemiological and animal studies indicate that helminth infection can suppress the development of allergic disease. This helminth-mediated protection appears to arise from some aspect of the regulatory response that develops during helminth infection. As regulatory responses are most developed during long-term infections, chronicity of infection could be an important determinant in the development of an

allergy-protective response. Further, helminth products appear to have the ability to suppress not only the development of type 2 immune responses, but also the effector phase of type 2 responses. However, there does not appear to be any evidence that the release of worm products is how helminths protect against allergic disease during an actual infection.

Specific Aims

1. Determine whether basophils become harder to activate over the course of chronic helminth infection in mice.

In order to determine whether basophil responsiveness decreases during chronic helminth infection, BALB/c mice were infected with the rodent filarial nematode *Litomosoides sigmodontis* and the trematode *Schistosoma mansoni*. Degree of basophil activation was determined by evaluating surface CD200R expression and intracellular IL-4 levels by flow cytometry. As basophils can amplify type 2 immune responses and also participate in the effector phase of immediate hypersensitivity reactions, reduced basophil functionality could be one way helminth infection protects against allergic disease.

2. Determine the mechanisms by which basophils become harder to activate in chronic helminth infection.

If basophil suppression develops in chronic helminth infection then elucidating that mechanism of suppression will be attempted. There are several ways reduced

basophil functionality could occur in helminth infections. For instance, it has been demonstrated that activating basophils by IgE receptor aggregation make them less able to become activated to subsequent stimulation (40-42). It is possible that repeated activation of helminth-sensitized basophils during infection leaves basophils less responsive. Additionally, the excretory/secretory product ES-62 derived from *A. viteae* has been demonstrated to directly inhibit mast cell responsiveness (39). It is possible that *L. sigmodontis* is releasing a similar ES product during infection that suppresses basophils. Alternatively, chronic helminth infection is known to induce a regulatory response characterized by increases in Foxp3 regulatory T cells and the production of anti-inflammatory cytokines such as IL-10 and TGF-β. However, it's not known whether any of these things have the capability of suppressing basophils or if they are doing so in a helminth infection.

3. Determine if eradication of helminth infection augments basophil histamine release in humans.

Since there are several differences between murine and human basophils (43), assessing whether basophil suppression also develops in humans infected with helminths is necessary. Human blood basophils release large amounts of stored histamine quickly after activation. Amounts of histamine released into supernatant can be easily measured using a competitive ELISA, making histamine measurement an ideal way to assess basophil activation. Helminth-infected children will be identified by examination of feces for the presence of intestinal helminth eggs, and blood will be obtained to assess

basophil responsiveness to anti-IgE. Changes in basophil responsiveness two weeks after anthelmintic treatment will be assessed in infected children to determine whether removal of helminths resulted in increased basophil responsiveness. Children from the United States will be used as an uninfected control group.

4. Optimize methods for evaluating murine mast cell activation.

Like basophils, mast cells are also important effector cells in allergic disease, capable of releasing mediators such as histamine and leukotriene C4. In fact, some studies suggest that mast cells are the primary effector cells in some murine allergy models (44), so determining whether mast cell releasability is also effected by helminth infection is of importance. Unfortunately, many of the techniques to measure mast cell activation rely on the ability to obtain very high numbers of cells. This is particularly problematic if mast cell functionality is to be assessed from experimental animals ex vivo. Methods of measuring murine mast cell activation that require the least number of mast cells will be identified. Also, if possible a rapid flow cytometric technique requiring low numbers of murine mast cells to assess activation will be established. Once these techniques have been identified and optimized, future work to determine whether murine mast cell responsiveness is altered in our helminth infection models can be accomplished.

Chapter 2: Chronic helminth infection reduces basophil responsiveness in an IL-10-dependent manner

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Running Title: "Chronic helminth infection suppresses basophils"

Currently in revision (The Journal of Immunology)

Abstract

Basophils play a key role in the development and effector phases of type 2 immune responses in both allergic diseases and helminth infections. This study shows that basophils become less responsive to IgE-mediated stimulation when mice are chronically infected with *Litomosoides sigmodontis*, a filarial nematode, and *Schistosoma mansoni,* a blood fluke. Although excretory/secretory products from microfilariae of *L. sigmodontis* suppressed basophils in vitro, transfer of microfilariae into mice did not result in basophil suppression. Rather, reduced basophil responsiveness, which required the presence of live helminths, was found to be dependent on host IL-10 and was accompanied by decreases in key IgE signaling molecules known to be downregulated by IL-10. Given the importance of basophils in the development of type 2 immune responses, these findings help explain the mechanism by which helminths protect against allergy and may have broad implications for understanding how helminth infections alter other disease states in people.

Introduction

Like allergic diseases, helminths induce type 2 immune responses characterized by eosinophilia, elevated IgE levels, and CD4+ T-cell production of IL-4, IL-5, and IL-13. In people with infrequent exposures to helminths, this immune response is often associated with allergic symptoms such as rash and pruritus during acute infection (45). Over time, though, allergic symptoms in patients chronically infected with helminths appear to decrease (45). The filarial nematode *Loa loa*, for example, causes less angioedema, hives, and itching in chronically-infected individuals living in endemic regions than in travelers with infections of shorter duration (46, 47). In addition to decreased allergic responses to the infection itself, chronic helminth infections are associated with decreased allergic responses to environmental allergens (35, 48-52). While several studies have investigated the mechanisms by which chronic helminth infection may suppress allergic reactivity, to date no studies have evaluated the effect chronic helminth infection has on basophil responsiveness. Like mast cells, basophils function as acute effector cells in allergic disease, releasing pre-formed histamine and other inflammatory mediators within minutes of allergen exposure, primarily through cross-linking of allergen-specific IgE molecules bound to high affinity IgE receptors on the cell surface.

In addition to being effector cells of allergic disease, basophils also help development of type 2 immune responses (19, 53-57). IL-4 is a principal driver of type 2 immunity, and studies have shown basophils to be major contributors of IL-4 in helminth and allergic diseases (58-62). Studies conducted in mice depleted of or deficient in

basophils have found decreased type 2 immune responses in both allergy and helminth models (14, 16, 18, 20, 61, 63, 64). In addition to amplifying established type 2 responses, there is also evidence that basophils, which can traffic to lymph nodes and present antigen to T-cells, may be involved in the initiation of type 2 responses (9, 14, 15, 61).

In this study, we tested the hypothesis that chronic helminth infection suppresses basophil responsiveness to IgE-mediated stimulation.

Materials and Methods

Animals and parasites

Female BALB/c mice (NCI Mouse Repository, Frederick, MD), BALB/c IL-10 deficient mice (Jackson Laboratory, Bar Harbor, ME), and BALB/c $J_H^{-/-}$ mice, (Taconic, Hudson, NY) were housed at The Uniformed Services University (USU) Center for Laboratory Animal Medicine. Blood used for basophil activation studies was obtained by retroorbital bleed. At study endpoints, mice were euthanized with carbon dioxide. All experiments were performed under protocols approved by the USU Institutional Animal Care and Use Committee.

For *Litomosoides sigmodontis* infections, L3-stage larvae were obtained by pleural lavage of jirds (*Meriones unguiculatus*, obtained from TRS Laboratory Inc., Athens, GA) that had been infected 4 days earlier by the bite of infectious mites as previously described (65). Forty L3-stage larvae were collected in RPMI and injected subcutaneously in the dorsal neck region into female BALB/c mice that were 5 to 12 weeks of age.

For *Schistosoma mansoni* infections, 6 to 10 week old female BALB/c mice were infected by exposing the tails to 30 – 40 *Schistosoma mansoni* cercariae (Puerto Rican strain) obtained from infected *Biomphalaria glabrata* snails provided by Fred Lewis (Biomedical Research Institute, Rockville, MD).

Parasite antigens

L. sigmodontis worm antigen (*Ls*Ag) and *S. mansoni* worm antigen (SWAP) were prepared from the PBS-soluble fraction of homogenized male and female adult worms harvested from infected animals as previously described (18, 66).

For worm excretory/secretory (E/S) products, adult male worms, adult female worms, and microfilariae were cultured separately in RPMI 1640 supplemented with HEPES, penicillin/streptomycin and glucose. Supernatants from cultures were collected every 24 hours while worms were still living. E/S products from each day were pooled and concentrated using 3,000 Kda MWCO Amicon Ultra centrifugal filter units (Millipore) and protein concentration measured using a BCA protein assay kit (Pierce, Rockford, IL). Concentrated ES products were stored at -20ºC until use.

Ls*Ag sensitization,* L. sigmodontis *implantation and microfilariae injection*

Mice 10 weeks of age were sensitized to *Ls*Ag by 3 intraperitoneal injections of 100 µg *Ls*Ag adsorbed to Imject Alum Adjuvant (Thermo Scientific). Injections were spaced 2 weeks apart. Three weeks after the last injection, adult worms for implantation and microfilariae were obtained from jirds that had been infected with *L. sigmodontis* for more than 8 weeks. After euthanizing infected jirds, adult worms were removed from the pleural cavity using dissecting probes and placed in RPMI to determine sex. Five to seven male worms or five to seven microfilaria-producing female worms were surgically implanted into the peritoneal cavity of anesthetized mice as previously described (67). Microfilariae were purified from the blood by diluting peripheral blood 1:2 with RPMI 1640 containing 25mM HEPES and 2.05mM glutamine (Mediatech, Inc.). Diluted blood

was layered on top of a 25% and 30% percoll/sucrose isosmotic solution and centrifuged for 35 minutes at 400xg. Microfilariae were washed with RPMI to remove any remaining percoll. Fifty thousand microfilariae were then injected into the jugular vein of *Ls*Ag-sensitized mice. After 2 weeks, blood was obtained from mice, plasma was removed and basophil activation was measured using flow cytometry.

Antibodies

The following antibodies were used to assess basophil activation by flow cytometry: anti-CD45b/B220 PerCP (RA3-6B2), anti-CD4 PerCP (RM4-5), anti-IgE FITC (R35-72), anti-IL-4 APC (11B11), anti-CD45 FITC (30-F11), anti-CD49b PE (HM ALPHA2) and anti-CD200R PE (OX-110). Anti-IgE (R35-72) and anti-FceRIα (MAR-1) were used for stimulation. Anti-DNP IgE (SPE-7) used to boost basophil surface IgE levels was purchased from Sigma-Aldrich. Anti-FceRIα (MAR-1) was purchased from eBioscience, anti-CD200R PE (OX-110) was purchased from AbD SeroTec and all other antibodies were purchased from BD Biosciences.

Antibodies used to measure expression levels of basophil signaling molecules by flow cytometry: anti-Akt AF647 (5G3), anti-PKC-α, anti-phospho-Akt PE (D9E), anti-phospho-STAT5 AF647 (C71E5), and anti-rabbit F(ab') AF647 all purchased from Cell Signaling Technology. Anti-FYN PE, anti-Syk PE, anti-STAT5 and FcγRIIb/CD16-2 (2.4G2) blocking Ab purchased from Santa Cruz Biotechnology, Inc.

Antibodies used for mouse IgE ELISAs: anti-mouse IgE (R35-72) for coating, mouse IgE (27-74) used as the standard and biotinylated anti-mouse IgE (R35-118) for detection were all purchased from BD Biosciences.

Measuring Basophil Activation by Flow Cytometry

Basophil activation was determined as previously described (18, 68) with minor modifications. Briefly, after collecting whole blood in heparinized tubes, blood was centrifuged and plasma was removed. Cells were washed twice with RPMI to ensure complete removal of plasma. After resuspending blood cells back to original volume using RPMI, cells were diluted 1:2 with RPMI and aliquoted 200 µl per condition. Diluted blood cells were stimulated with media alone, 1 µg/ml ionomycin (Calbiochem), several concentrations of either anti-IgE (0.0005, 0.002, 0.0078, 0.031, 0.125 and 0.5 µg/ml), or anti-FceRIα (0.4, 1.6, 6.25, 25 and 100 µg/ml) for 45 minutes before adding GolgiStop (containing monensin, BD Biosciences). Stimulation proceeded for 2 more hours. At the end of the stimulation period, cells were washed with PBS, RBCs were lysed and remaining cells fixed using a whole blood lysing reagent kit (Beckman Coulter). Cells were resuspended in 1% bovine serum albumin (BSA, Cohn V fraction, Sigma)/PBS at 4ºC overnight, and then surface stained with anti-IgE FITC, anti-CD4 PERCP, anti-B220 PERCP and, when CD200R was also evaluated, anti-CD200R PE. Cells were subsequently washed, permeabilized with Perm/Wash buffer (BD Biosciences), and then stained with anti-IL-4 APC. Cells were analyzed using a BD LSRII flow cytometer and FACSDiva software (BD Biosciences). In figure 1B, CD200R was measured without simultaneously measuring intracellular IL-4. For these assays, no GolgiStop was added and total stimulation time was reduced to 2 hours. Cut-off gates for CD200R-PE and IL-4-APC positivity were established using the fluorescence-minus-one (FMO) technique.

Measuring signaling molecules by flow cytometry

Blood was obtained from mice chronically infected with *L. sigmodontis* and age-matched uninfected controls, red blood cells were lysed and leukocytes were immediately fixed using a whole blood lysing kit (Beckman Coulter). After washing twice with PBS, cells were resuspended with 1% BSA/PBS with FcγRIIb/CD16-2 (2.4G2) blocking Ab and incubated overnight. Surface staining was performed by adding anti-IgE FITC (R35-72), anti-CD45b/B220 PerCP (RA3-6B2) and anti-CD4 PerCP (RM4-5) and incubating for 30 minutes at 4ºC. After washing cells twice with PBS, they were permeabilized with Perm/Wash buffer (BD Biosciences). Antibodies to signaling molecules were added to permeabilized cells and incubated for 30 minutes at 4ºC. Cells were washed 2 times with PBS before using an anti-rabbit secondary antibody for the detection of anti-stat5 and anti-PKC-α antibodies. After staining, cells were washed 2 times with PBS and fluorescence analyzed using a LSRII flow cytometer and FACSDiva 6.1.3 software (BD Biosciences). Titrations were performed to determine optimum concentrations of each antibody and BD CompBead compensation beads (BD Biosciences) were used for all colors to account for spectral overlap. Nonspecific binding of anti-rabbit secondary antibody was subtracted from MFIs obtained for stat5 and PKC-α expression. Infected and control samples were assayed at the same time to eliminate interday variability.

Worm-specific and total IgE ELISAs

ELISAs were performed using Costar half-area, high binding plates. IgG was removed from plasma using GammaBind Plus sepharose (GE Healthcare). For worm-specific IgE ELISAs, plates were coated with 20 µg/ml *Ls*Ag or SWAP diluted in PBS and incubated overnight at 4ºC. Plates were blocked with 5% BSA/PBS plus 0.05% tween. Plasma was added to the plate in duplicate at a starting dilution of 1:4, followed by further 3-fold dilutions made in 1% BSA/PBS plus 0.05% tween. Bound IgE antibody was detected by adding biotinylated anti-mouse IgE diluted in 1% BSA/PBS plus 0.05% tween, followed by adding AP-conjugated streptavidin that was diluted 1:1000 in 1% BSA/PBS plus 0.05% tween. Plates were developed by the addition of 4-nitrophenyl phosphate disodium (Sigma-Aldrich) in 0.1M carbonate buffer to the plate. Absorbances were read at 405nm using a Victor^3 V microplate reader from Perkin Elmer. Titers were calculated as the dilution that would yield an OD of 0.5 calculated by SoftMax Pro 4.6 (Molecular Devices, Inc). All samples were assayed the same day. For total IgE measurements, plates were coated with 10 µg/ml anti-IgE in PBS instead of worm antigen. All subsequent steps were identical to the worm-specific IgE ELISA. Total IgE concentration was determined from the IgE standard curve using WorkOut 2.0 ELISA software (Perkin Elmer).

