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Determining the Contribution of Basophil Accumulation in the Lymph Node

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Determining the Contribution of Basophil Accumulation in the Lymph Node

by
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Determining the Contribution of Basophil Accumulation in the Lymph Node

James Jung

Abstract

Basophils represent a rare cell population involved in allergic responses and are one of the primary effector cells for IgE-induced inflammation. However, the basis for which certain IgE-antigen interactions trigger basophil activation and the downstream mechanisms for basophil-enhanced inflammation remain unclear. We examined how IgE affinity and overall antigen-IgE/Fc epsilon receptor I (FcεRI) binding differentially relate to basophil activation. Using IgE-loaded murine basophils, we observed that high affinity antigens promoted enhanced basophil degranulation and IL-4 production compared to low affinity antigens, even when the relative concentrations of these antigens were adjusted to achieve the same equilibrium binding to IgE/FcεRI. Similarly, antigen exposure of basophils in the lymph node also lead to enhanced basophil degranulation using high affinity antigen compared to low affinity antigen after adjusting for antigen surface binding. Subcutaneous exposure of high affinity antigen, but not low affinity antigen, promoted systemic basophil activation at distal sites and increased susceptibility to anaphylaxis. We propose that the affinity of IgE for antigen is a primary determinant of basophil degranulation and is a crucial factor in susceptibility to severe IgE-mediated responses such as anaphylaxis.

We also determined whether basophils promote IgE-induced responses in the lymph node. Exposure to a variety of stimuli leads to the accumulation of basophils into the draining lymph node. There, basophils have been proposed to act as critical initiators of the type II adaptive

immune response, though more recent reports have challenged this hypothesis. Since then, a clear role for basophils in entering the lymph node has remained elusive. We investigated an alternative function for basophil accumulation into the lymph node: to participate in a local allergic inflammatory response. Through histological examination, basophils were observed to closely associate with dense inflammatory cell clusters comprised of eosinophils, alternatively activated macrophages, multinucleated giant cells, and a reorganized stromal cell population. These cell clusters increased upon antigen rechallenge. Testing basophil-deficient mice revealed that basophils were important contributors in both inflammatory cell recruitment and cell cluster formation. Moreover, IgE-induced activation of basophils was sufficient to promote the allergic inflammatory response in the lymph node. Behaviorally, basophils were observed to respond to incoming antigen by degranulating, producing IL-4, relocalizing closer to sites of antigen entry, and increasing in extended interactions with macrophages, suggesting important steps in the development of localized basophil-mediated responses. Finally, basophils associated with allergic inflammatory cell clusters that had formed in response to lymph node infiltration by the lymphatic-traversing helminth, *Brugia pahangi*, revealing the targeted and potentially defensive nature of the immune response. Altogether, we propose that a primary function for basophil entry into the lymph node is to participate in and promote a local allergic inflammatory response to protect against a perceived threat.

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Chapter 1: Introduction – Basophils in Allergic Disease

We live in an age where the capacity to reveal biology has grown exponentially and far beyond what the earliest cell biologists would have fathomed. From the invention of the basic light microscope to multi-photon fluorescent microscopy, the discovery of the double helix of DNA to the common-place genetic manipulations through CRISPR we have today, biological tools have advanced to a degree where an unprecedented number of previously impossible revelations, small and large, are truly at our fingertips. This is excitingly true for immunological research, which has tremendous medical implications, certainly for developing protective strategies against infectious agents, but also in helping to treat a broad spectrum of ailments, ranging from autoimmunity and allergy to cancer, cardiovascular disease and neurodegeneration. In light of all of this, the intention of this dissertation is to inform the reader on one particular area of immunology, and of note, a historically, particularly enigmatic one that has greatly benefited by the era we live in today: basophil biology. I will discuss how our understanding has historically advanced, what the field stands now, and the future direction of basophil research.

Early History of Basophil Biology

In 1879, the German scientist and physician, Paul Ehrlich, published a technique for the microscopic identification of several leukocyte populations through the differential staining of intracellular granules using basic and acidic aniline coal tar dyes (Ehrlich, 1879a, 1879b). Through this research, he was able to identify the granulocytes that we now know today as neutrophils, eosinophils, mast cells, and basophils. In his earlier studies, he had first discovered that certain granulated connective tissue cells, which he named mast cells, possessed acidic granules that stained with basic dye. After examining the blood of a patient with myeloid leukemia, he had found an additional population of granulocytes that also stained with basic

dye, that he called “blood mast cells.” We now refer to this cell population as basophils and regard them as a distinct lineage of cells compared to mast cells, eosinophils, and neutrophils. To Ehrlich’s credit, he had believed that both mast cells and basophils were two distinct populations, though much controversy had been generated over this for years thereafter (Kay, 2016). About fifteen years after Ehrlich had discovered “blood mast cells,” Kanthack and Hardy would further characterize them as the “finely granular basophil cell” and were the first to describe basophils as having a certain “explosive nature” in response to certain chemical stimuli. (Kanthack & Hardy, 1894) (Steiner, Huber, Harrer, & Himly, 2016). A clearer and more mechanistic understanding of basophil function would remain elusive for more than fifty years.

In the early 1900’s, the biological phenomena of immediate hypersensitivity was discovered, which would later impact a resurgence on the research and understanding of basophils. In 1902, Portier and Richet published a report detailing their study testing the effects of recurrent injection of sea anemone toxin into dogs. Since it was highly regarded that the immune system was protective, the hypothesis was that the injection of toxin would provide additional resistance to future exposure; however, rather than becoming protected, the dogs became severely hypersensitive. Where small amounts of toxin could be tolerated in the first injection, the same amount would cause rapid death after a secondary exposure. This phenomena was termed, “anaphylaxis” meaning “against protection” (Portier & Richet, 1902). It was later revealed that this anti-protective response could be transferred through blood serum. In 1919, Ramirez reported a peculiar case of sudden asthma in a patient who previously had no history of the disease, but had recently been given a blood transfusion. After the blood transfusion, the patient suffered an asthma attack after sitting in a horse-drawn carriage. Remarkably, it was discovered that the blood donor also had a history of hypersensitivity to horses. Because of this link, it was postulated that whatever led to the hypersensitivity reaction was found in and could be transferred through blood (M. Ramirez, 1919). A few years later, Prausnitz and Kustner directly

tested on themselves whether hypersensitivity could be transferred through serum. Kustner, who was suffering from a severe fish allergy, had his serum injected subcutaneously into Prausnitz. One day later, the serum injection was followed up by a local exposure to fish, leading to an immediate cutaneous inflammatory reaction (Prausnitz & Kustner, 1921). These early observations would later become foundational to our understanding of what we now term as immediate type I hypersensitivity, for which the antibody isotype IgE would later be identified as the crucial and mysterious “anaphylactic” serum factor, and for which mast cells and basophils would be understood to be its primary effector cells (Blank, Falcone, & Nilsson, 2013).