Passive sensitization with DNP-specific IgE

Mice were injected with 30 µg anti-DNP IgE intravenously and with 50 µg intraperitoneally two times. Injections were two weeks apart. Blood was obtained 5 days

after last injection, plasma was removed and basophil activation assays by flow cytometry were performed.

Splenocyte and Whole Blood Cultures

For splenocyte cultures, after obtaining single cell suspensions of splenocytes from mice chronically infected with L. sigmodontis or age-matched uninfected controls, red blood cells were lysed with ACK lysis buffer (Quality Biological, Inc.) and cells were plated in Iscove's Dulbecco modified medium (Mediatech) containing 10% fetal calf serum (Valley Biomedical), 1% L-glutamine (Mediatech), 1% insulin-transferrin-selenium medium (Invitrogen Inc.) and 80 µg/ml gentamicin (Quality Biological, Inc.) at a concentration of $2x10^6$/ml and were incubated with either media, 0.05 µg/ml PMA (Sigma-Aldrich) with 1 µg/ml ionomycin (Calbiochem), 5 µg/ml anti-CD3 with 2 µg/ml anti-CD28 (both from eBioscience) or 20 µg/ml *Ls*Ag and cultured at 37°C, 5% CO_2, After 3 days, culture supernatants were collected and stored at -80ºC until IL-10 ELISAs were performed.

For whole blood cultures, after removing plasma, blood cells were thoroughly washed 2 times with RPMI, and aliquoted 500ul per condition into 48-well cell culture plates (Corning Incorporated). Blood was stimulated with media and several concentrations of anti-IgE (0.0001, 0.0005, 0.002, 0.0078, 0.031 and 0.125 µg/ml) for 2 hours at 37°C, 5% CO_2. Samples were centrifuged at 400xg for 5 minutes, supernatants collected and stored at -80 ºC until IL-4 ELISAs were performed.

For whole blood cultures with worm E/S products, plasma from mouse whole blood was removed and whole blood cells were incubated with 30 µg/ml E/S products or

BSA diluted in RPMI for 7 hours. Blood containing E/S products was then diluted 1:2 from the original blood volume and aliquoted 200 µl per condition. Basophil activation was performed using flow cytometry as described earlier.

Mouse IL-10 and IL-4 ELISAs

IL-10 and IL-4 ELISAs were performed using Costar half-area, high binding plates. Mouse IL-10 BD OptEIA ELISA kits and OptEIA ELISA reagents were purchased from BD Biosciences. Mouse IL-4 ELISAs were purchased from eBiosciences, Inc. ELISAs were performed using manufacturer's reagents following the protocols provided with each kit.

Statistical Analyses

All statistics were performed using GraphPad Prism 4.03. Statistical significance between multiple groups was determined using the Kruskal-Wallis test, followed by Dunn's multiple comparison post test. Either paired or unpaired 2-tailed Student's t test was used for comparisons between two groups. For basophil activation curves when more than two groups were compared, one-way ANOVA was performed for each stimulation concentration, followed by Bonferroni correction. For basophil activation curves when only two groups were compared, 2-tailed Student's t test was performed for each stimulation concentration. For all tests, *p*-values less than 0.05 were considered significant.

Results

Basophils from mice chronically infected with Litomosoides sigmodontis *are less responsive to* Ls*Ag than basophils from acutely infected mice*

To determine whether basophils become less responsive to worm antigen over the course of a filarial infection, BALB/c mice were infected with 40 L3-stage *L. sigmodontis* larvae. After obtaining whole blood from uninfected, acutely infected, and chronically infected mice, plasma was removed from blood cells by multiple washes. Blood cells were then stimulated with several concentrations of *Ls*Ag in order to develop dose-response basophil activation curves to *Ls*Ag. Basophil activation was determined by measuring surface CD200R expression and intracellular IL-4 production by flow cytometry (Fig. 2A). Consistent with prior work we have done (18), total and *Ls*Ag-specific IgE levels are low and undetectable, respectively, in uninfected animals (Figs. 2B, 2C). *Ls*Ag-specific IgE levels are measurable by 8 weeks of infection, and levels of both total and *Ls*Ag-specific IgE increase throughout the course of infection.

One of the principal mechanisms of basophil activation is by aggregation of surface high affinity IgE in response to cross-linking of receptor-bound IgE antibodies by specific antigen. As expected, basophils from uninfected animals, which lack specific antibodies against *Ls*Ag, do not become activated when stimulated with *Ls*Ag (Figs. 2D, 2E). The highest percentage of basophils activated in response to *Ls*Ag was observed in acutely infected mice, in which a maximal percentage of basophils became activated even at the lowest *Ls*Ag concentration tested (Figs. 2D, 2E). In contrast, basophils from chronically infected mice never achieved the same frequencies of activation, even at the highest *Ls*Ag

concentration tested. Results were consistent when measuring basophil activation by either surface CD200R or intracellular IL-4 expression (Figs. 2D, 2E). Similar results were obtained when the data was analyzed by evaluating changes in CD200R and IL-4 expression on a per cell basis by MFI (Figs. 3A, 3B), suggesting that not only do greater percentages of basophils become activated in response to *Ls*Ag during the acute stage of infection, but that the amplitude of activation of individual basophils is greater at eight weeks than at thirteen weeks. Interestingly, the decrease in basophil responsiveness to *Ls*Ag at thirteen weeks occurred despite the fact that more *Ls*-specific IgE is present at that timepoint (Fig. 2C).

To confirm that the changes observed by flow cytometry reflected actual changes in basophil functionality, we assessed IL-4 release from basophils by measuring IL-4 in supernatants of whole blood cells after 2 hour in vitro stimulation with *Ls*Ag. As seen in figure 1F, there is a significant decrease in IL-4 release from whole blood of chronically infected mice after *Ls*Ag stimulation as compared to acutely infected mice ($p = 0.008$, Fig. 2F). Since basophils are the only circulating cells capable of releasing IL-4 within 2 hours of activation (68, 69), and as the percentages of blood cells that were basophils was equivalent at 8 and 13 weeks post-infection (1.1% vs. 0.9%, $p = 0.133$, data not shown), these results confirm that basophil responsiveness is suppressed in chronic *L. sigmodontis* infection.

Figure 2. Responsiveness of basophils from L. sigmodontis*-infected mice to LsAg stimulation.*

(A)Gating strategy for flow cytometric assessment of basophil activation. Initial gating by forward and side scatter (left panel). Basophils identified as CD4-B220-IgE+ peripheral blood cells (2nd panel). Right panels demonstrate CD200R and IL-4 staining after incubation of whole blood with media and 0.031 μg/ml anti-IgE. Levels of total IgE (B) and LsAg-specific IgE (C) were measured in the plasma of uninfected (n = 6), 8 week (n = 6), and 13 week (n = 6) L. sigmodontis-infected BALB/c mice. Percentages of basophils that stain positively for CD200R (D) and IL-4 (E) after stimulation with several concentrations of LsAg as measured by flow cytometry and after subtracting media levels. Each data point represents the mean of at least three independent experiments (statistically significant differences are shown between 8 week and 13 week-infected mice). (F) Supernatant IL-4 concentrations from whole blood after stimulation with 2 μg/ml LsAg for 2 hours (F). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 2. Responsiveness of basophils from L. sigmodontis*-infected mice to LsAg stimulation.*

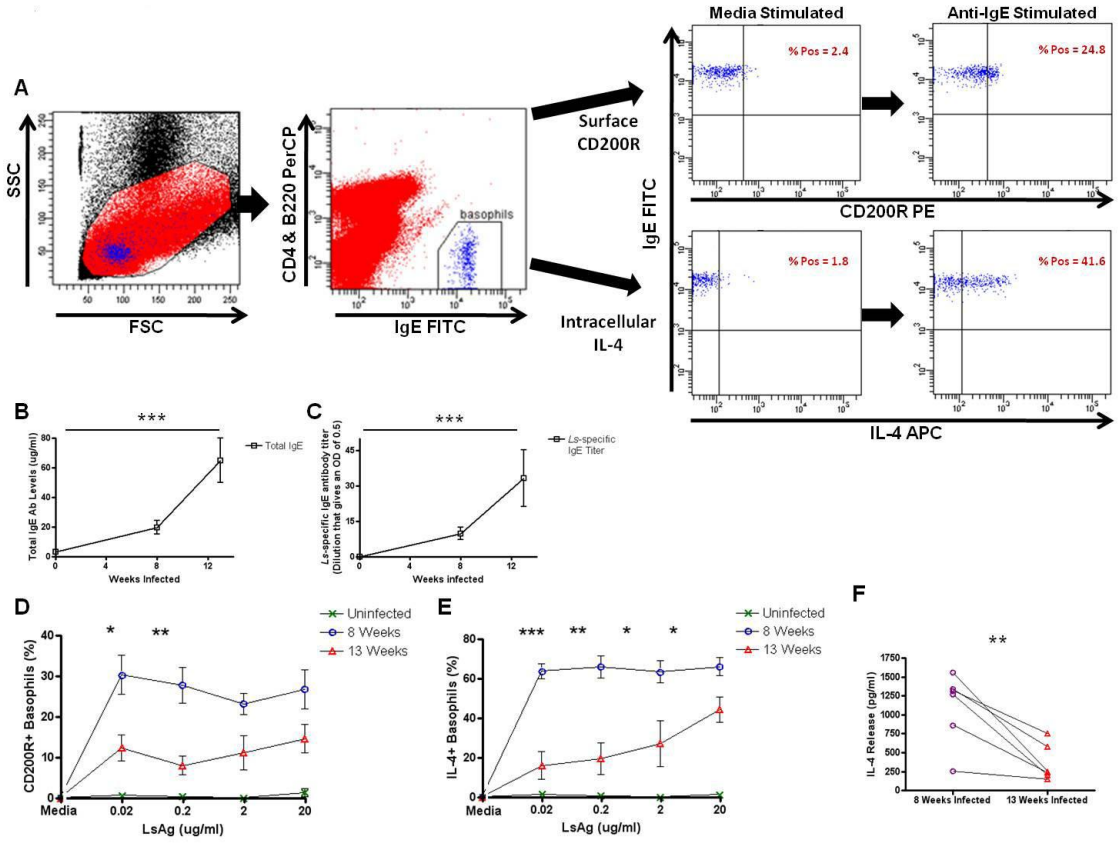
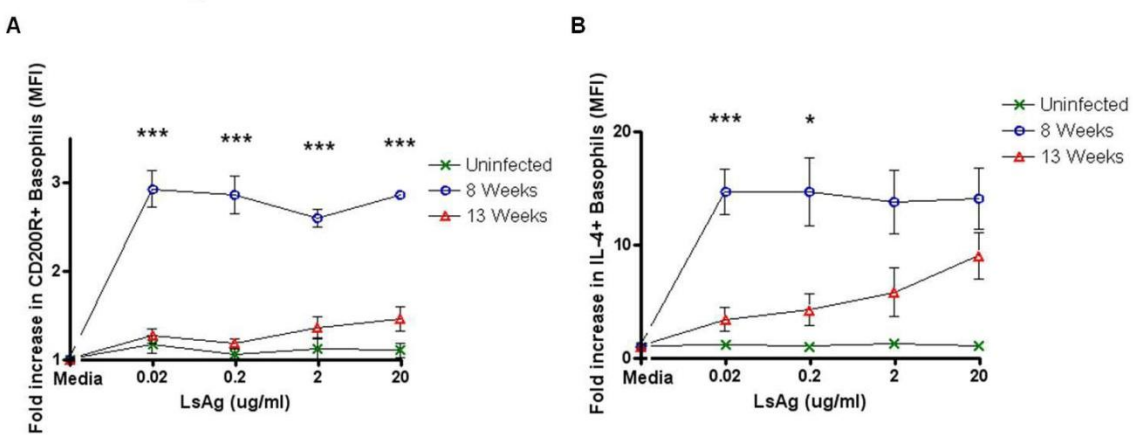


Figure 3. Responsiveness of basophils from L. sigmodontis*-infected mice to LsAg stimulation (MFI measurements).*

MFI of basophils that stain positively for CD200R (*A*) and IL-4 (*B*) after stimulation with several concentrations of *Ls*Ag as measured by flow cytometry and after dividing media levels. Each data point represents the mean of at least three independent experiments. (* $p < 0.05$, *** $p < 0.001$).

Figure 3. Responsiveness of basophils from L. sigmodontis*-infected mice to LsAg stimulation (MFI measurements).*



Basophils from chronically infected mice are less responsive to anti-IgE stimulation than basophils from acutely infected mice and uninfected mice

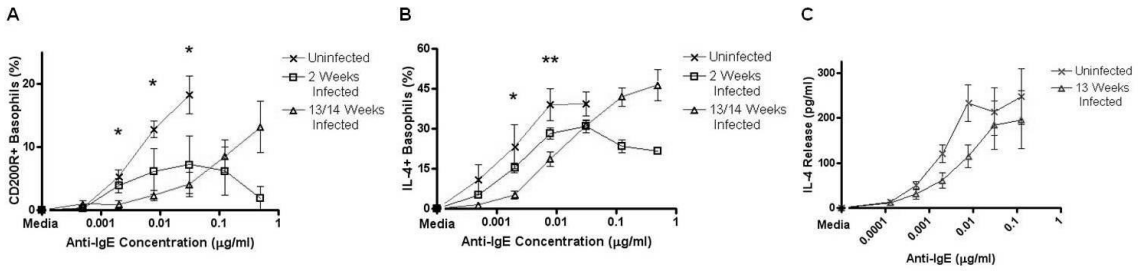
To determine whether reduced basophil responsiveness was an antigen-specific phenomenon or if overall basophil responsiveness becomes downmodulated by chronic filaria infection, basophil activation curves in response to increasing IgE cross-linking were assessed in uninfected, acutely infected and chronically infected mice. Basophils from chronically infected mice were less responsive to anti-IgE stimulation as measured by both surface CD200R (Fig. 4A) and intracellular IL-4 (Fig. 4B) expression. Significantly lower percentages of basophils from chronically infected mice expressed CD200R than uninfected mice in response to stimulation with 0.002, 0.0078 and 0.031 µg/ml anti-IgE (mean percentage of CD200R+ basophils from uninfected mice = 5.28, 12.78, 18.28 vs. 0.93, 2.33, 4.08 from chronically infected mice, $p < 0.05$ for all concentrations, Fig. 4A). Percentages of basophils expressing intracellular IL-4 were also significantly lower in chronically infected mice than uninfected mice when stimulating with 0.002 and 0.0078 µg/ml anti-IgE (mean percentage of IL-4+ basophils from uninfected mice = 23.12, 39.02 vs. 5.16, 18.63 from chronically infected mice, $p < 0.05$, $p < 0.01$, Fig. 4B). Basophils from two week infected mice demonstrated an intermediate basophil response phenotype, though the differences between these mice and uninfected mice were not statistically significant.

This right shift in basophil activation curves was also observed when measuring IL-4 release from whole blood stimulated with anti-IgE (Fig. 4C). As basophils are the only whole blood cells that release IL-4 within two hours of activation, this data confirms the flow cytometric data. Given that blood of 13 week-infected mice had on average 1.5-

Figure 4. Responsiveness of basophils from L. sigmodontis*-infected mice to anti-IgE stimulation.*

Percentages of basophils that stain positively for CD200R (*A*) and IL-4 (*B*) in response to increasing concentrations of anti-IgE as assessed by flow cytometry after subtracting media levels (statistical significance between uninfected and 13/14 week infected groups). (*C*) Supernatant IL-4 concentrations from whole blood after 2 hour stimulation with several concentrations of anti-IgE (*C*). Each data point represents the mean of at least four independent experiments. (* $p < 0.05$, ** $p < 0.01$).

Figure 4. Responsiveness of basophils from L. sigmodontis*-infected mice to anti-IgE stimulation.*



2 times as many basophils as uninfected animals, the decreased IL-4 release from blood of chronically infected animals reflects a substantial decrease in basophil responsiveness.

Basophils from mice chronically infected with Schistosoma mansoni *are less responsive to SWAP and anti-IgE stimulation than basophils from acutely infected and uninfected mice*

To determine whether basophil suppression is filaria-specific or a phenomenon that occurs with other helminthiases, we evaluated basophil responses in mice infected with *Schistosoma mansoni*, a blood fluke that establishes chronic infection in BALB/c mice. Since a prior study with *Schistosoma mansoni* infection in mice demonstrated that it takes 16 weeks for Th2 CD4+ T-cells to develop substantial hyporesponsiveness (70), mice were infected for 16 weeks before performing basophil activation assays.

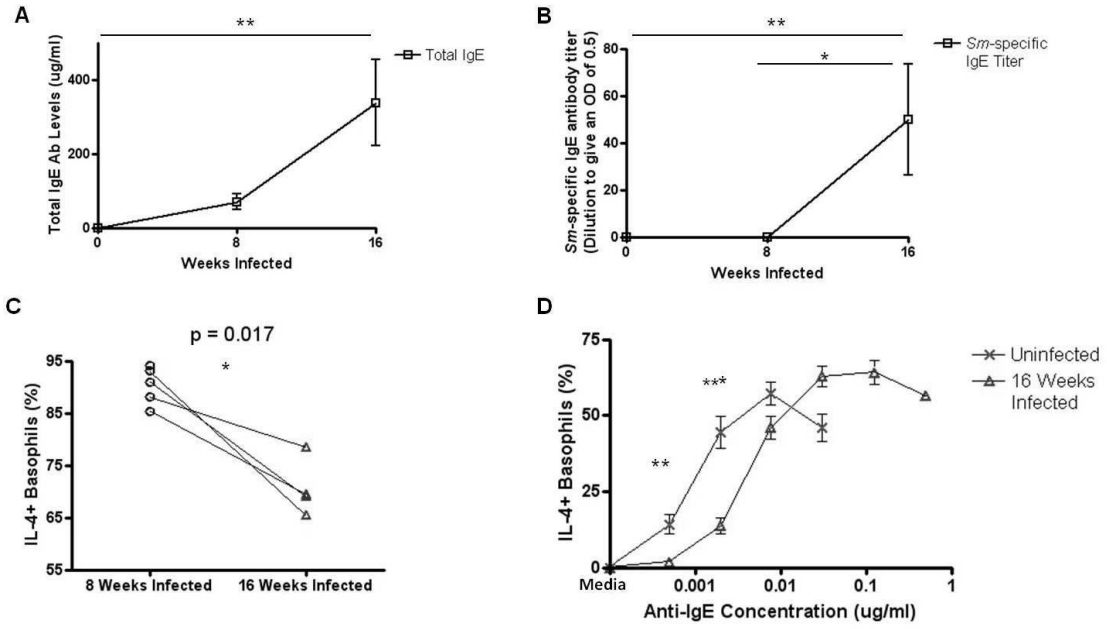
The effect of *Schistosoma mansoni* infection on basophil functionality mirrored that of *L. sigmodontis*. As with *L. sigmodontis,* levels of total and parasite-specific IgE were greater in chronic infection than at the 8 week timepoint (Figs. 5A, 5B). Despite the higher specific IgE concentration at 16 weeks, basophils of chronically infected mice became less activated in response to SWAP than basophils of 8-week infected mice (p = 0.017, Fig. 5C). Additionally, basophil activation curves of 16 week infected animals were significantly right-shifted in comparison to those of uninfected controls after stimulating with anti-IgE (mean percentage of IL-4+ basophils after stimulation with 0.0005 and 0.002 µg/ml anti-IgE from uninfected mice = 14.1, 44.5 vs. 1.9, 13.6 from chronically infected mice, p = 0.008, p = 0.0007, Fig. 5D).

These data suggest that reduced basophil responsiveness may be a phenomenon that occurs in many chronic helminth infections.

Figure 5. Responsiveness of basophils from Schistosoma mansoni*-infected mice.*

Levels of total IgE (*A*) and SWAP-specific IgE (*B*) measured in the plasma of uninfected ($n = 5$), 8 week infected ($n = 5$) and 16 week infected ($n = 5$) BALB/c mice. Percentages of basophils that stain positively for IL-4 in response to 2 μg/ml SWAP (*C*) and to several concentrations of anti-IgE (*D*) from uninfected ($n = 5$) and 16 week infected ($n = 5$) mice after subtracting media stimulation levels. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Figure 5. Responsiveness of basophils from Schistosoma mansoni*-infected mice.*



Reduced basophil responsiveness is not due to increased levels of surface IgE

Since basophils from chronically helminth-infected mice have more IgE on their surface than basophils from uninfected ($p < 0.001$) and even acutely infected mice (Fig. 6A), we tested whether increased surface IgE levels can account for changes in basophil responsiveness to IgE cross-linking by anti-IgE antibody. In order to increase IgE levels on the surface of basophils, uninfected BALB/c mice were injected with an IgE monoclonal antibody to DNP and basophil responsiveness to anti-IgE stimulation was compared between IgE injected and control mice.

Surface IgE levels were significantly increased in mice that were injected with DNP IgE ($p = 0.024$, Fig. 6B) and this IgE was functional and of sufficient quantity for basophils to become activated when stimulated with DNP-HSA ($p = 0.0054$, Fig. 6C). As seen in figure 6D, basophils with increased surface IgE levels were as sensitive to activation with anti-IgE, if not more so, than basophils from control mice (Fig. 6D).

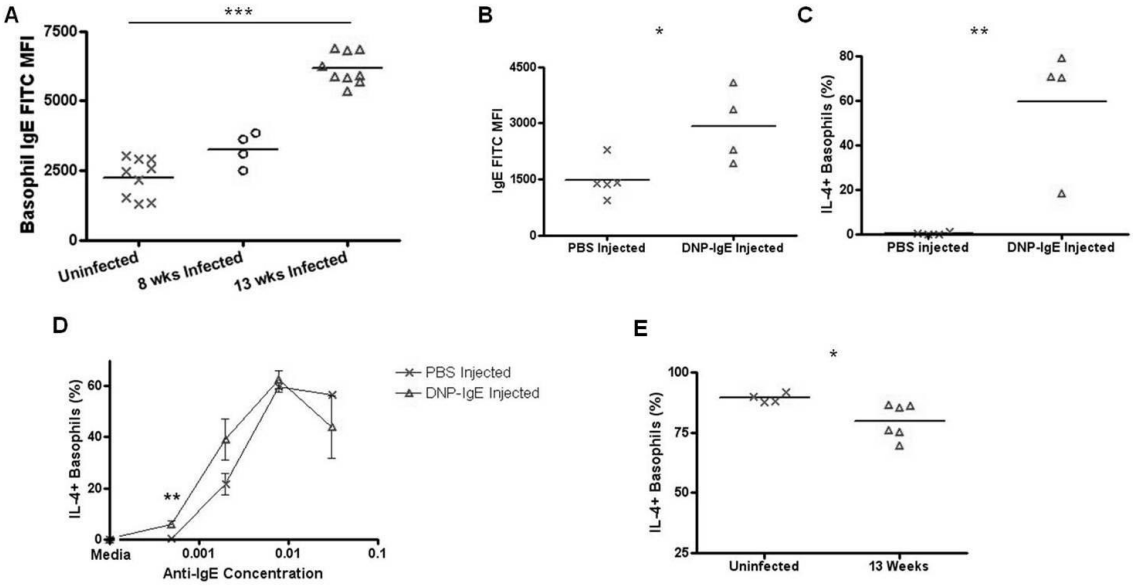
Additionally, basophils from chronically infected mice became significantly less activated ($p = 0.033$) than basophils from uninfected mice when stimulated with ionomycin (Fig. 6E), a calcium ionophore that bypasses surface IgE to cause activation of the cell.

Together, these data indicate that decreases in basophil responsiveness during chronic *Litomosoides* infection are not due to increased surface IgE levels.

Figure 6. Activation curves of basophils with different surface IgE levels.

(A) Relative surface IgE levels of uninfected, 8 week infected, and 13 week L. sigmodontis-infected BALB/c mice as assessed by mean fluorescence intensity staining of IgE on basophils. (B) Relative basophil surface IgE levels after injection of BALB/c mice with DNP IgE MAb, assessed by measuring IgE FITC MFI by flow cytometry. (C) Percentages of basophils from PBS-injected and DNP-IgE injected mice that stain positively for IL-4 after stimulation with 2 μg/ml DNP-HSA. (D) Percentages of basophils from PBS-injected (n = 5) and DNP IgE-injected (n = 4) mice that stain positively for IL-4 after stimulation with several concentrations of anti-IgE (E) Activation of basophils from uninfected and 13 week L.s.-infected mice measured by flow cytometry after stimulation with 1 μg/ml ionomycin. (* $p < 0.05$, **, $p < 0.01$, *** $p < 0.001$).

Figure 6. Activation curves of basophils with different surface IgE levels.



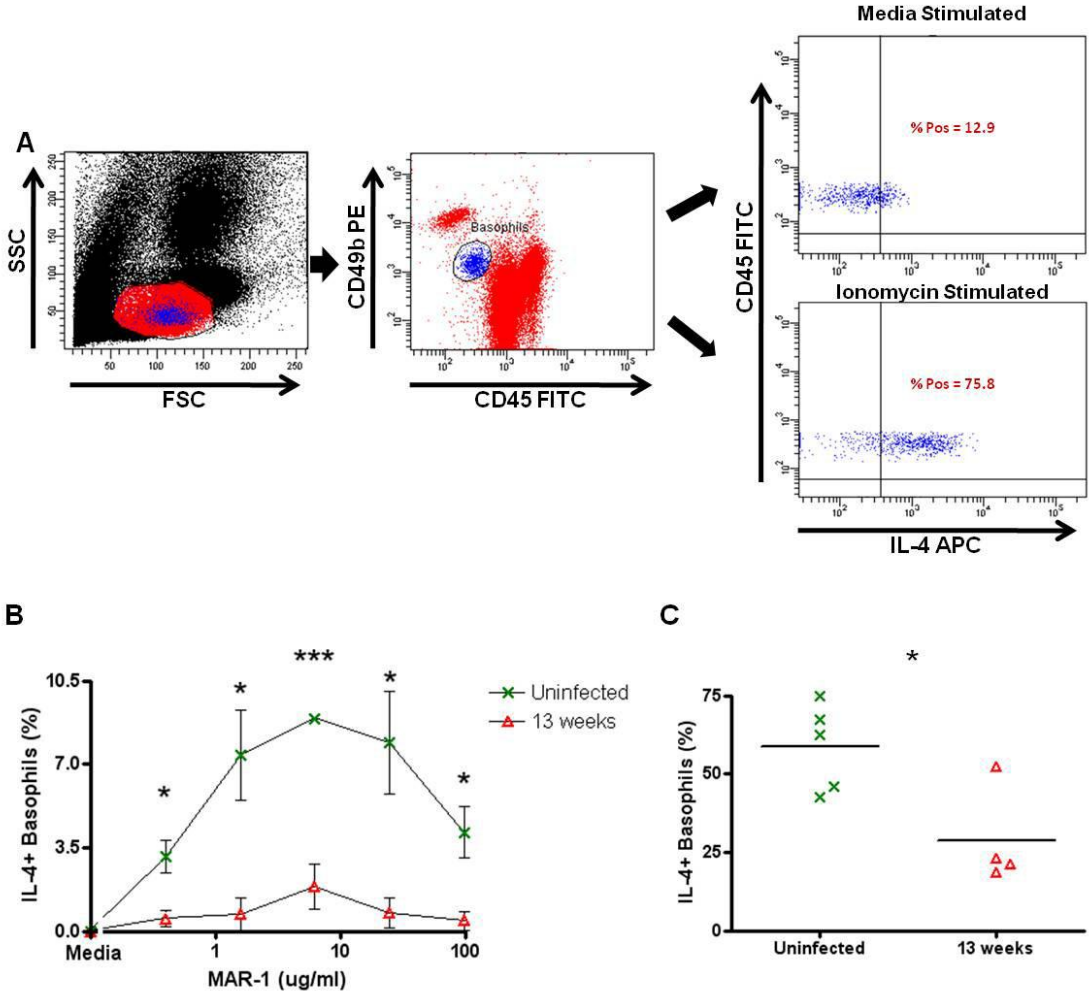
Repeated basophil activation through IgE receptors is not the cause of reduced basophil responsiveness

As previous studies have demonstrated that IgE-mediated activation of basophils reduces their responsiveness to subsequent IgE cross-linking events (40-42), we hypothesized that repeated antibody-mediated activation could be responsible for the induction of basophil hyporesponsiveness during chronic helminth infection. To test this, we measured basophil responsiveness to anti-FcεRIα and ionomycin stimulation in uninfected and chronically infected $J_H^{-/-}$ mice. As $J_H^{-/-}$ mice cannot generate worm-specific IgE or IgG during helminth infection (71), their basophils cannot undergo antibody mediated activation. Additionally, prior work we have conducted has shown that *Ls*Ag does not activate basophils in the absence of specific antibody (18). Because we could not use surface IgE staining as a marker for basophils in $J_H^{-/-}$ mice, basophils were identified by flow cytometry as being $CD49b^{hi}$ and $CD45^{lo}$ (72) and then assessed for IL-4 expression by intracellular flow cytometry (Fig. 7A). Comparing the basophil response curves to anti-FcεRIα stimulation from $J_H^{-/-}$ chronically infected and age-matched uninfected controls revealed that basophils from chronically infected $J_H^{-/-}$ mice still become less responsive to anti-FcεRIα when infected with *L. sigmodontis* despite the absence of repeated antibody-mediated activation (mean percentage of IL-4+ basophils after stimulation with 0.4, 1.6, 6.25, 25 and 100 µg/ml anti-FcεRIα from uninfected mice = 3.1, 7.4, 8.9, 7.9, 4.1 vs. 0.53, 0.7, 1.9, 1.9, 0.44 from chronically infected mice, $p = 0.028$, $p = 0.035$, $p = 0.0003$, $p = 0.013$, $p = 0.013$, Fig. 7B). Basophils from chronically infected $J_H^{-/-}$ mice also became less activated than uninfected $J_H^{-/-}$ controls in response to ionomycin stimulation ($p = 0.02$, Fig. 7C).