Although it was not until the 1960’s and 1970’s that mast cells and basophils were formally linked to immediate hypersensitivity reactions, evidence for their role in the response was building since the early 1900’s. In 1910, Dale and Laidlaw tested the physiological effects of histamine (which cells synthesize by decarboxylating the amino acid histidine) upon injection into various animals including frogs, guinea pigs, cats, dogs, and rabbits. What followed were a set of fairly dramatic events, one of which included the induced labor and stillbirths of a pregnant cat. Nonetheless, the study was quite comprehensive in detailing what we know today as anaphylactic shock. Dale and Laidlaw would later postulate that the response of anaphylactic shock in other systems may be due to poisoning by histamine (Dale & Laidlaw, 1910). Moreover, in the same set of experiments, it was observed that the injection of histamine was associated with bronchial, uterine, and intestinal muscle contraction and that exposure of the intestine to histamine induced smooth muscle constriction. The latter was of particular importance because a year prior it was observed that intestines isolated from sensitized guinea pigs contracted after the addition of the sensitized antigen (Schultz, 1910). Thus, histamine became linked to the immediate hypersensitivity reaction. In the 1950’s it was discovered that the major sources of histamine in tissue and blood were mast cells and basophils respectively (Graham, Lowry, Wheelwright, Lenz, & Parish, 1955; Riley & West, 1953). About ten years later,

Kimishige and Teruko Ishizaka (husband and wife, respectively), using the same cutaneous hypersensitivity technique of Prausnitz and Kustner (PK reaction), were able to identify a non-IgG, non-IgA gamma globulin fraction, termed gamma-E (for Erythema), which when injected into rabbits, led to the production of antiserum that could block a ragweed-specific immediate response (K. Ishizaka & Ishizaka, 1966, 1967). Similarly, Johansson and Bennich could also block the PK reaction using myeloma-derived IgE (Johansson & Bennich, 1967; Stanworth, Humphrey, Bennich, & Johansson, 1967). Both groups would later agree to name this new immunoglobulin serum factor that was responsible for the immediate hypersensitivity responses observed, IgE. Finally, linking everything together, in the 1970's, the Ishizakas and other research associates, discovered that IgE bound to basophils and mast cells, and that the crosslinking of IgE using an anti-IgE antibody or ragweed antigen using basophils of allergic donors could cause the release of histamine (K. Ishizaka, Tomioka, & Ishizaka, 1970; T. Ishizaka, De Bernardo, Tomioka, Lichtenstein, & Ishizaka, 1972; T. Ishizaka, Ishizaka, Johansson, & Bennich, 1969). Hence, by the 1970's, mast cells and basophils, due to their production of histamine and effector capacity of IgE, became closely linked to the immediate hypersensitivity reaction, a primary component of allergic responses.

Human Allergy and Parasitic Infection

Although the goal of this introduction is not to provide a comprehensive understanding of allergic inflammation, it may be helpful to provide a brief overview so as to create a better context in which to understand basophil biology.

Defined elsewhere, an allergy is an abnormal immune response directed against a non-infectious environmental substance (allergen) (Galli, Tsai, & Piliponsky, 2008). Allergic responses can manifest in many organs and can range from acute to persistent, mild to life

threatening. Some of these responses include allergic rhinitis (hay fever), asthma, atopic dermatitis (eczema), urticaria (hives), and anaphylaxis. Immunologically, these responses are characterized by the involvement of allergen-specific IgE and/or Th2-polarized T cells (Th2 cells). As previously discussed, IgE is critically involved in initiating immediate allergic reactions (more often referred to as immediate type hypersensitivity). On the other hand, Th2 cells greatly contribute to the delayed inflammation that follows hours and days after the initial response by secreting cytokines such as IL-3, IL-4, IL-5, IL-9, and IL-13 which promote an allergic inflammatory environment. Apart from IgE-secreting B cells (plasma cells) and Th2 cells, other classical leukocyte contributors prevalent in allergic inflammation include mast cells, basophils, eosinophils, type 2 innate lymphoid cells (ILC2s), and alternatively-activated macrophages (AAMs). Non-leukocyte, tissue-specific cells also participate in allergic responses after stimulation by allergy-associated signals. These populations include epithelial cells, secretory cells, neurons, fibroblasts, and smooth muscle. Extensive research is being done to elucidate the multifaceted roles that each of these cellular participants have on the allergic response (Galli et al., 2008; Locksley, 2010).

The classical events that occur during allergic inflammation can generally be divided into three stages: early, late, and chronic. The early stage reaction, often termed immediate type hypersensitivity, occurs within minutes. Depending on the route of allergen exposure, early responses can manifest in sneezing, nasal discharge, itchiness, coughing, vomiting, diarrhea, hives and in the more severe circumstances, difficulty breathing (associated with acute asthmatic reactions) and a drop in blood pressure (involving anaphylaxis). This reaction is primarily mediated through mast cells, already stationed at exposure sites, which then become activated through the crosslinking of IgE and its receptor, FcεRI by specific allergen. Once activated, they immediately release pro-inflammatory mediators stored within intracellular vesicles during a process termed degranulation. These preformed-mediators include the

previously mentioned histamine, proteases, and some cytokines such as TNF-alpha. Additionally, mast cells rapidly synthesize various lipid-derived inflammatory mediators, which include the prostaglandins and leukotrienes. These mediators then act on various cells populations such as the vascular endothelium (leading to vasodilation and vascular permeability), secretory cells (causing mucus production), smooth muscle (causing airway constriction, wheezing), and sensory neurons (causing sneezing, itching, and coughing). These inflammatory mediators also enhance the recruitment of various leukocytes such Th2 cells which then contribute to the late stage of the response. The late stage of the response represents a delayed and more prolonged period of inflammation that occurs hours after the initial perturbation. The delay is a consequence of various slowly-produced mediators released by mast cells and additionally recruited inflammatory cells such as Th2 cells, eosinophils, and basophils. In most cases, the inflammatory process resolves after 1-2 days, but when allergic stimuli persist (chronic exposure), then a more severe stage of the inflammatory process occurs, termed chronic allergic inflammation. This stage involves the long-lasting and enhanced accumulation of various inflammatory cells (Th2 cells, eosinophils, mast cells, etc), significant changes in tissue-specific cell populations (altered fibroblasts, increased smooth muscle and secretory cells, etc), excessive extracellular matrix deposition (fibrosis), and the impeded homeostatic function of afflicted organs (decreased lung function, increased airway sensitivity to stimuli, increased propensity to skin infection, etc). Allergic inflammation, indeed, is an intricate, powerful, and even dangerous immunological mechanism (Galli et al., 2008).