Figure 7. Basophil responsiveness in L. sigmodontis*-infected J_H-/- BALB/c mice.*

*(*A*) Gating strategy for flow cytometric assessment of basophil activation in J_H-/- BALB/c mice. Basophils identified as CD49b^{hi} and CD45^{lo}. Right panels demonstrate IL-4 staining after incubation of whole blood with media and 1 µg/ml ionomycin. (*B*) Percentages of basophils from uninfected and* L. sigmodontis*-infected J_H-/- BALB/c mice that stain positive for IL-4 after stimulating with several concentrations of anti-FcRIα after subtraction of media stimulation levels (*n = *4 per data point) (*C*) Percentages of basophils from uninfected and 13 week infected J_H-/- mice in response to 1 µg/ml ionomycin. (** p < 0.05, *** p < 0.001).*

Figure 7. Basophil responsiveness in L. sigmodontis*-infected J_H-/- BALB/c mice.*



In a second experiment, BALB/c mice were sensitized to parasite antigen by three injections of *Ls*Ag adsorbed to alum. Functional evidence for specific antibody came from the observation that basophils of sensitized mice became activated when exposed to either *Ls*Ag ($p < 0.05$) or microfilarial antigen (Fig. 8A). To test whether basophil hyporesponsiveness is associated with chronic exposure of helminth-sensitized basophils to helminths, three weeks after sensitization mice had either adult male worms or adult female worms surgically implanted into the peritoneal cavity or had microfilariae injected into the jugular vein. Control mice had sham surgery with no worms implanted. Implantation of adult female worms, as well as direct injection of microfilariae, resulted in circulating microfilaremia for at least two weeks. After 14 days, blood was obtained to compare basophil responsiveness to anti-IgE stimulation.

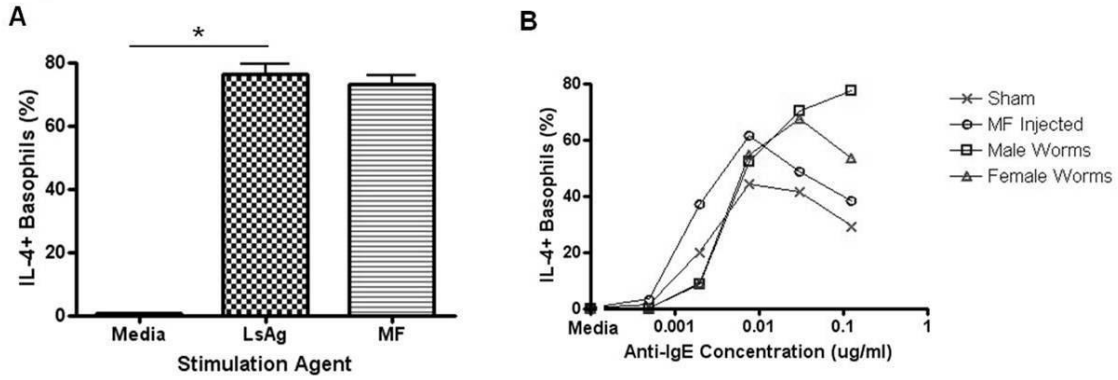
As seen in Figure 8B, after 14 days *Ls*Ag-sensitized basophils exposed to adult male and female worms had little or no reduced responsiveness to anti-IgE stimulation. *Ls*Ag-sensitized basophils exposed to microfilariae actually seemed to become slightly more responsive to anti-IgE stimulation (Fig. 8B).

These data indicate the reduced basophil responsiveness which develops in chronic helminth infection is not due to chronic antibody-mediated activation of basophils.

Figure 8. Basophil responsiveness following adult worm implantation or microfilaria transfusion in LsAg-sensitized BALB/c mice.

BALB/c mice were sensitized to worm antigens by three injections of 100 μg *Ls*Ag+alum. (*A*) Percentages of basophils from *Ls*Ag-sensitized mice that are IL-4+ after stimulation with media, 2 μg/ml *Ls*Ag or 2 μg/ml microfilaria antigen three weeks after sensitization as assessed by flow cytometry. (*B*) Percentages of basophils that stain IL-4 positive after stimulation with several concentrations of anti-IgE as assessed by flow cytometry after subtracting media stimulation levels fourteen days after intraperitoneal implantation of 5-7 adult male worms, intraperitoneal implantation of 5-7 adult female worms, or intravenous injection of 50,000 microfilariae. Each data point represents the mean of four independent experiments. (* $p < 0.05$).

Figure 8. Basophil responsiveness following adult worm implantation or microfilaria transfusion in LsAg-sensitized BALB/c mice.



Incubation of basophils with high concentrations of microfilaria E/S products results in reduced basophil responsiveness, but in vivo microfilariae do not suppress basophil responsiveness

Since a previous study has demonstrated that a filarial excretory/secretory product from *Acanthocheilonema vitae* can suppress bone marrow derived mast cells (BMMCs) (39), we tested whether E/S products from *L. sigmodontis* could suppress basophils. After incubating whole blood with E/S products from different life cycle stages of *L. sigmodontis*, basophils were stimulated with several concentrations of anti-IgE and basophil activation assessed by measuring IL-4 expression with intracellular flow cytometry.

No changes in basophil responsiveness to anti-IgE were observed after incubating whole blood with E/S products from adult male or adult female worms (Fig. 9A). However, basophil responsiveness to anti-IgE stimulation was reduced after incubating whole blood with E/S products from microfilariae (mean percentage of IL-4+ basophils after stimulation with 0.002 and 0.0078 µg/ml anti-IgE from whole blood incubated with BSA = 21.1, 33.5 vs. 3.4, 10.1 from whole blood incubated with microfilaria E/S products, $p < 0.001$, $p < 0.01$, Fig. 9A).

To determine whether microfilariae were responsible for inducing basophil hyporesponsiveness in vivo, 50,000 microfilariae in RPMI were injected intravenously into BALB/c mice and, after 11 days, basophil responsiveness assessed by stimulating basophils with several concentrations of anti-IgE.

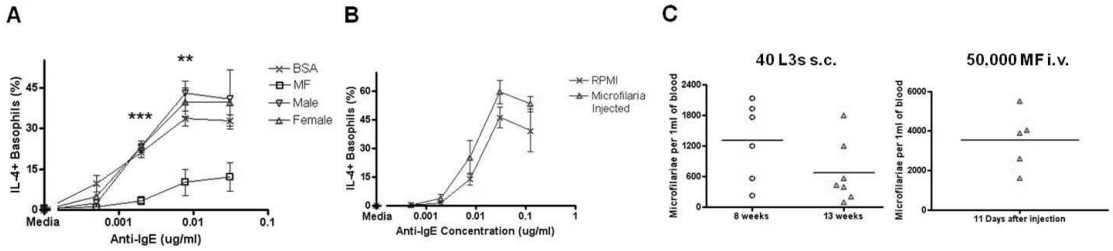
Injection of microfilariae did not reduce basophil responsiveness to IgE cross-linking (Fig. 9B). In fact, basophils seemed more responsive to anti-IgE stimulation after

microfilariae injection. Importantly, microfilaria counts when blood was obtained for basophil activation studies were equal to or higher than microfilaria counts from blood obtained from mice with chronic infections (Fig. 9C). Thus, the lack of basophil responsiveness was not due to insufficient numbers of microfilariae in the injection study. Further evidence that products secreted from microfilariae are not the main drivers of basophil hyporesponsiveness comes from the observation that in natural *L. sigmodontis* infections basophils are more suppressed at 13 weeks than 8 weeks (Figs. 2D, 2E), even though microfilaria counts are lower at 13 weeks than 8 weeks (Fig. 9C). In total, these results suggest that E/S products from microfilaria have the capability to suppress basophil responsiveness, but likely are not the main cause of basophil hyporesponsiveness in chronic helminth infection.

Figure 9. Effects of L. sigmodontis *Excretory/Secretory products on basophil responsiveness.*

(*A*) E/S products from different life cycle stages of *L. sigmodontis* were incubated with whole blood containing basophils for 7 hours and then stimulated with several concentrations of anti-IgE (each data point represents mean of at least two independent experiments). Degree of basophil activation was assessed by measuring basophil IL-4 positivity by flow cytometry (only showing statistical significant difference between the BSA control and microfilaria E/S incubated conditions). (*B*) Percentages of basophils that stain positively for IL-4 in response to several concentrations of anti-IgE eleven days after i.v. injection of 50,000 microfilairae in RPMI or RPMI only ($n = 5$ for each group). Microfilaria levels in mice 8 weeks and 13 weeks after s.c. injection of 40 L3-stage larvae and 11 days after injection of 50,000 microfilairae (*C*). (** $p < 0.01$, *** $p < 0.001$).

Figure 9. Effects of L. sigmodontis *Excretory/Secretory products on basophil responsiveness.*



IL-10 generated during helminth infection is important for development of reduced basophil responsiveness

IL-10 is an anti-inflammatory cytokine that is produced during helminth infections and plays a role in immune and cellular hyporesponsiveness (73-77). To test whether IL-10 generated during helminth infection was responsible for reduced basophil responsiveness, IL-10 deficient BALB/c mice were infected with *L. sigmodontis* and basophil responsiveness to anti-IgE, *Ls*Ag and ionomycin compared with uninfected IL-10 deficient BALB/c age-matched controls.

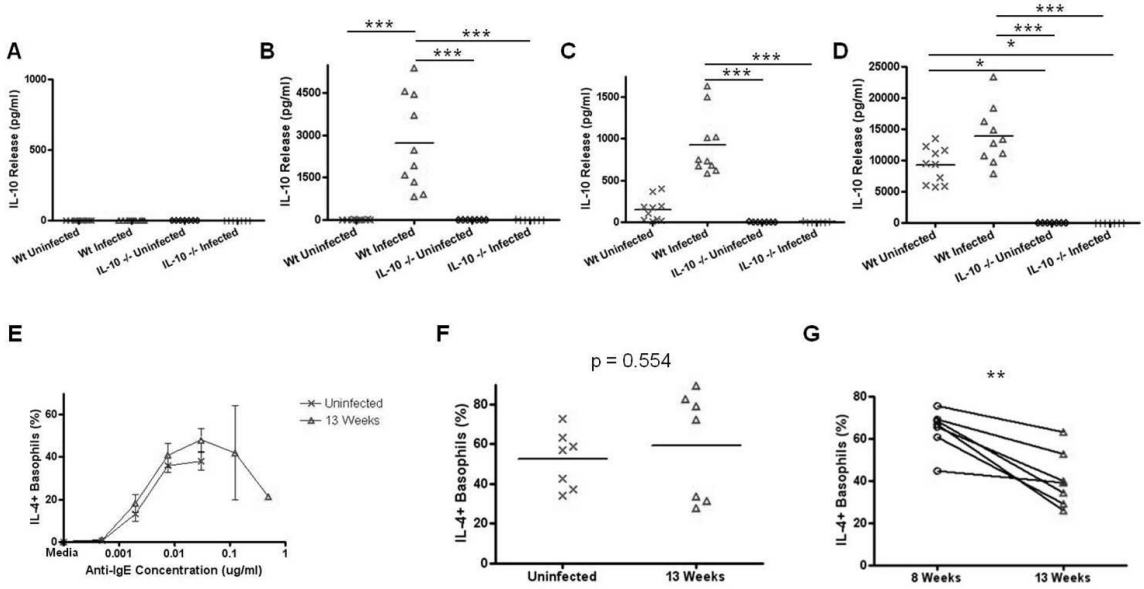
Whereas no splenocyte populations spontaneously released measurable quantities of IL-10 (Fig. 10A), splenocytes from chronically infected WT BALB/c mice produced significant amounts of IL-10 ($p < 0.001$) in response to *Ls*Ag compared to WT uninfected controls (Fig. 10B). These splenocyte cultures also produced more IL-10 on average than cultures from uninfected mice in response to polyclonal activation (Figs. 10C, 10D). No IL-10 was detected from splenocytes obtained from IL-10 deficient BALB/c mice under any stimulation conditions (Figs. 10A-D).

As seen in figures 10E and 10F, the reduced basophil responsiveness to IgE cross-linking and ionomycin stimulation previously observed in chronic helminth infections was abrogated in IL-10 deficient BALB/c mice infected with *L. sigmodontis* (Figs. 10E, 10F). Interestingly, basophils from chronically infected IL-10 deficient BALB/c mice still had slightly reduced responsiveness to *Ls*Ag (mean IL-4+ basophils in response to 2 µg/ml *Ls*Ag 64.3% at 8 wks vs. 40.5% at 13 wks, $p = 0.003$, Fig. 10G), although not to the same degree as basophils from chronically infected WT mice (mean IL-4+ basophils in response to 2 µg/ml *Ls*Ag 63.3% at 8 wks vs. 26.9% at 13 wks, $p = 0.036$, Fig. 2E).

Figure 10. Decreased basophil responsiveness to IgE-mediated stimulation is dependent on IL-10.

IL-10 concentrations from supernatants of splenocytes from uninfected or 13 week infected WT BALB/c mice and uninfected or 13 week infected IL-10 deficient BALB/c mice after stimulation with (*A*) media, (*B*) *Ls*Ag, (*C*) PMA/ionomycin, or (*D*) anti-CD3/CD28 as measured by ELISA. Percentages of basophils from uninfected ($n = 7$) and 13 week infected ($n = 7$) IL-10 deficient BALB/c mice that stain positively for IL-4 after stimulation with (*E*) several concentrations of anti-IgE, (*F*) ionomycin or (*G*) *Ls*Ag after subtracting media stimulation levels. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 10. Decreased basophil responsiveness to IgE-mediated stimulation is dependent on IL-10.



These results demonstrate that IL-10 is responsible for much of the reduced basophil responsiveness observed in chronic *L. sigmodontis* infection.

Helminth infection decreases Syk, Akt, and STAT5 expression in basophils

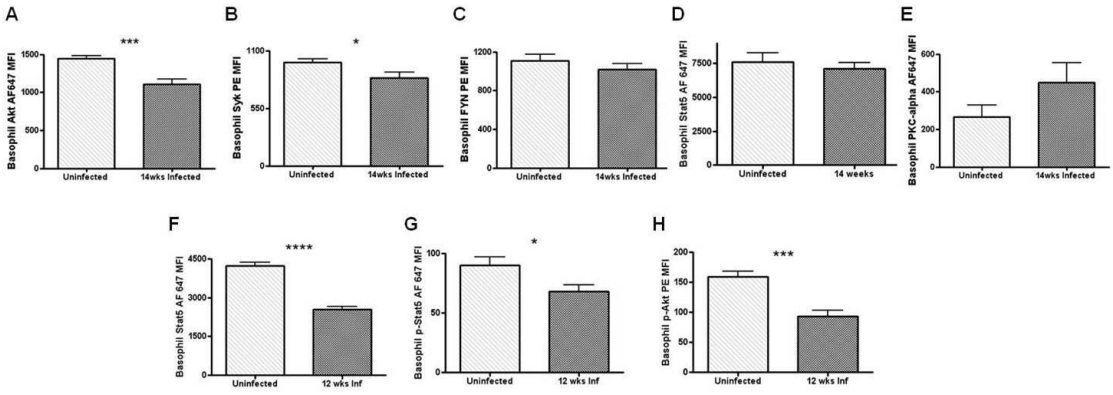
We assessed the expression of signal transduction molecules in basophils from WT BALB/c mice chronically infected with *L. sigmodontis* and age-matched uninfected controls by flow cytometry. Expression levels of Akt ($p = 0.001$) and Syk ($p = 0.033$) from mice chronically infected with *L. sigmodontis* were significantly lower compared to uninfected controls (Figs. 11A, 11B). Fyn expression in basophils from chronically infected mice was also slightly lower on average than uninfected controls (Fig. 11C), but this difference did not reach statistical significance. No decrease in baseline STAT5 expression ($p = 0.453$) could be detected in basophils of chronically infected mice (Fig. 11D). However, expression levels of STAT5 ($p < 0.0001$) and phosphorylated STAT5 ($p = 0.049$) in basophils of chronically infected mice were significantly lower than in basophils of uninfected controls after stimulation for 10 minutes with 0.0078 μg/ml anti-IgE (Figs. 11F, 11G). Basophil phosphorylated Akt expression levels ($p = 0.0008$) were also lower after anti-IgE stimulation (Fig. 11H).