The physiological function for allergic inflammation (also called type II inflammation) has most commonly been viewed as a protective response to macroparasites (e.g. helminths or ticks) and other non-infectious, yet noxious environmental substances (e.g. venoms or irritants) (Marichal et al., 2013; Palm, Rosenstein, & Medzhitov, 2012). This is in contrast to type I inflammation which is typically directed against viruses, bacteria, fungi, and protozoa and involves Th1- and

Th17-associated cells and cytokines. Evidence for a role of allergic inflammation in protection from macroparasites comes from studies in both humans and mice. In humans, parasitic infection is highly correlated with hallmarks of allergic inflammation such as enhanced IgE levels, Th2-associated cytokines, and eosinophilia (Estambale, Simonsen, Vennervald, Knight, & Bwayo, 1995; Mahanty, Abrams, King, Limaye, & Nutman, 1992; Mahanty et al., 1993; McCarthy, Ottesen, & Nutman, 1994; R. M. Ramirez, Ceballos, Alarcon de Noya, Noya, & Bianco, 1996; Rossi, Takahashi, Partel, Teodoro, & da Silva, 1993). These correlative studies suggest a potential role for allergic inflammation in protection from parasites. In further confirmation, numerous murine studies have directly demonstrated the benefit of many allergic components in protection from parasitic infection. Some of these components which may provide protection in differing circumstances include Th2 cells, IgE, mast cells, eosinophils, alternatively activated macrophages, innate lymphoid cells, and, as will be discussed in detail here, basophils (J. E. Allen & Maizels, 2011; Anthony, Rutitzky, Urban, Stadecker, & Gause, 2007; Spits & Di Santo, 2011).

The downstream events of type II immune responses may provide protection from parasites through expulsion, containment, and healing (Palm et al., 2012). In brief, expulsion is the first line of active defense and occurs at barrier sites (mucosal and skin) through reactions that trigger the early removal of the parasite. These reactions include enhanced mucus production, increased bowel movements, coughing, and even itching (in the case of ectoparasites). It is noteworthy to mention that while allergic inflammation appears to be an important protective response to multicellular parasites, many of these organisms actively produce mediators to modulate or dampen the host response and thereby maintain survival. Host containment mechanisms are often triggered when the parasite is not expelled. During this process, the immune system attempts to contain the invading organism. These containment responses typically result in the formation of Th2-associated granulomas, which are complex and

organized conglomerates of numerous cells which surround the parasite and include Th2 cells, eosinophils, macrophages (including alternatively activated macrophages), and stromal cell populations. Infection with large multicellular parasites commonly involves digestive enzymes and physical forces that lead to significant tissue damage as the organism penetrates and sometimes traverses through numerous tissues. Allergic inflammation may counteract this damage by triggering mechanisms that promote tissue regeneration. However, in some cases, the chronic stimulation of this healing pathway may lead to fibrosis, a condition of excessive connective tissue deposition, which can hinder organ function. Taken together, these responses to allergen may provide protective or homeostatic benefit from many multicellular parasites, but when misdirected against harmless environmental substances, these responses become known as allergic disorders.

For some diseases, the excessive inflammation and damage associated with the parasite actually occurs after death of the organism, such as in the case of lymphatic filariasis (Dreyer, Noroes, Figueredo-Silva, & Piessens, 2000). Because of the link between parasite infection and reduced incidence of allergic disorders (and other inflammatory disorders) in developing nations, it has been postulated that certain parasitic infections may provide host benefit against these disorders. Therefore, when the parasite burden is not excessive, it is possible that, similar to our intestinal bacteria, helminth infection may also have a mutualistic role (Locksley, 2010).

Association of Basophils with Human Allergic Inflammation and Infection

Though basophils were connected to IgE-mediated allergic responses in the 1970's, many studies before and after that time have separately demonstrated an association for basophils with allergic inflammation and certain parasitic infections.

Historically, perhaps the most well associated site for inflammation and basophil involvement has been the skin. By the 1950's, Baer and Yanowitz had examined leukocyte infiltrate of inflamed skin that was patch-tested with antigen that individuals had a known allergy for. Interestingly, basophils, in particular, were found to have accumulated in the blister fluid of these allergen-exposed individuals (or sites of poison ivy dermatitis) significantly more than in the untreated, normal skin of other patients (Baer & Yanowitz, 1952). In the 1960's and 1970's similar experiments were done that confirmed the observation of enhanced basophil accumulation. Wolf-Jurgensen applied dinitrochlorobenzene to the skin of patients already allergic to the reagent while Dvorak, having recently developed a poison ivy sensitivity, applied urushiol (the primary irritant in poison oak and poison ivy) to himself and then examined leukocyte accumulation on his own biopsies (Dvorak & Mihm, 1972; Wolf-Jürgensen, 1966). As a side note, while the immune response to urushiol is often believed to be Th1-associated, data suggests that urushiol-induced inflammation carries a Th2-associated signature as well. (B. Liu et al., 2019; Lopez, Kalergis, Becker, Garbarino, & De Ioannes, 1998; Nickoloff, Fivenson, Kunkel, Strieter, & Turka, 1994; Ryan & Gerberick, 1999). In 1976, Askenase and Atwood, rather than testing a previously acquired allergy, instead sensitized subjects with the protein keyhole limpet hemocyanin (KLH) followed by re-exposure one week later. They observed an accumulation of basophils at the site of exposure, peaking 3 days later (Askenase & Atwood, 1976). Interestingly, Dvorak had previously classified this delayed form of inflammation "cutaneous basophil hypersensitivity," having studied it more thoroughly in guinea pigs. He had observed that this response was characterized by notably high numbers of basophils, and was qualitatively distinct from a different form of delayed inflammation which was inducible through initial priming with Complete Freund's Adjuvant (a mixture of mineral oil and inactivated mycobacteria) (Dvorak et al., 1970; Dvorak & Mihm, 1972; Richerson, Dvorak, & Leskowitz, 1970). Given what we now know, it is likely that the immune phenotype of the skin inflammation observed in all the mentioned studies was polarized toward an allergic inflammatory phenotype

rather than a Th1 or Th17 phenotype (such as psoriasis). Though the tools were lacking at the time to test whether basophils do actually enhance allergic inflammation, basophils were nonetheless found to be associated with and implicated in the pathogenesis of the response. It was not until the mid 2000's, that Dr. Karasuyama would finally test the role of basophils in promoting allergic skin inflammation (discussed later) (Mukai et al., 2005; Obata et al., 2007).

Aside from experimentally-induced dermatitis, many other skin disorders are associated with basophil accumulation. Not surprisingly, chronic idiopathic urticaria (CIU), a condition of persistent and systemic hives, is highly associated with basophil accumulation (Ito et al., 2011). In this condition, the immune and cytokine profile from the skin of CIU patients resemble that of affected skin from those given a cutaneous allergen challenge (Ying, Kikuchi, Meng, Kay, & Kaplan, 2002). Other skin conditions that lead to basophil accumulation include atopic dermatitis, the most common form of eczema; eosinophilic pustular folliculitis, a condition that appears physically similar to acne but is characterized by high numbers of eosinophils; prurigo, characterized by numerous small and severely-itchy bumps; bullous pemphigoid, a fluid-filled blistering disorder; Henoch-schonlein purpura, a disease that causes blood vessel inflammation and leakage, including in the skin; and even lepromatous leprosy, a form of mycobacterial-induced leprosy that involves a Th2-associated response (Ito et al., 2011; Otsuka, Miyagawa-Hayashino, Walls, Miyachi, & Kabashima, 2012; Otsuka et al., 2013; Ugajin, Takahashi, Miyagishi, Takayama, & Yokozeki, 2015). Other skin disorders more associated with non-allergic inflammation such as psoriasis, a Th17-associated inflammation; and tuberculoid leprosy, a form of leprosy that invokes a Th1-associated response, do not have increased basophils accumulation (Ito et al., 2011; Otsuka et al., 2013). Interestingly, of the basophil-associated skin disorders studied by Ito, et al., urticaria, prurigo, bullous pemphigold, and eosinophilic pustular folliculitis exhibited relatively dense numbers of basophils, whereas atopic dermatitis, though certainly being basophil-associated, displayed far fewer basophils per area of

skin (Ito et al., 2011). It remains unclear why some of these conditions exhibit heavier basophil densities than others.