Because ES-62, a filarial E/S product, has been shown to suppress BMMCs in vitro by decreasing PKC-α expression (39), we also evaluated expression of PKC-α in basophils of mice chronically infected with *L. sigmodontis*. As seen in figure 11E, no reduction in PKC-α expression was observed in basophils of chronically infected mice (Fig. 11E).

Figure 11. Alterations in signal transduction molecules in basophils of mice chronically infected with L. sigmodontis.

Expression levels of key signaling molecules were assessed by flow cytometry. After identifying basophils as CD4-B220-IgE+ peripheral blood cells, baseline (*A*) Akt, (*B*) Syk, (*C*) FYN, (*D*) STAT5, and (*E*) PKC-alpha MFI levels were measured from chronically infected BALB/c mice and age-matched uninfected BALB/c controls. Basophils from chronically infected mice and age-matched controls were also stimulated for 10 minutes with 0.0078μg/ml anti-IgE and then MFI levels of (*F*) phospho-Akt, (*G*) STAT5, and (*H*) phospho-STAT5 measured by flow cytometry. Each data point represents the mean of at least 4 independent experiments. (* $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$).

Figure 11. Alterations in signal transduction molecules in basophils of mice chronically infected with L. sigmodontis.



Basophil suppression requires active helminth infection

While *L. sigmodontis* can produce patent infections in BALB/c mice, the adult worms and microfilariae eventually die by 16-20 weeks. To determine whether helminth infection permanently reduces basophil responsiveness, basophil activation assays were performed 22 weeks post infection, a timepoint when both adult worms and microfilariae are no longer present.

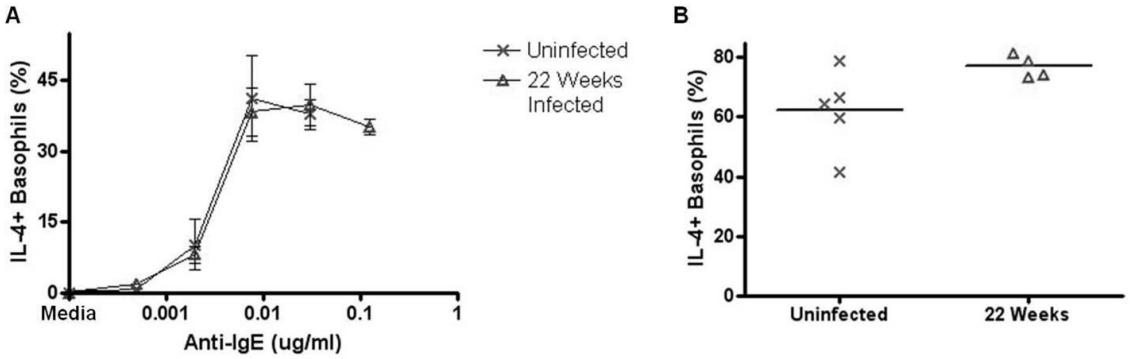
No adult worms were found in the pleural cavity and no microfilariae were found in the blood of mice inoculated with *L. sigmodontis* 22 weeks prior. The basophil response curve to IgE cross-linking of mice that no longer had worms present was identical to that of uninfected controls (Fig. 12A). Basophils from 22 week infected mice also no longer had reduced basophil activation when stimulated with ionomycin (Fig. 12B).

These data suggest that active worm infections are needed to reduce basophil responsiveness and that basophil responsiveness, which is reduced in chronic helminth infections, returns to normal after death of the worms.

Figure 12. Basophil responsiveness in BALB/c mice after clearance of L. sigmodontis *infection.*

Basophils from mice that were infected 22 weeks prior but no longer actively infected (n = 4) and uninfected control mice (n = 5) were stimulated with several concentrations of anti-IgE (*A*) or 1μg/ml ionomycin (*B*). To assess activation, percentages of IL-4+ basophils were measured using flow cytometry after media was subtracted out.

Figure 12. Basophil responsiveness in BALB/c mice after clearance of L. sigmodontis *infection.*



Discussion

This study demonstrates that chronic helminth infection suppresses basophil responsiveness to both IgE and non-IgE mediated stimuli. This phenomenon was observed with *L. sigmodontis* and *S. mansoni* infections. Given that these worms belong to different phyla (Nematoda and Platyhelminthes, respectively), these results suggest that basophil suppression may be a common immunologic phenotype in many chronic helminth infections.

Basophil suppression appeared to be due primarily to IL-10 as basophils of mice deficient in this cytokine did not exhibit alterations in their activation curves. Additionally, intracellular flow cytometry revealed decreases in basophil expression of Syk and Akt as well as decreased total STAT5, phosphorylated STAT5, and phosphorylated Akt in response to IgE-mediated activation. These findings are consistent with IL-10 mediated suppression, as IL-10 suppresses IgE signaling in mast cells by decreasing expression of these same molecules (73). The reduced expression of Syk is particularly noteworthy since there is substantial evidence that Syk may be the principle signaling molecule responsible for fine-tuning basophil responsiveness (78). IL-10 production during chronic helminthiasis is well documented and has been shown to contribute to the highly immune regulated state observed in these infections (79-81). Important sources of IL-10 during filaria infection include CD4+CD25- T-cells, CD4+CD25+T-regs, CD8+ T-cells, B-cells, monocytes, and NK cells (82). Our data expands the known suppressive capabilities of IL-10 since this is the first study to find that IL-10 plays a role in basophil suppression. It is important to note that while IL-10 is

an important cause of basophil suppression in the *L. sigmodontis* model, it is probably not the only mechanism since some decreased responsiveness to *Ls*Ag developed in chronically infected IL-10 deficient mice.

Recently, ES-62, a secreted phosphorylcholine-containing excretory/secretory product of the filarial worm *Acanthocheilonema viteae*, was shown to directly inhibit mast cell function in vitro by reducing intracellular levels of PKCα, a molecule implicated in a non-canonical IgE-signaling pathway (39). While we observed that E/S products from *L. sigmodontis* microfilariae inhibit basophil responsiveness in vitro, direct injection of microfilariae into mice did not result in basophil suppression and intracellular levels of PKCα were not diminished in basophils of chronically infected animals. These findings suggest that while E/S products of *L. sigmodontis* microfilariae have the capability to downmodulate basophil function, they likely do not do so in vivo during chronic infection.

Another mechanism that did not play a large role in basophil suppression was repeated IgE mediated stimulation. While IgE-mediated activation can cause negative feedback signals which suppress subsequent IgE-mediated signaling (41), this process is not required for basophil suppression during filaria infection since basophil suppression developed in chronically-infected antibody-deficient mice.

Interestingly, suppression of basophil function required the presence of living worms as basophil responsiveness returned to baseline after all worms had died. This requirement for active worm infection is consistent with studies in humans in which decreased cellular proliferation and cytokine responses to parasite antigens reverse after therapy (83, 84).

The finding that chronic helminth infections suppress basophil responsiveness has important clinical implications. Recently, basophils have become increasingly recognized as being important mediators of allergic disease. In terms of acute effector function, basophils release histamine and leukotriene C4 after becoming activated. These molecules induce classic allergy symptoms by increasing vascular permeability, mucus secretion, and smooth muscle contraction (10, 85). While the contribution basophils make to acute inflammatory responses likely varies between different diseases, a recent clinical study demonstrated that basophils may be responsible for the majority of allergic symptoms that occur after intranasal allergen challenge of individuals with cat allergy (86). Similarly, two murine studies have identified basophils as the principal effector cells of chronic allergic inflammation (20, 60). One of the mysteries regarding helminth infections and allergy has been the observation that, in contrast to its utility in developed countries, allergen-specific IgE has poor predictive value for allergic disease in developing countries with high rates of helminth disease (87). Our discovery that chronic helminth infections can suppress IgE-mediated activation of basophils provides an explanation for this finding.

In addition to contributing to acute allergic inflammation, basophils also play prominent roles in amplifying, and possibly initiating, the type 2 immune responses that drive allergic disease (9, 14-16, 18, 20, 61, 63, 64, 72, 88). Thus, suppression of basophil responsiveness may be a principal mechanism by which chronic helminth infections protect against development of allergic diseases. Interestingly, some animal studies have implicated IL-10 as being important for helminth-mediated protection against allergic disease (89, 90). Our finding that basophil suppression in chronic *L. sigmodontis*

infection is due to IL-10 provides a downstream mechanism explaining how helminth-induced IL-10 can block allergic inflammation.

Changes in basophil responsiveness may also have important implications for susceptibility to helminths, as there is increasing evidence that basophils play a role in protective immunity against some of these infections (91, 92). Although basophils do not protect against primary murine filariasis (18), basophil depletion or deficiency results in impaired expulsion of the intestinal nematode *Trichuris muris* (14) and inhibits protection against reinfection by the hookworm *Nippostrongylus brasiliensis* (63) (20). Thus, it is possible that in certain helminth infections reduced basophil responsiveness may play a role in enabling parasite survival.

Finally, it is interesting to note that basophils have recently been implicated as contributing to the pathogenesis of lupus nephritis. In that study, basophils were shown to contribute to disease by amplifying production of autoantibodies in an IL-4 and IgE-dependent manner (93). As with allergy, worm infections are associated with protection against autoimmune disease in both human studies and animal models. If basophils play a role in mediating pathology in certain autoimmune diseases, then it is possible that basophil suppression could be one of the mechanisms by which helminths protect against autoimmunity.

In summary, this study demonstrates that chronic helminth infections reduce basophil responsiveness in an IL-10-dependent manner through reduction of key IgE signaling molecules. Given the prominent roles basophils play in the development and effector phases of type 2 responses, the protective role they may have against certain helminth infections, and their possible contribution to autoimmune disease pathogenesis,

this finding has broad implications for understanding how helminth infections alter disease manifestations in people.

Acknowledgments:

We thank Dr. Cara Olsen for help with statistical analyses and Karen Wolcott and Kateryna Lund at the Uniformed Services University Biomedical Instrumentation Center for valuable assistance with flow cytometry.

Chapter 3: Helminth infection is associated with decreased basophil responsiveness in humans

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Currently in submission (Journal of Allergy and Clinical Immunology)

To the Editor:

Helminth infections and allergic diseases are characterized by increases in IgE and type 2 cytokines such as IL-4. Despite their common immunopathogenesis, epidemiological studies reveal an inverse relationship between the prevalences of these diseases (45, 74) and a number of animal experiments have demonstrated that helminth infections can actively protect against the development of allergy (74). While several mechanisms have been proposed, the pathophysiology underlying this phenomenon remains unclear (74).

Recently, we demonstrated that chronic helminth infections suppress basophil responsiveness to IgE-mediated activation in mice (94). This phenomenon may be a principal mechanism underlying helminth-mediated protection against allergy as basophils are increasingly recognized as being functionally important in allergic diseases. Basophils participate in the effector phase of allergic responses by releasing acute inflammatory mediators such as histamine after IgE-mediated activation and, through the release of IL-4, they also play prominent roles in the development of the type 2 responses that drive allergic diseases (95, 96). Given the many differences between murine and human basophils (43, 96), in this study we sought to determine whether basophil suppression also occurs in humans infected with helminths. To evaluate this, we compared baseline basophil histamine release between helminth-infected children from a helminth-endemic area in Ecuador to uninfected children in the U.S., and we evaluated basophil function in helminth-infected children before and after anthelmintic treatment. Studies on Ecuadoran children were approved by the Institutional Review Boards (IRBs) at the Universidad San Francisco de Quito, Ecuador, and at the Uniformed Services

University (USU). Studies on American children were approved by the IRBs at the National Human Genome Research Institute, NIH, and at USU.

Twenty-eight children aged 8 – 14 years with intestinal helminth infections were identified by parasitologic examination of stool samples in a rural community in Esmeraldas Province, Ecuador. *Ascaris lumbricoides* and *Trichuris trichiura* eggs were found in the stool of all 28 children and *Hymenolepis nana* eggs were found in the stool of 2 children. Eight healthy, uninfected children aged 5 – 13 years were recruited in Maryland, U.S. Blood from children was collected in heparinized tubes and centrifuged. After centrifugation, plasma was carefully removed and blood cells were washed twice with PBS. Blood cells were resuspended to the original volume using PBS, diluted with Histamine Release Buffer (Beckman Coulter, Inc.) and stimulated for 30 minutes with seven four-fold concentrations of anti-IgE (0.0005 µg/ml to 2 µg/ml, Sigma-Aldrich) and ionomycin (5 µg/ml, Calbiochem). Stimulated blood was centrifuged for 10 minutes at 400 x g, supernatant was acylated, and histamine levels determined using a competitive histamine ELISA (Beckman Coulter, Inc.). Percentage of total histamine released was calculated by dividing the amount of histamine released into supernatant by the amount of histamine in a lysed aliquot of blood. Infected children were then treated orally with three daily 800 mg doses of albendazole and a single dose of ivermectin at 0.2 mg/kg. Two weeks after therapy, histamine release from blood basophils was measured again from 22 of the treated children using identical stimulation conditions. A two week timepoint was chosen as basophil half-life is estimated to be between 5-8 days (97). Samples from 5 infected children did not respond to anti-IgE stimulation so were excluded from experiments where anti-IgE stimulation was used.

Two-tailed Mann-Whitney test was used to determine statistical significance between unpaired groups and two-tailed Wilcoxon signed ranked test was used to determine statistical significance between paired samples. GraphPad Prism version 4.03 was used for all statistical analyses.

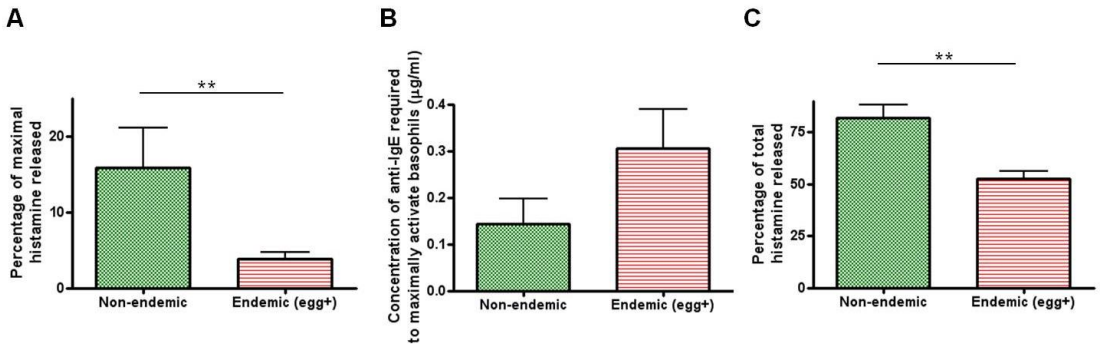
As seen in figure 13, *A*, the lowest concentration of anti-IgE tested (0.0005 µg/ml) was sufficient to induce histamine release from basophils of non-endemic children but not from those of endemic children with helminth infections (mean percentage of maximal histamine release, non-endemic children 15.8 % vs. endemic [egg +] 3.8 % P = 0.006). Similarly, the concentrations of anti-IgE required to maximally activate basophils was higher on average for infected children, although this difference was not statistically significant (mean concentration of anti-IgE, non-endemic 0.14 µg/ml vs. endemic [egg +] 0.30 µg/ml, Fig. 13, *B*). Basophils from non-endemic and uninfected children also released a greater percentage of their total histamine stores than basophils from infected children after ionomycin stimulation (mean percentage of total histamine release, non-endemic 81.8 % vs. endemic [egg +] 52.7 % P = 0.0015, Fig. 13, *C*).

To evaluate whether helminth infections play a role in suppressing basophil function in helminth-infected children, histamine release of infected endemic children was measured 2 weeks after anthelmintic therapy. As seen in figure 14, substantial increases in histamine release developed in endemic children after their infections were treated. Two weeks after treatment basophils of endemic children released more of their.

Figure 13. Blood basophil histamine release from uninfected U.S. children and helminth-infected children living in an endemic area of Ecuador.

(A). Histamine released after 0.0005 µg/ml anti-IgE stimulation. (B). Concentration of anti-IgE required to maximally activate basophils. (C). Histamine released after stimulation with 5 µg/ml ionomycin. Panels A,B endemic children n=23 and non-endemic n=8. Panel C endemic children n=28 and non-endemic n=8. (**, $P < 0.01$).

Figure 13. Blood basophil histamine release from uninfected U.S. children and helminth-infected children living in an endemic area of Ecuador.



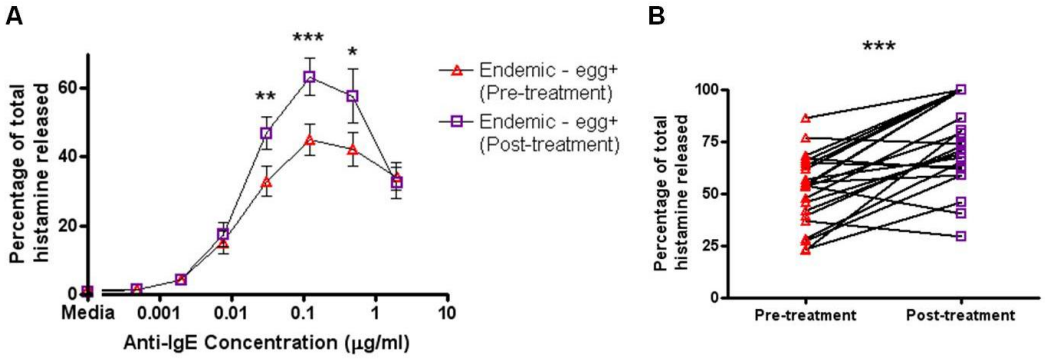
total histamine in response to 0.031, 0.125, and 0.5 μg/ml anti-IgE (mean percentage of total histamine release, pre-treatment 32.8, 45.0, 42.1 % vs. post-treatment 46.9, 63.3, 57.7 % respectively $P = 0.014$, $P = 0.002$, $P = 0.0007$, Fig. 14, *A*). Similarly, basophils from children 2 weeks after treatment released more histamine after ionomycin stimulation (mean percentage of total histamine release, pre-treatment 53.4 % vs. post-treatment 74.9 % $P = 0.0003$, Fig. 14, *B*).

These findings suggest that the ability of basophils to respond to both IgE-dependent and IgE-independent activation is suppressed during human intestinal helminth infection. This suppression appears to require the continuous presence of helminths as basophil histamine releasability increases within two weeks of the elimination of helminths. While the mechanism of basophil suppression requires further study, it could have important clinical consequences. First, as basophils have been shown to be important for protection against intestinal helminth infections in some murine models (20), basophil suppression may help worms to survive in hosts. Second, since basophils function as effector cells of allergy and are involved in the development of type 2 immune responses, reduced basophil functionality may be a mechanism by which helminths protect against allergic diseases.

Figure 14. Blood basophil histamine release from infected children before (n=22) and 2 weeks after anthelmintic treatment (n=22).

(A). Histamine released after stimulation with several concentrations of anti-IgE. (B). Histamine released after stimulation with 5 µg/ml ionomycin. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 14. Blood basophil histamine release from infected children before (n=22) and 2 weeks after anthelmintic treatment (n=22).



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Acknowledgments

This work was supported by grants R073QS (E.M.) from the Uniformed Services University of the Health Sciences and 072405/Z/03/Z (P.J.C.) from the Wellcome Trust. The participation of study mothers and children in the Esmeraldas Province of Ecuador is gratefully acknowledged.

Chapter 4: Histamine release and surface CD200R1 staining as sensitive methods for assessing murine mast cell activation

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Keywords: mast cell; flow cytometry; CD200R1; histamine; β-hexosaminidase; IgE

Currently in revision (Journal of Immunological Methods)

Abstract

Mast cells are important effector cells of allergy and are involved in the pathology of many other diseases. Measurement of β-hexosaminidase activity, the most commonly used method for evaluation of murine mast cell activity, requires a large number of cells and thus is of limited utility for studying mast cells in mouse models of disease. In this study we evaluated the sensitivity of histamine release as compared to β-hexosaminidase activity in the measurement of mast cell activation. Whereas a minimum of 6×10^4 mast cells per ml were required to detect slight increases in β-hexosaminidase activity after anti-IgE and ionomycin stimulation, substantial increases in histamine release could be detected under the same activating conditions with as few as 480 mast cells per ml. These findings demonstrate that measurement of histamine release is substantially more sensitive than assessment of β-hexosaminidase activity for detecting mast cell activation. Additionally, we describe a novel flow cytometric method for detecting murine mast cell activation. When using 7.5×10^5 peritoneal cells per condition and gating on IgE+ c-kit+ cells, mast cell expression of surface CD200R1 increased after both IgE and non IgE-mediated activation. This flow cytometric procedure was uncomplicated and rapid, with increases in surface CD200R1 expression appearing after as little as 30 minutes of stimulation time. Measuring histamine release and surface CD200R1 expression are sensitive approaches for detection of murine mast cell activation. Further, both approaches can be done on unpurified peritoneal cell populations. By requiring low numbers of cells, these approaches are ideal for investigating murine mast cell activation in animal models of disease.

Introduction

Mast cells, originally identified by Paul Ehrlich in 1878, are bone marrow-derived cells that reside in tissues throughout the body (98). They typically become activated after surface bound IgE is crosslinked by multivalent antigen, which results in rapid release of inflammatory mediators such as histamine and leukotriene C4 and induces classical allergy symptoms (10, 98, 99). Mast cells can also become activated by pathways that do not involve IgE and have the ability to release a wide range of cytokines and inflammatory molecules. In addition to being key effector cells of allergy, mast cells have become increasingly recognized as playing important roles in helminth infections, bacterial infections, autoimmunity, neurologic disorders, and vascular diseases (21, 99). Identifying easy and rapid techniques to measure activation of murine mast cells could be of substantial benefit for investigations involving disease models in mice.

Currently, the most common approach taken to assess murine mast cell activation is measurement of β-hexosaminidase activity. In this assay, purified mast cells are stimulated in vitro, supernatants are collected, and colored product is measured after addition of a nominally-priced β-hexosaminidase substrate. Although this approach is relatively simple and inexpensive, a major drawback is that it requires high numbers of mast cells. Typical protocols for the measurement of β-hexosaminidase activity require concentrations of 6×10^5 mast cells/ml per condition tested (100-102). While obtaining such numbers of mast cells is not an issue for in vitro experiments utilizing cells lines or bone-marrow derived mast cells, it is limiting for experiments evaluating the function of mast cells obtained directly from mice. In this study, we tested whether histamine release

was a more sensitive measure of murine mast cell activation than β-hexosaminidase assay.

Additionally, we developed a flow cytometric method for measuring murine mast cell activation. While there are numerous flow cytometric methods for detecting activated human mast cells, including measuring surface expression of CD35, CD63, CD69, LAMP-1, and LAMP-2 (103-105), there is no widely used flow cytometric assay in place for detection of murine mast cell activation. Recently, we demonstrated that surface expression of CD200R1, an inhibitory receptor belonging to the imunoglobulin superfamily, can be utilized as an activation marker of basophils (68). As both basophils and mast cells can be activated through cross-linking of surface IgE and as a prior study demonstrated that murine mast cells express CD200R1 (106), in this study we evaluated whether CD200R1 can be utilized for detection of murine mast cell activation.

Our results demonstrate that histamine is a far more sensitive measure of murine mast cell activation than β-hexosaminidase and that CD200R1 can be used as a marker of mast cell activation.

Materials and Methods

Animals

Female BALB/c mice were obtained from the NCI Mouse Repository (Frederick, MD) and housed at the Uniformed Services University (USU) Center for Laboratory Medicine. At study endpoints, mice were euthanized with carbon dioxide and cells used for mast cell activation studies obtained by peritoneal lavage. All experiments were performed in accordance with protocols approved by the USU Institutional Animal Care and Use Committee.

Collection of peritoneal cells

Peritoneal cells were collected as previously described (107, 108). Briefly, after soaking the ventral surface with 70% ethanol, fur-bearing skin and subcutaneous tissue was dissected away. Approximately 5 ml 1 x HBSS (Invitrogen, CA) was injected into the peritoneal cavity and abdomen was manually agitated for 1 minute. After carefully cutting a 5 mm hole in the peritoneum, 1 x HBSS and cells were removed with a transfer pipette. The peritoneal cavity was then washed 2-3 more times with 1 x HBSS. If significant red blood cell contamination existed, red blood cells were lysed with 0.8% ammonium chloride (NH_4Cl).

Isolation of peritoneal mast cells

After centrifugation of peritoneal cells at 400 x g for 10 min, supernatant was thoroughly removed and the cell pellet resuspended in 70% percoll (GE Healthcare) solution and overlayed with RPMI (Mediatech Inc., VA), as previously described (108, 109). Cells were centrifuged for 15 minutes at 580 x g. Non mast cells were carefully removed from the Percoll/RPMI interface along with most of the remaining supernatant without disturbing the mast cell pellet, which was then resuspended in RPMI.

Determining mast cell purity

Flow cytometric enumeration of mast cells:

Total numbers of peritoneal cells were enumerated by counting non red cells using a hemocytometer. Cells were then fixed with fixative solution from a whole blood lysing reagent kit (Beckman Coulter, CA) containing 9.25% formaldehyde and 3.75% methanol. Cells were washed and resuspended in 250 µl 1% bovine serum albumin (BSA, Cohn V fraction, Sigma-Aldrich, MO) in 1x PBS and incubated for 1 hour or overnight at 4ºC. Cells were surface stained with anti-CD117 APC (2B8, BD Bioscience, CA) and anti-IgE FITC (R35-72, BD Biosciences) and incubated at 4ºC for 30 minutes. After washing cells twice with 1x PBS, cells were resuspended in a final volume of 250 µl 1x PBS. Cell were then analyzed using a BD LSRII flow cytometer and FACSDiva software (BD Biosciences) and the percentage of peritoneal cells that were mast cells determined by measuring the percentage of total cells that were c-kit+IgE+. For all flow cytometry

experiments, optimal concentrations of antibody used for staining were determined by individual antibody titration experiments.

Cytospins:

Thirty thousand to seventy-five thousand cells were centrifuged at 500 RPM for 5 minutes onto slides using a cytofunnel and then allowed to air dry:

For May Grünwald/Giemsa staining, slides were placed in May Grünwald stain (Sigma-Aldrich) for 5 minutes, then 1 x PBS, pH 7.2 for 2 minutes, followed by Giemsa stain (Sigma-Aldrich) diluted 1:20 in distilled, deionized water for 18 minutes. Slides were removed from 1:20 diluted Giemsa stain and briefly dipped in distilled, deionized water and allowed to air dry.

Toluidine blue staining was conducted as previously described (110). Briefly, slides were placed in Mota's fixative for 10 minutes, fixative was removed by rinsing with 66% ethanol, and then slides were washed by dipping in distilled, deionized water. Slides were stained with acid toluidine solution for 10 minutes followed by dipping in distilled, deionized water. Slides were rinsed with 66% ethanol and dipped in distilled, deionized water and then rinsed with 100% ethanol and dipped in distilled, deionized water.

Measuring mast cell activation by flow cytometry

After counting total numbers of peritoneal cells using a hemocytometer, peritoneal cells were diluted with RPMI to a concentration of $3.75x10^6$/ml and 200 µl of cells were aliquoted per tube (750,000 cells/tube). Cells were incubated for 15 minutes at 37ºC and then stimulated with either several concentrations of anti-IgE (0.0078, 0.031, 0.125, 0.5, 2 µg/ml, R35-72, BD Biosciences), 0.5 µg/ml ionomycin, or 0.5 µM fMLP. Cells were incubated at 37ºC for 0.5, 1, 2, 3 or 4 hours for timecourse experiments and for 3 hours for all other experiments. At the end of the incubation period, cells were fixed with 100 µl of fixative solution from a whole blood lysing reagent kit (Beckman Coulter, CA), containing 9.25% formaldehyde and 3.75% methanol. Cells were washed and resuspended in 250 µl 1% bovine serum albumin (BSA, Cohn V fraction, Sigma-Aldrich) in 1 x PBS and blocked for 1 hour or overnight at 4ºC. After incubation with 1% BSA, cells were surface stained with anti-CD117 APC, anti-IgE FITC, and anti-CD200R1 (OX-110, AbdSerotec) for 30 minutes at 4ºC. After washing cells twice with 1 x PBS, cells were resuspended to a final volume of 250 µl with 1 x PBS and analyzed using a BD LSRII flow cytometer and FACSDiva software (BD Biosciences). Gates for CD200R1 positivity were established using the fluorescence minus 1 (FMO) technique (111).

Measuring histamine concentration and β-hexosaminidase activity

Peritoneal cells and purified mast cells were adjusted to correct starting concentrations using Histamine Release Buffer (Beckman Coulter). Cells were incubated at 37ºC for 15 minutes and then stimulated for 30 minutes with media, 1 µg/ml

ionomycin (CalBiochem, CA), and 0.5 µg/ml anti-IgE at 37ºC. At the end of the incubation period, cells were centrifuged at 400 x g for 10 minutes.

For histamine measurement, 150 µl supernatant was acylated and histamine concentration measured using a competitive ELISA (EIA Histamine assay, Beckman Coulter) per the manufacterer's directions. Concentration of histamine in acylated supernatants was determined from a standard curve after reading absorbances at 405 nm using a Victor^3 V plate reader (PerkinElmer, MA).

For β-hexosaminidase activity determination, 50 µl of supernatant was incubated with 200 µl of 1mM 4-nitrophenyl N-acetyl-β-D-glucosaminide in 0.05M citrate acid buffer (pH 4.6) for 1 hour at 37 ºC as previously described (112). Reaction was terminated with 0.1M sodium carbonate buffer. Activity of β-hexosaminidase was determined from a standard curve generated with 4-nitrophenol in 0.05M citrate acid buffer after measuring absorbances at 405 nm using Victor^3 V plate reader.