Basophils are also associated with human allergic inflammation within the nose, esophagus, lungs, and the parotid glands of the neck. In a similar manner to the allergen-induced dermatitis, as previously described, nasal and lung mucosal regions were also tested for basophil recruitment in individuals known to have allergen-associated sinusitis or asthma. In these experiments, allergic individuals were found to have increased basophil recruitment after challenge in nasal and bronchial biopsies as well as bronchial alveolar lavage or sputum. (Gauvreau et al., 2000; KleinJan et al., 2000; Nouri-Aria et al., 2001; Schroeder, Lichtenstein, Roche, Xiao, & Liu, 2001). Increased basophil numbers in lung sputum or biopsies have also been observed in patients suffering from asthma attacks, with increased basophil counts associated with asthma severity (Kepley, McFeeley, Oliver, & Lipscomb, 2001; I. Kimura et al., 1975). Interestingly, one recent study revealed that in Chronic Obstructive Pulmonary Disease (COPD), a lung disease that is not typically associated with allergic inflammation, the severity of the disease correlated with increased basophil accumulation in the lung and presentation of regionalized type II inflammatory cell clusters comprised of eosinophils, basophils, and Th2 cells (Jogdand et al., 2020). Eosinophilic esophagitis, a chronic and degenerative inflammatory disorder of the esophagus associated with high eosinophil accumulation, has also been investigated for basophil accumulation. Studies on this disease have revealed an increase in esophageal basophils in patients with the disease compared to controls (Chehade et al., 2008; Iwakura et al., 2015; Noti et al., 2013). Finally, basophils have also been shown to infiltrate inflamed tissue in patients with Kimura's Disease. Kimura's disease is an allergic inflammatory disorder of the soft tissue, generally of the head and neck, and sometimes presents with severe tumor-like outgrowths (often involving inflamed lymph nodes). In one particular study, basophils were histologically revealed to infiltrate the inflamed parotid gland tissue as well as associated

lymphoid-like regions (Nonaka et al., 2017). While recent mice studies have suggested a function for basophils in eosinophilic esophagitis and COPD (discussed later) (Noti et al., 2013; Shibata et al., 2018a), whether basophils can contribute to asthma and the allergic inflammation involved in Kimura's Disease, including the lymphadenitis, has yet to be revealed.

Biting and skin dwelling parasites have also been associated with basophil recruitment, suggesting a protective role for basophils in these circumstances rather than merely a pathogenic role in inflammation. An early implication for the protective role of basophils came in the 1970's by Allen who had been studying the immune response to ticks (J. R. Allen, 1973). These experiments were originally founded on much earlier studies dating back to the 1930's when Trager had first characterized the inflammatory response to ticks in guinea pigs (Trager, 1939). Trager had observed that in a secondary infection, tick attachment and feeding was reduced. Moreover, tissue sections of the bite site had revealed the accumulation of a leukocyte mass and a thickened epithelium underneath. He insightfully noted that "by the fourth day, before the tick larvae has been able to engorge, it is effectively walled off from its source of supply of food." In the 1970's, Allen performed a more detailed histological analysis of leukocyte infiltrate and found that part of the "leukocyte mass" was an accumulation of basophils. Though these observations were originally found in guinea pigs, later investigations would show that infections of humans with ticks also led to an accumulation of basophils (Ito et al., 2011; R. Kimura, Sugita, Ito, Goto, & Yamamoto, 2017; Nakahigashi, Otsuka, Tomari, Miyachi, & Kabashima, 2013). In addition to ticks, basophils have also been associated with the bites of bed bugs and the rashes of scabies, caused by the skin-burrowing mite *Sarcoptes scabiei*. In a recent 2019 study, scabies infected skin was shown to have high infiltration of basophils, eosinophils, and alternatively activated macrophages as well as high expression of TSLP, an epithelial-derived cytokine that can initiate and amplify many aspects of allergic inflammation (Hashimoto, Satoh, & Yokozeki, 2019). Interestingly, one study, published in the 1970's, had

reported on a patient who lacked both eosinophils and basophils. This patient was found to also have an abnormal and widespread infection of scabies, suggesting a protective role for basophils and eosinophils in the infection (Juhlin & Michaelsson, 1977). Finally, other than biting insects, ringworm, though not a worm, but rather an infection caused by a fungus, was also found to involve basophil accumulation. Whether basophils are actually important in protection against fungal infections or mites has yet to be more conclusively investigated, but mouse models have suggested a role for basophils in tick infections (discussed in detail later) (Wada et al., 2010).

Basophils are heavily associated with helminth infection in animal models, but human data has been lacking. Infection of mice with the parasitic helminths, *Nippostrongylus.brasiliensis, Heligmosomoides polygyrus bakeri*, and *Schistosoma mansoni* has been observed to induce the migration and accumulation of basophils into the lung, intestine, and liver, respectively (C. Schwartz, Oeser, Prazeres da Costa, Layland, & Voehringer, 2014; C. Schwartz, Turqueti-Neves, et al., 2014; Sullivan et al., 2011; Voehringer, Shinkai, & Locksley, 2004). However, to my knowledge, no human data has been reported examining basophil counts in helminth-infected tissue compared to healthy tissue of non-infected individuals. Some studies had investigated blood basophilia as a potential indicator of basophil responsiveness to helminth infection, but these studies did not establish a correlation between infection and blood basophilia in humans, despite a positive correlation in rodent models (Conboy & Stromberg, 1991; Harper, Genta, Gam, London, & Neva, 1984; Herbst et al., 2012; Lindor, Wassom, & Gleich, 1983; Mitre & Nutman, 2003, 2006; Ogilvie, Askenase, & Rose, 1980; Ogilvie, Hesketh, & Rose, 1978; Okada, Nawa, Horii, Kitamura, & Arizono, 1997; Roth & Levy, 1980; Rothwell & Love, 1975). Nonetheless, lack of blood basophilia ought not to be equated with a lack of tissue recruitment or function during helminth infection. Blood basophils taken from helminth-infected individuals, have been shown to possess surface-bound, anti-helminth IgE and respond to a re-

exposure of helminthic antigen in vitro by degranulating and producing IL-4 (Falcone et al., 1996; Genta et al., 1983; Gonzalez-Munoz, Garate, Puente, Subirats, & Moneo, 1999; Mitre, Taylor, Kubofcik, & Nutman, 2004; Nielsen et al., 1994; Ottesen et al., 1979). Additionally, basophils have been demonstrated to enhance allergic inflammation and helminth defense in numerous more recent murine studies (discussed in detail later). Therefore, though direct evidence is lacking regarding the involvement of basophils in human helminth infection, the role of basophils in protection against such parasites is strongly implicated.