Fold increase in histamine release and 4-nitrophenol was calculated by dividing media stimulation levels.

The extent of degranulation after LsAg stimulation was calculated as a percentage of total histamine amounts by dividing the amount of histamine in supernatant by the amount of histamine in a lysed aliquot of cells.

Infection of mice with Litomosoides sigmodontis

BALB/c mice were injected subcutaneously with L3-stage larvae in RPMI obtained from the pleural cavity of Mongolian jirds (*Meriones unguiculatus*, TRS Laboratory Inc., Athens, GA) that had been infected by the bite of infectious mites 4 days earlier as previously described (65).

Statistical Analyses

Two-tailed, paired Student's t test was used to determine statistical significance between two groups. All statistics were performed using GraphPad Prism 4.03. P-values less than 0.05 were considered significant.

Results

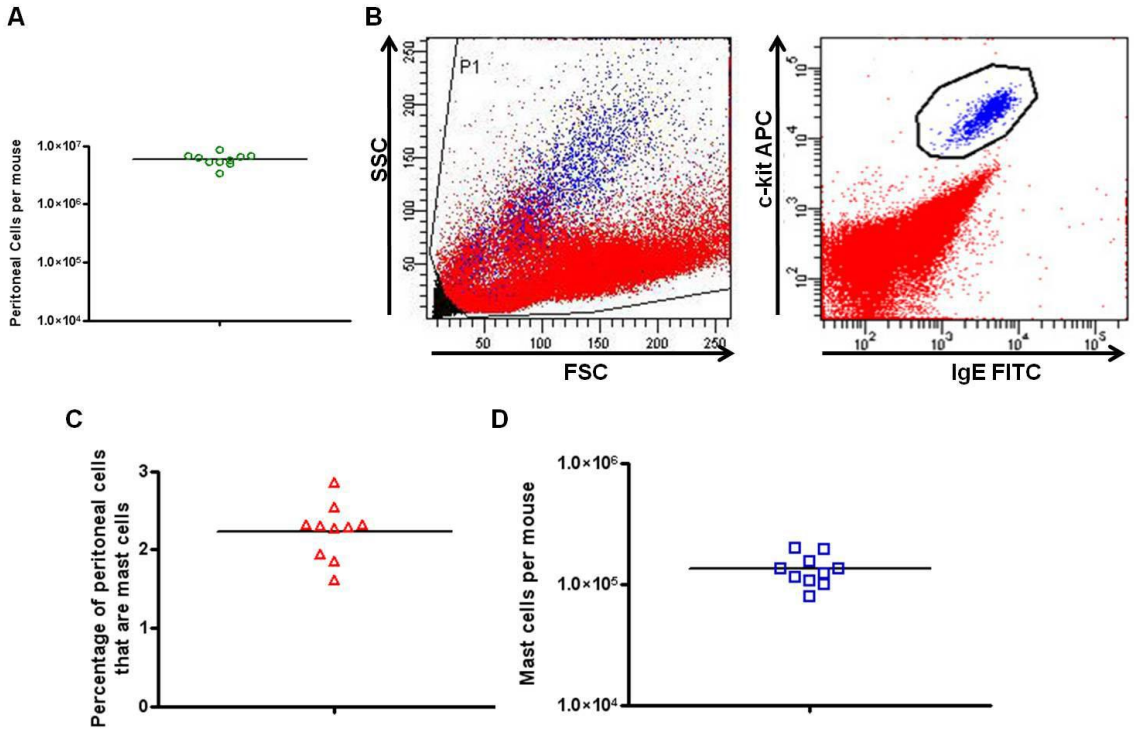
Identification and enumeration of peritoneal cells and peritoneal mast cells

Peritoneal cells were obtained from individual BALB/c mice by lavage of the peritoneal cavity with cold 1 x HBSS. Total peritoneal cells from each mouse were enumerated using a hemocytometer and averaged 5.95×10^6 per mouse (Fig. 15A). Mast cells were identified as IgE+ c-kit+ cells by flow cytometry (Fig. 15B). The mean percentage of peritoneal cells that were mast cells was 2.23% (Fig. 15C), resulting in a total of 132,685 mast cells on average per mouse (Fig. 15D).

Figure 15. Identification and enumeration of peritoneal cells and peritoneal mast cells.

A. Numbers of total peritoneal cells from individual mice after lavaging the peritoneal cavity and counting cells using a hemocytometer. B. Gating strategy to identify mast cells. After initial forward and side scatter gating (left panel), mast cells were identified as IgE+ and c-kit+ cells (right panel). C. Percentages of peritoneal cells that were mast cells determined by flow cytometry. D. Total calculated number of peritoneal mast cells per mouse.

Figure 15. Identification and enumeration of peritoneal cells and peritoneal mast cells.



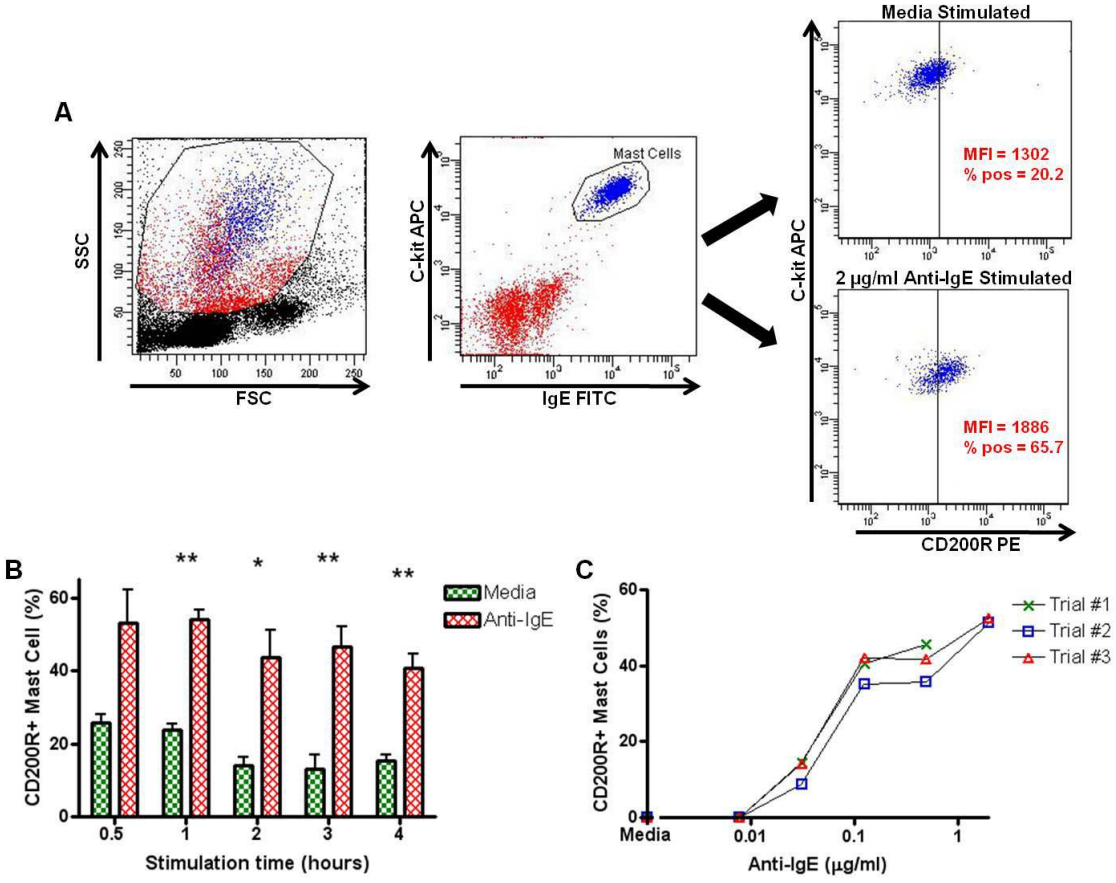
Mast cell surface CD200R1 time-course expression following activation with different anti-IgE doses

Recently, we demonstrated that surface expression of CD200R1, an inhibitory receptor belonging to the immunoglobulin superfamily, can be utilized as a marker of basophil activation (68). To ascertain whether CD200R1 could also be used as a murine mast cell activation marker, peritoneal cells were stimulated with anti-IgE for various amounts of time and CD200R1 expression on mast cells evaluated by flow cytometry. Peritoneal mast cells were identified as IgE+ c-kit+ cells (Fig. 16A left and middle panel). As seen in the right panels of figure 16A, the percentage of mast cells expressing CD200R1 markedly increases after activation with anti-IgE. Similarly, mean fluorescence intensity of surface CD200R1 staining of all mast cells also increases substantially. To evaluate the kinetics of CD200R1 upregulation on mast cells after IgE-stimulation, surface CD200R1 expression was measured on mast cells after various incubation periods. Surface CD200R1 expression was upregulated in as little as 30 minutes following stimulation with 0.125 μg/ml anti-IgE (mean percentage of CD200R1+ mast cells = 25.8 after media incubation vs. 53.0 when stimulated with anti-IgE, Fig. 16B). CD200R1 remained upregulated for as long as 4 hours after stimulation and significant differences between media stimulation and anti-IgE stimulation were found when stimulating for 1, 2, 3 and 4 hours (mean percentages of CD200R1+ mast cells after media incubation = 23.7, 13.9, 12.9, 15.3 vs. 54.0, 43.5, 46.5, 40.8 after stimulation with anti-IgE, $p = 0.001$, $p = 0.03$, $p = 0.006$, $p = 0.01$, Fig. 16B). Expression of CD200R1 also increased in a dose-dependent manner in response to 0.0078, 0.031, 0.125, 0.5, and 2.0 μg/ml anti-IgE stimulation (Fig. 16C).

Figure 16. Mast cell surface CD200R1 expression following activation with anti-IgE.

A. Gating strategy for measuring mast cell activation by flow cytometry. After initial forward and side scatter gating (left panel), mast cells were identified as IgE+ and c-kit+ cells (second panel) and activation of peritoneal mast cells was assessed by measuring expression of surface CD200R1 after media and anti-IgE stimulation (right panels). B. Percentages of mast cells expressing CD200R1 after stimulation with media and 0.125 μg/ml anti-IgE for 0.5, 1, 2, 3, and 4 hours (n = 3 per condition). Means +/- SEMs are shown. C. Percentages of mast cells expressing CD200R1 in response to stimulation with 0.0078, 0.031, 0.125, 0.5, and 2.0 μg/ml anti-IgE for 3 hours and after subtracting media stimulation levels. * = $p < 0.05$, ** = $p < 0.01$.

Figure 16. Mast cell surface CD200R1 expression following activation with anti-IgE.



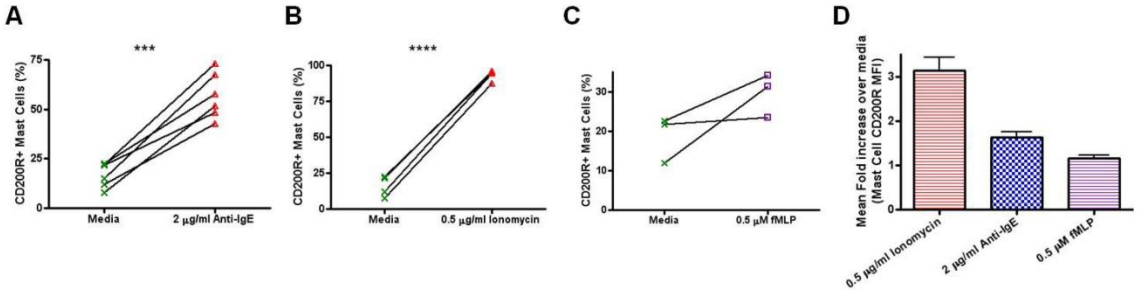
Increased surface CD200R1 expression following IgE-mediated and non IgE-mediated stimulation

While significant increases in CD200R1 expression were observed after stimulation with anti-IgE (mean percentage of CD200R1+ mast cells incubated with media = 16.9 vs. 57.0 when stimulated with 2 µg/ml anti-IgE, $p = 0.0003$, Fig. 17A), increases in the percentage of mast cells expressing CD200R1 also occurred after IgE-independent stimulation of cells (mean percentage of CD200R1+ mast cells stimulated with media = 17.2 vs. 93.7 when stimulated with 0.5 µg/ml ionomycin, $p < 0.0001$, Fig. 17B and mean percentage of CD200R1+ mast cells stimulated with media = 18.8 vs. 29.7 when stimulated with 0.5 µM fMLP, Fig. 17C). These trends were also observed when assessing changes in CD200R1 expression on a per cell basis by MFI (Fig. 17D).

Figure 17. Increased surface CD200R1 expression following IgE-mediated and non IgE-mediated stimulation.

Percentages of mast cells expressing surface CD200R1 after stimulation with (A) 2 µg/ml anti-IgE, (B) 0.5 µg/ml ionomycin, and (C) 0.5 µM fMLP. D. Fold increase in MFI over media of mast cells expressing CD200R1 after stimulating with 0.5 µg/ml ionomycin, 2 µg/ml anti-IgE, and 0.5 µM fMLP (n = at least 3 independent experiments). Showing means +/- SEMs, *** = $p < 0.001$, **** = $p < 0.0001$.

Figure 17. Increased surface CD200R1 expression following IgE-mediated and non IgE-mediated stimulation.



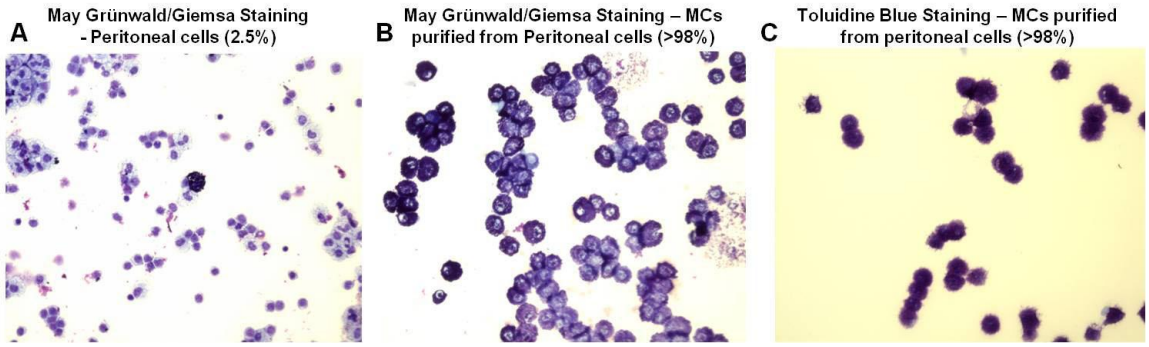
70% Percoll gradient results in a highly purified population of peritoneal mast cells

Use of a percoll gradient to purify peritoneal mast cells has been previously described (108, 109). As seen in figure 18, this approach results in a highly purified population of mast cells. Peritoneal cells were obtained by lavage of the peritoneal cavity of BALB/c mice. Mast cells were determined to be 2.5% of all peritoneal cells by May Grünwald/Giemsa staining of cytospins (Fig. 18A). A 70% percoll gradient was used to isolate mast cells from other peritoneal cells. Mast cell purity was measured as greater than 98% by both May Grünwald/Giemsa staining (Fig. 18B) and toluidine blue staining (Fig. 18C).