Basophil Development and Expansion

Basophil development occurs in the bone marrow where mature basophils are generated and released into the blood stream. To generate basophils, hematopoietic stem cells (HSCs) in the bone marrow give rise to common myeloid progenitors (CMPs) which can then differentiate into granulocyte-monocyte progenitors (GMPs). GMPs can became basophil-mast cell progenitors (BMPs or BMCPs), which can either lead to basophil or mast cell differentiation depending on the transcription factors induced (Sankaran & Weiss, 2015; Sasaki, Kurotaki, & Tamura, 2016; M. Wang et al., 2016). Differentiated basophils leave the bone marrow as fully matured leukocytes and, under normal homeostatic conditions, are estimated to live for approximately 60 hours (Ohnmacht & Voehringer, 2009). Of note, some reports have also observed progenitor cells, capable of differentiating into basophils, in the spleen as well (Arinobu et al., 2005). Nonetheless, most studies have focused on the bone marrow as a major site of basophil production.

Numerous transcription factors and signaling components have been discovered to be regulators of basophil differentiation, but three key transcription factors are GATA Binding Factor 2 (GATA2), CCAAT-enhancer binding protein alpha (C/EBPα), and microphthalmia-

associated transcription factor (MITF). Prior to differentiation into either basophils or mast cell progenitors, BMPs selectively express either C/EBPα or MITF. In order for GMPs to differentiate toward a BMP pathway, they must express GATA2. GATA2 deficiency ultimately prevents the production of both mast cells and basophils (Iwasaki et al., 2006; Y. Li, Qi, Liu, & Huang, 2015). Of note, the relative timing of GATA2 expression may be important for the transition of GMPs toward an eosinophil- or BMP-directed stage (Iwasaki et al., 2006). Two recent reports had revealed that GATA2 expression relied upon both STAT5 and IRF8 (Y. Li et al., 2015; Sasaki et al., 2015). While STAT5 was shown to bind to the promoter and an intronic sequence GATA2, it is less clear how IRF8 may influences GATA2 expression (Y. Li et al., 2015). After GMPs have differentiated further toward a BMP stage, the exclusive expression of MITF and C/EPBα determines whether the progenitors will further mature down mast cell or basophil-directed pathways. The expression of MITF leads to and is required for a mast cell-committed fate, while high expression of C/EPBα leads to and is required for a basophil-committed fate (Arinobu et al., 2005; Iwasaki et al., 2006; Qi et al., 2013). Exclusive expression of these two transcription factors is due to MITF and C/EPBα having direct antagonistic activity, so the expression of one leads to the suppression of the other (Qi et al., 2013). Other factors known to contribute to basophil development, though their mechanistic role is less clear, include distal-promoter derived Runt-related transcription factor 1 (P1-RUNX1), GATA-binding protein 1 (GATA1), and promyelocytic leukemia zinc finger (PLZF) (Mukai et al., 2012; Nei et al., 2013; S. Zhang et al., 2019). In contrast to the transcription factors required for the generation of basophils, Ikaros family zinc finger protein 1 (Ikaros), was found to play a role in reducing basophil production, seemingly by suppressing C/EPBα expression (Rao, Smuda, Gregory, Min, & Brown, 2013). Certainly, the hematopoietic process leading to the differentiation and production of basophils is complex and much more research is needed to better understand the pathway.

In contrast to the homeostatic production of basophils, certain conditions lead to the enhancement of basophil differentiation, resulting in basophilia. As previously mentioned, numerous parasite infections in animal models have demonstrated basophilia after infection. For mice in particular, infection with *N. brasiliensis, H. polygyrus bakeri, Stronglyoides venezuelensis, S. mansoni, and Litomosoides sigmodontis* result in basophilia. At homeostatic levels, basophils generally remain below 1% of total leukocytes, but after infection, the frequency of basophils can increase more than ten-fold (Herbst et al., 2012; Lantz et al., 1998; Lantz et al., 2008; C. Schwartz, Turqueti-Neves, et al., 2014; Shen et al., 2008; Torrero, Hubner, Larson, Karasuyama, & Mitre, 2010). This increase is likely an additional defense mechanism to increase the number of effector cells capable of responding to infection. The most extensively studied signaling molecule important for basophil expansion has been the cytokine, IL-3. Interestingly, IL-3 was once termed histamine-producing cell-stimulating factor (HCSF) because it could enhance the production of histamine-producing progenitors from hematopoietic cells (Ihle et al., 1983). In studies with *S. venezuelensis*, *N. brasiliensis*, and *H. polygyrus*, basophilia was found to be strongly dependent upon IL-3. Moreover, injection of IL-3 or IL-3 complexes, in the absence of infection, led to basophilia as well. The receptor for IL-3 is a heterodimer comprised of the IL-3R-alpha chain (CD123) and a covalently linked beta chain (CD131). The CD131 beta chain is not unique to the IL-3 receptor, but rather is shared among numerous cytokine receptors including GM-CSF and IL-5 (Geijsen, Koenderman, & Coffer, 2001). Worth noting, in mice but not humans, an additional, IL-3 specific beta chain also exists ($\beta_{IL\text{-}3}$) that pairs with the alpha chain, though its unique contribution is unclear (Geijsen et al., 2001; Hara & Miyajima, 1992). The primary source of IL-3 appears to be T cells (Shen et al., 2008). Although the mechanism for how IL-3 induces basophilia is not clear, STAT5 and GATA2 are likely involved. STAT5 is an important transcription factor that is activated through IL-3 receptor signaling. As previously mentioned, GATA2, required for basophil and mast cell production from the bone marrow had been shown to be a direct target of STAT5 (Y. Li et al.,

2015). Although IL-3 is not important for homeostatic basophil production, it seems plausible that under conditions of basophilia, IL-3 acts to promote GATA2 transcription through STAT5, leading to an increase in the differentiation of BMPs. If IL-3 is acting directly on bone marrow progenitors, then this most likely is occurring during or after the GMP to BMP transition, since expression of the IL-3 receptor appears to increase between the GMP and BMP stages. On a final note, additional cytokines that may influence blood basophilia include GM-CSF, NGF, and TGF-β. GM-CSF, when injected in vivo along with IL-3, was found to enhance basophil differentiation (Schneider et al., 2009). NGF and TGF-β have also been shown to enhance the differentiation of basophils from bone marrow in vitro, but the relevance of these in vivo is less clear (Sillaber et al., 1992; Tsuda et al., 1991).