Figure 18. A 70% Percoll gradient results in a highly purified population of peritoneal mast cells.

A. Cytospin of peritoneal cells stained with May Grünwald/Giemsa stain. Mast cells determined to be 2.5% of all peritoneal cells. B. Cytospin of May Grünwald/Giemsa stainied mast cells purified from the peritoneal cavity with percoll. Mast cells determined to be >98% of all cells. C. Additional staining of purified mast cells with Toluidine Blue Stain. Percentage of cells that were mast cells was identical to May Grünwald/Giemsa stainied cells.

Figure 18. A 70% Percoll gradient results in a highly purified population of peritoneal mast cells.



Histamine release is a more sensitive test of mast cell activation than β-hexosaminidase activity

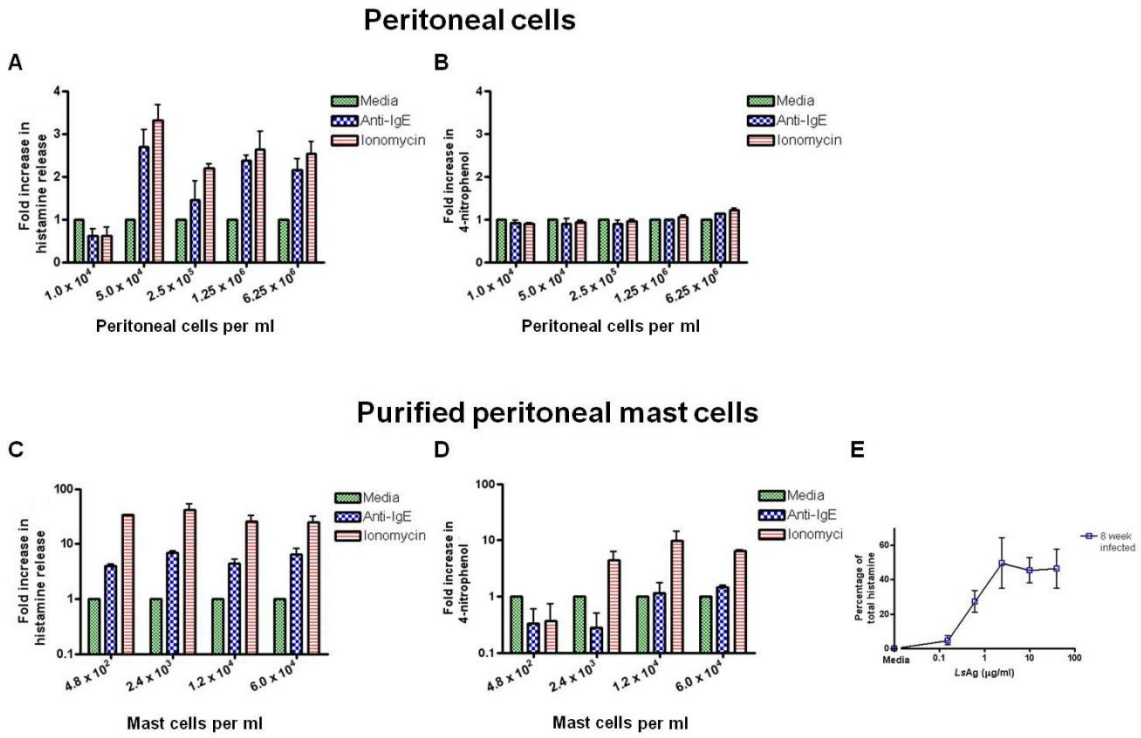
Sensitivities of two mast cell degranulation assays were compared since ex vivo assays are often limited by cell number. Histamine release and β-hexosaminidase activity were measured in peritoneal cells and purified peritoneal mast cells after 30 min of incubation with media, 0.5 µg/ml anti-IgE, or 1 µg/ml ionomycin. Histamine release in response to both anti-IgE and ionomycin stimulation could be detected from as few as 5 x 10^4 peritoneal cells/ml (mean concentration of histamine release when stimulated with media = 47.2 nM vs. 118.8 nM when stimulated with anti-IgE and 150.5 nM when stimulated with ionomycin, Fig. 19A). However, only slight increases in β-hexosaminidase activity could be detected even when concentrations of 6.25 x 10^6 peritoneal cells/ml were used (mean concentration of 4-nitrophenol when stimulated with media = 57.2 µM vs. 64.5 µM when stimulated with 0.5 µg/ml anti-IgE and 69.2 µM when stimulated with 1 µg/ml ionomycin, Fig. 19B).

The same assays were then performed on purified mast cells. Increases in histamine concentration could be detected after anti-IgE and ionomycin stimulation at concentrations of mast cells as low as 480 mast cells/ml (mean concentration of histamine release when stimulated with media = 3.4 nM vs. 13.2 nM when stimulated with anti-IgE and 114.6 nM when stimulated with ionomycin, Fig. 19C). While increases in β-hexosaminidase activity over media were detected after ionomycin stimulation with 6 x 10^4, 1.2 x 10^4, and 2.4 x 10^3 mast cells/ml, changes in β-hexosaminidase activity after anti-IgE stimulation could only be detected with the highest concentration of 6 x 10^4 mast

Figure 19. Histamine release is a more sensitive test of mast cell activation than β-hexosaminidase activity.

(A) Histamine release and (B) β-hexosaminidase activity measured in the supernatants of peritoneal cells after 30 minute stimulation with with media, 0.5 µg/ml anti-IgE, and 1 µg/ml ionomycin. (C) Histamine release and (D) β-hexosaminidase activity measured in the supernatants of purified peritoneal mast cells after 30 minute stimulation with media, 0.5 µg/ml anti-IgE, and 1 µg/ml ionomycin. E. Histamine release from 4 x 10^3 mast cells/ml purified from the peritoneal cavity of mice infected for 8 weeks with *Litomosoides sigmodontis* after stimulation with LsAg. For each panel, n = at least 3 independent experiments per condition. Means +/- SEMs are shown.

Figure 19. Histamine release is a more sensitive test of mast cell activation than β-hexosaminidase activity.



cells per ml (mean concentration of 4-nitrophenol when stimulated with media = 3.9 µM vs. 5.5 µM when stimulated with 0.5 µg/ml anti-IgE, Fig. 19D). Histamine release was also a sensitive method for detecting activation of mast cells that had become sensitized to worm antigen during infection of mice with the filarial nematode *Litomosoides sigmodontis*. This infection sensitizes mast cells and basophils to LsAg by inducing high levels of LsAg-specific IgE (113). As seen in figure 19E, mast cell response curves to LsAg were generated with as few as 4×10^3 mast cells/ml (Fig. 19E).

Discussion

Mast cells are effector cells of allergic disease (10, 98, 99), are involved in the pathology of many other diseases (99), and can play critical roles in host defense (22, 23, 114-119). Their activation status is a diagnostic criterion of some diseases like mastocytosis (103-105) and may be important for the progression or control of other diseases. In this study, we found that histamine release and surface CD200R1 staining are sensitive methods for detection of murine mast cell activation.

Comparison studies clearly demonstrated that histamine release was far more sensitive than β-hexosaminidase assay for detecting activation of in vitro stimulated mast cells. Histamine release after anti-IgE and ionomycin stimulation could be measured from as few as 480 purified mast cells per ml, while the minimum concentration of mast cells required to detect an increase in β-hexosaminidase activity after identical stimulation was 6×10^4 per ml. Typical protocols assessing β-hexosaminidase activity require a concentration of 6×10^5 mast cells per ml (100-102) in order to observe significant activation of cells – a concentration that requires 1,250 times more mast cells than histamine release assays would require. Given that the average mouse we analyzed had 132,000 mast cells in its peritoneum (Fig. 1D), ex-vivo studies would require pooling cells from several mice to study mast cell activation if β-hexosaminidase assays were used, whereas numerous histamine release studies could be conducted using the mast cells obtained from just one mouse. Additionally, our results also demonstrate that histamine release can be used to study mast cell activation using non-purified peritoneal cells. Use of freshly obtained peritoneal cells has the advantage of decreasing ex vivo

manipulation of cells, though it would require measurement of percentages of mast cells in each peritoneal cell population to make comparisons between different mice.

In this study we also developed a flow cytometric method for measuring murine mast cell activation. When using 7.5×10^5 peritoneal cells per condition and gating on IgE+ c-kit+ cells, mast cell expression of surface CD200R1 increased after IgE and non IgE-mediated activation. This procedure was uncomplicated and quick, with increased CD200R1 appearing in as little as 30 minutes of stimulation time. This flow cytometric technique does not require mast cell purification, and has the advantage of enumerating mast cells while measuring their activation.

Acknowledgments

The authors thank Karen Wolcott and Kateryna Lund at the Uniformed Services University Biomedical Instrumentation Center for assistance with flow cytometry. This work was supported by National Institutes of Allergy and Infectious Diseases, National Institutes of Health Grant R01AI076522.

Chapter 5: Discussion and Future Directions

Specific Aim #1 & #2

In our study utilizing murine models of filariasis and schistosomiasis, we observed that basophils from chronically infected mice were less responsive to IgE-mediated stimulation. We elucidated the mechanism of basophil suppression to be dependent on host-derived IL-10, demonstrating IL-10-mediated basophil suppression for the first time.

Determining the cellular source of IL-10 responsible for basophil suppression is a potential future project. If the cell type responsible for IL-10 production is identified, it might be possible to elicit or augment IL-10 production from it. Since some organisms are capable of producing an IL-10 homologue (120), our experiment using IL-10-deficient mice allowed us to conclude that IL-10 produced by the host was suppressing basophils. Many cell types are capable of producing IL-10 in the context of helminth infection. For example, in chronic helminth infections, increased levels of alternatively activated macrophages and CD4+Foxp3+ T cells are observed. Either of these cell types could potentially be responsible for basophil suppression since they are both capable of producing IL-10. Presumably the IL-10 produced by B cells is dispensable for basophil suppression to develop in helminth infections as infected J_H-/- mice, which lack antibodies and B cells, still developed reduced basophil functionality. Other studies have demonstrated that CD4+CD25- T cells are the main IL-10-producing cells in filarial infected individuals (82). It is quite possible that no one cell type produces enough IL-10 to suppress basophils in chronic helminth infections, rather it is the collective IL-10 from several cell types that lead to their reduced responsiveness.

Another future project could be to determine whether IL-10-mediated basophil suppression acquired during chronic helminth infection protects in a murine allergy model. Since basophils function as allergy effector cells, in theory IL-10-mediated basophil suppression should reduce allergic symptoms. However, it is also possible that helminth infection does not reduce basophil functionality enough to make a difference during allergic responses. Cell transfer techniques could be utilized to more effectively demonstrate the clinical significance of helminth-mediated basophil suppression. To perform this difficult experiment, wild-type mice and IL-10-deficient mice would be sensitized to an allergen and then infected with *L. sigmodontis*. When these mice are chronically infected, basophils would be purified from them (and uninfected controls) and transferred into a basophil knockout mouse. Mice would then be challenged with the allergen they were previously sensitized to. If recipient mice that got basophils from wild-type mice are protected from allergy symptoms, but recipient mice that got basophils from IL-10 deficient mice are not, it indicates that helminth infection can protect against allergic disease by suppressing basophils in an IL-10-dependent manner.

We determined that *L. sigmodontis* microfilaria excretory/secretory products can suppress basophils, which could lead to additional work to uncover the mechanism. Determining how microfilariae E/S products suppress basophil function might be important for the development of novel therapeutics to treat severe allergies. For example, if it was determined that one particular protein in the E/S product was directly suppressing basophils, that specific protein could be given to patients to reduce allergic responses. In fact, another group demonstrated that E/S product from a different filarial worm, *A. viteae*, can directly suppress mast cells by reducing levels of the signaling

molecule PKC-α (39). We did not look at the expression of PKC-α in basophils suppressed by our microfilaria E/S product, but it would be a relatively easy experiment. Helminths can also produce TGF-β homologues (121), which could potentially suppress basophils. Experiments could be performed to first determine if the anti-inflammatory cytokine TGF-β is present in microfilaria E/S products. This could be accomplished by ELISA or by using a reporter cell line that can recognize TGF-β activity (121). If the presence of this cytokine is confirmed, then experiments using TGF-β blocking antibodies could be added to basophil microfilaria E/S product cultures to see if it is in fact responsible for the observed reduced basophil functionality. In addition to determining the mechanism of basophil suppression, whether microfilaria E/S products suppress other cells such as mast cells could be investigated. Using the techniques described in chapter 4, microfilaria E/S product-mediated suppression of mast cells could be investigated. After incubating mast cells obtained from the peritoneal cavity with microfilaria E/S products, mast cells would be stimulated with IgE and non IgE-mediated stimuli. Histamine release could be measured by ELISA and surface CD200R1 expression measured by flow cytometry. To determine whether cellular suppression by microfilaria E/S products could be protective against allergic disease, an allergy model could be established by sensitizing mice to OVA. When they are appropriately sensitized, mice would be injected with microfilaria E/S products to determine whether effector cell suppression is sufficient to protect against anaphylaxis. Alternatively, assessment of whether basophil and or mast cell suppression during the sensitization protocol decreases type 2 immune responses could be accomplished. Microfilaria E/S

would be given during the sensitization protocol and then production of IgE and type 2 cytokines could be measured.

We also determined that reduced basophil functionality was dependent on the presence of live helminths. Unfortunately this indicates that past helminth infections may not offer any protection to current or future allergies.

Specific Aim #3

We are able to conclude that our findings in mice are applicable to humans. That is because we determined that human basophils from uninfected, non-endemic volunteers were more responsive to stimulation than basophils from infected, endemic volunteers. Additionally, the ability of basophils to release histamine increased after two weeks of anthelmintic treatment. Consistent with our work in mice, we determined that basophil suppression ended when helminths were removed. While this reduced functionality was statistically significant, it was not a profound reduction in basophil responsiveness. Future studies may examine a longer duration of treatment and alterations to other basophil functionality parameters, such as cytokine production. However, a difficulty with treatment studies in highly endemic areas is the possibility of reinfection during the course of treatment. An alternative experimental design would be to assess basophil functionality in helminth infected individuals immigrating to the United States. Infected individuals would then be treated and since they would be residing in the United States, should stay helminth free. Further work could be done to determine whether the mechanism of basophil suppression in humans is also consistent with studies done in

mice. Expression of intracellular basophil signaling molecules could be assessed by either western blot or flow cytometry to accomplish this.

Specific Aim #4

We were successful in establishing sensitive methods for measuring murine mast cell activation, making ex vivo assessment of activation possible. The measurement of histamine release required far fewer cells than the more commonly used β-hexosiminidase assay. Flow cytometric assessment of surface CD200R1 expression also required few cells and was relatively quick and easy. These methods of mast cell activation measurement were sensitive enough that purification of mast cells was not required.

Measuring histamine release and surface CD200R1 expression can be used to determine whether mast cells are suppressed during helminth infection. If mast cells do have reduced functionality during helminth infection, determining the mechanism of that suppression could lead to the discovery of novel allergy therapeutics. Since some studies indicate that mast cells play a role in protective immunity against certain helminth species, mast cell suppression may be one method helminths use to prevent elimination. Targeting how helminths suppress mast cells could then be useful in helminth vaccine development.

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