Other factors that have been shown to increase blood basophil levels are interleukin-33 (IL-33) and thymic stromal lymphopoietin (TSLP) (Schneider et al., 2009; Siracusa et al., 2011). Interestingly, these proteins, along with IL-25, are considered a class of cytokines termed "alarmins." These molecules are released from many non-leukocyte cells, particularly epithelial cells in response to various stimuli and act as signals to initiate or enhance an immune response (Yang, Han, & Oppenheim, 2017). Of particular note, TSLP-induced basophilia was found to be independent of IL-3, as basophils still increased in IL-3 receptor knockout mice after the injection of TSLP (Siracusa et al., 2011). Moreover, infection of *Trichinella spiralis* in IL-3 receptor knockout mice led to an early basophilia that could be inhibited by blocking TSLP (though the basophilia did not occur in wild-type mice), also suggesting an alternative, IL-3-independent means of enhanced basophil differentiation (Giacomin et al., 2012). Further mechanistic details of how IL-33 or TSLP enhances basophil abundance in the blood has yet to be determined. It is worth mentioning that TSLP and IL-3 led to different transcriptional signatures after being used in vitro to differentiate basophils from bone marrow cells. IL-3-differentiated basophils appeared significantly larger, were better at degranulation, and had

increased expression of mast cell protease 7 (Mcpt-7) transcripts compared with TSLP-differentiated basophils. On the other hand, TSLP-differentiated basophils had higher surface expression of IL-3, IL-18, and IL-33 receptors, and could respond better to these signals in producing various cytokines such as IL-4, IL-6, CCL3, CCL4, CCL12, CXCL12 (Siracusa et al., 2011). Though it is unclear how representative bone marrow-derived basophils are of basophils in vivo, these experiments still suggest that, given the differential transcriptional profile and propensity of effector molecule secretion, there may be heterogeneity in the basophils produced from the bone marrow, depending on the inducing agent. Apart from the cytokines mentioned, whether other factors lead to increased basophil hematopoiesis is still an area of potential investigation.

Basophil Recruitment into Peripheral Tissues and Chemotaxis

Under homeostatic conditions, basophils circulate through the bloodstream, but numerous stimuli at peripheral sites can cause basophils to be recruited from the blood into the tissue(s) where they perform various effector functions. As described above, many conditions in humans can lead to basophil recruitment such as immediate hypersensitivity responses, allergic skin diseases, and ectoparasitic infections. Though less clear in humans, basophil accumulation into peripheral tissues has been clearly observed in mice upon infection with endoparasites (C. Schwartz, Oeser, et al., 2014; C. Schwartz, Turqueti-Neves, et al., 2014; Sullivan et al., 2011; Voehringer et al., 2004). The actual process of effective basophil recruitment into the peripheral tissue involves numerous steps including basophil adhesion to the endothelial wall at the target site, transendothelial migration, trans-basement membrane migration directly beyond the endothelial wall, and chemotaxis to the targeted site within the accumulated tissue. Significant research has been done in vitro to infer what receptors and ligands are involved in the recruitment process, but unfortunately, very little information actually exists in vivo as to what

receptors and ligands are most critical. Nonetheless, I will attempt to highlight the key mediators discussed in the literature regarding their role (or potential role) in basophil recruitment.

The most well studied factor involved in the accumulation of basophils into peripheral sites is IL-3. Numerous in vitro studies have revealed that this cytokine enhanced basophil adhesion to endothelium. A classic experiment done in the 1990s demonstrated that exposure of basophils to IL-3 enhanced their adhesion to human endothelial cells derived from human umbilical cord vein endothelial cells (HUVECs). Basophil adhesion was reduced by monoclonal antibodies directed against CD18 (integrin-beta 2), CD11a, or CD11b (Bochner et al., 1990). Within the more physiological context of actively flowing blood, proteins involved in leukocyte rolling and adhesion are especially important in overcoming sheer force. Under sheer force conditions, pre-incubation of HUVECs with IL-3 provided enhanced basophil adhesion in a CD49d, CD49e, P-selectin, P-selectin glycoprotein ligand 1, and CCR7-dependent manner (Lim et al., 2006). Within the bloodstream, certain chemokines such as eotaxin, RANTES, and SDF-1 may stimulate human basophils to undergo transendothelial migration. Once again, IL-3 was found to significantly enhance transendothelial migration across HUVECs in combination with eotaxin (Iikura et al., 2004). When cells leave the blood stream via the endothelium, they often must also cross a basement membrane before entering deeper into the tissue. Certain enzymes such as matrix metalloproteases are involved in the process of passing through this barrier. Transmigration assays performed in Matrigel®, a basement membrane matrix, demonstrated that IL-3 enabled the migration of basophils through the gel. This effect was enhanced by the addition of other chemotactic factors such as RANTES, IL-8, MCP-1, eotaxin, PDG2, 5-oxo-ETE, and platelet activating factor (PAF), though IL-3 was needed in addition to these chemokines for transmigration to occur. This IL-3-enhanced transmigration appeared dependent upon CD18 (beta 2 integrin) and matrix metalloproteinase 8 (Suzukawa et al., 2006). Once basophils have transmigrated through the endothelium and basement membrane, various

chemokines and adhesion molecules presumably control their localization in the tissue. IL-3, without the addition of other stimulants, could induce basophil migration across a porous membrane, though it was not clear whether IL-3 acted directly or indirectly (e.g. secretion or expression of other molecules by basophils) to induce migration (Tanimoto, Takahashi, & Kimura, 1992; Yamaguchi, Hirai, Shoji, et al., 1992). Altogether, in vitro evidence strongly suggests that IL-3 may be involved in multiple steps of basophil recruitment into peripheral tissue.

More recent studies have suggested the in vivo importance of IL-3 for the recruitment of basophils into peripheral organs. IL-3 has been shown to be important for lymph node accumulation of basophils upon exposure of mice to papain, a papaya derived serine protease; calcipotriol, an irritant that induces atopic dermatitis-like inflammation; and *N. brasiliensis* (Kim et al., 2013; S. Kim et al., 2010; Leyva-Castillo, Hener, Michea, et al., 2013; Shen et al., 2008; Sokol, Barton, Farr, & Medzhitov, 2008). IL-3 is also important for the accumulation of basophils into the lungs of mice infected with *N. brasiliensis* (Shen et al., 2008). Within the context of tick infection, T cells and IL-3 were required for rapid basophil accumulation into the skin after secondary infection. The injection of IL-3 into T cell deficient mice restored the accumulation of basophils into the skin (Ohta et al., 2017). Interestingly, in the calcipotriol model, while IL-3 was important for basophil accumulation into the lymph node, it appeared unnecessary for basophil accumulation in the skin, the primary site of irritant exposure; TSLP, however, was required. (Siracusa et al., 2011). This suggests that IL-3-independent mechanisms also exist for basophil recruitment into peripheral sites. One caveat to all these studies, however, is that the accumulation of basophils into peripheral tissues is inevitably also dependent upon cell survival, which IL-3 likely enhances (mentioned later). It has yet to be determined to what extent IL-3 directly enhances basophil accumulation through tissue recruitment versus indirect mechanisms like cell survival.

Although IL-3 appears to be the most widely studied stimulus for basophil recruitment, many other molecules have also been observed to play some role in the process of recruitment, whether in adhesion, transendothelial migration, or chemotaxis. I want to briefly mention a few particularly interesting highlights and caveats. First, basophils have very high and selective expression for the histamine H4 receptor, which has been shown in vitro to enhance migration of basophils (Mommert et al., 2016). Presumably, this may allow basophils to relocalize quickly in response to the activation of other basophils or mast cells in the tissue. Second, basophils are also highly responsive to prostaglandin D2 and leukotriene B4 (LTB4), lipid inflammatory mediators that are rapidly secreted from various cells after stimulation (Iikura et al., 2005; Reese et al., 2007). Injection of mice with chitin, a common structural component in fungi, helminths, insects, and crustaceans, induced basophil accumulation in the tissue that was dependent upon LTB4 (seemingly generated from macrophages) and its receptor, leukotriene B4 receptor 1 (BLT1) (Reese et al., 2007). Third, many reports using human basophils have revealed a role for eotaxins, a class of chemokines normally associated with eosinophil recruitment, for the migration of basophils (Menzies-Gow et al., 2002; Uguccioni et al., 1997). Indeed, experimentally injected eotaxin into human skin led to the recruitment of basophils to the site of injection (Menzies-Gow et al., 2002). Perplexingly, while human basophils express CCR3, a major receptor for eotaxins, this receptor is not found on murine basophils. (Voehringer et al., 2004). CXCR4 has also been shown important for basophil recruitment into the lymph node and skin (Hayes et al., 2020; Pellefigues et al., 2018). Stimulation of mouse basophils through IL-3 and TSLP resulted in CXCR4, stored in high quantities within the cell, to be transported to the plasma membrane (Hayes et al., 2020). Lastly, activation of the FcεRI/IgE complex is also known to contribute to adhesion and, to an extent, chemotaxis. In a model of skin inflammation, activation of basophils circulating in the bloodstream through FcεRI led to an increase in basophil adhesion to the endothelial walls where they were thought to secrete IL-4 to enhance

VCAM-1 expression (Cheng et al., 2015). This enhanced adhesion may have been mediated through activation of integrins such CD18 (beta 2 integrin) (Bochner, MacGlashan, Marcotte, & Schleimer, 1989). Interestingly, FcεRI-crosslinking enhanced the sensitivity of basophils to some chemokines as well, which may contribute to basophil localization when already within the inflamed tissue (Suzukawa et al., 2005). The contexts in which crosslinking of FcεRI on basophils leads to enhanced adhesion versus migration is an area of potential investigation.

Survival in Peripheral Tissue

Upon basophil accumulation into peripheral tissue, it is expected that certain factors may extend or even shorten the life span of the basophil. Nonetheless, while it is known that under homeostatic conditions, basophils circulate in the blood and survive for up to 60 hours (2.5 days), data regarding survival in the peripheral, inflamed tissue, has not been as clear (Ohnmacht & Voehringer, 2009). Nonetheless, numerous studies have tested the effects of IL-3, which has a clear effect on enhancing longevity in basophils in vitro. A classic study in the 1990s revealed that when human basophils were purified and cultured for one week, not surprisingly, only about 10% remained viable. However, with the addition of IL-3, almost 70% of basophils were still viable after one week of culture (Yamaguchi, Hirai, Morita, et al., 1992). Many other tested cytokines have not shown a similar enhancement of survival. GM-CSF could extend survival to a small extent, while IL-4, G-CSF, and M-CSF could not potently enhance survival (Yamaguchi, Hirai, Morita, et al., 1992). Among IL-2, IL-3, IL-4, IL-7, IL-9, IL-13, IL-15, IL-18, and TSLP, only IL-3 could prevent pro-caspase cleavage and activation, an event important for apoptosis, in basophils (Didichenko, Spiegl, Brunner, & Dahinden, 2008). The potent pro-survival effect of IL-3 appeared to be independent of the PI3K/AKT pathway but dependent upon PIM1, leading to the enhanced expression of various BCL-2 family anti-apoptotic proteins. Interestingly, this same study demonstrated that basophils constitutively

express relatively high amounts of BCL-2 compared to eosinophils and neutrophils, allowing basophils to survive longer than the two other granulocytes without exogenously-added survival factors, at least in vitro (Didichenko et al., 2008). In addition to merely extending survival, IL-3 may also be able to protect against external signals that induce cell death as well. Type 1 interferons (interferon-alpha and beta), Fas Ligand, and TRAIL were found to induce apoptosis of human basophils, but IL-3 could protect against this induced apoptosis (Hagmann, Odermatt, Kaufmann, Dahinden, & Fux, 2017). Though in vitro evidence for the role of IL-3 in enhancing basophil survival has been fairly strong, in vivo data has been less clear. Shen *et al*. found that while T cell derived IL-3 was crucial for basophil production after infection with N. brasiliensis, there was no difference in survival of basophils between wild-type mice and mice with IL-3-deficient T cells. As a caveat though, these results were derived from basophils in the bone marrow and liver, whereas active sites of infection for this parasite are normally considered to be the lung and intestines. Moreover, there was a significant decrease in the number of basophils at the lung in the IL-3 deficient mice; however, interpretation of these results is difficult because loss of basophils at the site could have been due to either a decrease in recruitment, blood basophil numbers, or survival (Shen et al., 2008). Therefore, the effect of IL-3 on basophil survival in vivo still remains unclear, though in vitro data strongly suggests that, at least in some circumstances, IL-3 may enhance survival at the site of inflammation.

Basophil-Derived Effector Molecules

Over the years, basophils have been reported to produce countless immune mediators. For the purposes of this dissertation, I will briefly highlight several prominent mediators that have been described in the literature, some of which will be discussed in greater detail later.

Basophils store numerous inflammatory mediators within intracellular vesicles. Activation of basophils induces a process termed degranulation, causing the intracellular vesicles to fuse with the plasma membrane of the cell, thereby releasing the inflammatory mediators to the extracellular environment. One key inflammatory mediator is histamine, generated from the decarboxylation of the amino acid histidine through the enzyme histidine decarboxylase, which is highly expressed in basophils. Histamine is most well-known for its contribution to the acute wheal and flare reaction of an inflammatory response, as it induces vascular permeability and vasodilation by acting on the vascular smooth muscle and endothelium. In addition to basophils, histamine can act on other cell types, with many additional functions that extend beyond the immune system (Thurmond, Gelfand, & Dunford, 2008). Another class of inflammatory mediators include the serine proteases, such as mast cell proteases 8 and 11 (MCPT-8 and MCPT-11). Despite their names, these proteases are primarily expressed in basophils rather than mast cells. In a model of basophil-dependent skin inflammation, MCPT-11 contributed to increased skin thickness, vascular permeability, and the accumulation of eosinophils, neutrophils, and monocyte/macrophages, over the course of many days. MCPT-11 also promoted the migration of basophils, eosinophils, and macrophages through the cleavage of an unknown serum factor (Iki et al., 2016). MCPT-8 appears to have a similar function as MCPT-11; intradermal injection of MCPT-8 induced a transient cutaneous inflammatory response, leading to enhanced skin swelling, vascular permeability, and inflammatory cell recruitment. Curiously, while mouse basophils express these two proteases, human basophils do not. Human basophils do express other proteases not found in mice that may functionally replace MCPT-8 and 11. One study proposed that alpha-tryptase and beta-tryptase in human basophils may be functionally similar to MCPT-11 (Iki et al., 2016); granzyme B in human basophils shares structural similarity with MCPT-8 in the critical region determining substrate specificity, suggesting that it may be able to functionally replace MCPT-8 in human basophils (Lutzelschwab, Huang, Kullberg, Aveskogh, & Hellman, 1998; Tschopp et al., 2006). Within

minutes of activation and degranulation, basophils will rapidly release lipid-derived mediators, in particular, cysteinyl leukotrienes (D. W. MacGlashan, Jr., Peters, Warner, & Lichtenstein, 1986). A key mediator of this class, cysteinyl leukotriene C4 (LTC4), is produced when phospholipase A cleaves phospholipids into arachidonic acid, which is then converted into leukotriene A4 (LTA4) through the arachidonate 5-lipoxygenase (5-LO) enzyme. LTA4 is converted into LTC4 by the enzyme LTC4 synthase (M. Liu & Yokomizo, 2015). Although the production and release of these lipid-derived mediators is not as quick as degranulation, it still occurs rapidly (Creticos et al., 1984; D. Wang, Clement, Smitz, De Waele, & Derde, 1995). Once released, these lipid-derived mediators further amplify the allergic response by enhancing vascular permeability, eosinophil recruitment, bronchoconstriction, and mucus production (M. Liu & Yokomizo, 2015).

Basophils also produce and release numerous cytokines upon activation. The most well-known of these basophil-derived cytokines has been IL-4. This cytokine can signal through two different heterodimeric receptors. The type I receptor is comprised of IL-4 receptor alpha and a common gamma chain, while the type II receptor is comprised of IL-4 receptor alpha and IL-13 receptor alpha 1. The type I receptor exclusively binds IL-4 while the type II receptor can bind both IL-4 and IL-13 (Gour & Wills-Karp, 2015). Functionally, IL-4 contributes to the development of allergic inflammation. In the peripheral tissue, IL-4 can act on the vascular endothelium to enhance cell recruitment, and can also promote macrophage polarization toward an anti-helminth or wound healing phenotype (Cheng et al., 2015; Egawa et al., 2013). IL-4 is important for the generation of the adaptive allergic immune response as well. It is required for B cells to class switch to IgE, and may also play an important role in Th2 cell differentiation or recruitment into the peripheral tissue (Prout, Kyle, Ronchese, & Le Gros, 2018; Voehringer, Reese, Huang, Shinkai, & Locksley, 2006). Given its broad impact on allergic inflammation, IL-4 has been a cytokine of great interest. In the early 1990s, basophils were discovered to be one of the primary producers of IL-4, sparking interest in the study of basophils. Initial reports showed that

mouse-derived, splenic, non-B, non-T cells could secrete large amounts of IL-4 after FcεRI crosslinking (Bensasson, Legros, Conrad, Finkelman, & Paul, 1990). Purification of these FcεRI+ cells revealed an enrichment for basophils, based on characteristics such as histamine production, alcian blue-staining granules, and appearance by electron microscopy (Seder et al., 1991). IL-4 production by human basophils was confirmed in subsequent studies (Brunner, Heusser, & Dahinden, 1993; D. MacGlashan, Jr. et al., 1994). Genetic reporters for IL-4 have allowed for tracking of IL-4 producing cells in vivo. The development of 4get mice, which contain a bicistronic IRES-eGFP reporter between the IL-4 stop codon and 3'-UTR, has allowed for tracking of the IL-4 transcript. These mice were used to show that eosinophils, Th2 cells, and basophils are the primary populations that produce IL-4 transcript upon infection. (Min et al., 2004; Voehringer et al., 2004). Interestingly, IL-4 has been found to be heavily regulated at the post-transcriptional level. Basophils constitutively express high levels of IL-4 mRNA, but only translate IL-4 after receiving a stimulatory signal, such as FcεRI/IgE crosslinking (K. Mohrs, Wakil, Killeen, Locksley, & Mohrs, 2005). This pool of IL-4 transcript most likely allows the cell to synthesize IL-4 protein much more rapidly when needed. Using IL-4 reporters that more faithfully reflect protein translation, such as the KN2 reporter, Th2 and basophils were determined to be the major producers of IL-4 at the peripheral, inflamed tissues of parasite-infected mice (Sullivan et al., 2011). Following these reports, numerous papers have explored the functions of basophil-derived IL-4, which will be discussed in detail later.

In addition to IL-4, other cytokines, such as IL-13 and TNF-alpha, may also be produced by basophils. Though Th2 and type 2 innate lymphoid cells are considered the major producers of IL-13 in vivo, multiple studies have reported that basophils produce IL-13 in vitro, suggesting that under some circumstances, basophils may also produce IL-13 in vivo (Devouassoux, Foster, Scott, Metcalfe, & Prussin, 1999; Eglite, Pluss, & Dahinden, 2000; Gibbs et al., 1996; Gibbs, Haas, Wolff, & Grabbe, 2000; H. Li, Sim, & Alam, 1996; Liang et al., 2011). Broadly, IL-

13 is known to drive goblet cell hyperplasia, smooth muscle contraction, mucus production, and eosinophil recruitment (Bao & Reinhardt, 2015), but the specific role of basophil-derived IL-13 is unknown. Basophils have also been reported to produce TNF-alpha, which may be protective against bacteria during infection or sepsis (Falkencrone et al., 2013; Piliponsky et al., 2019). Based on gene expression data from the Immunological Genome Project, as well as numerous other reports, basophils appear to highly express RNA transcripts for macrophage colony stimulating factor (M-CSF), hepatocyte growth factor (HGF), and the chemokines CCL3 and CCL4 as well (Cohen et al., 2018; Dwyer, Barrett, Austen, & Immunological Genome Project, 2016). While it is not exactly clear how these cytokines contribute to the effector functions of basophils, it is likely that these functions extend beyond the basic induction of inflammation through increased vascular permeability and cell recruitment, and may involve more intricate events such as macrophage differentiation and tissue regeneration (Nakamura, Sakai, Nakamura, & Matsumoto, 2011; Ushach & Zlotnik, 2016).

Basophil Activation and Signaling

Basophils are able to respond to a broad range of external stimuli, including allergens, pathogen-associated products, cytokines, activated complement components, proteases, and even contact-dependent signals. The most well-studied cause of basophil activation is the crosslinking of the high affinity IgE receptor, FcεRI. The high affinity IgE receptor (FcεRI) is typically found as a tetramer consisting of three components: (1) an alpha chain, which binds IgE on the surface of the cell (2) two gamma chains, which make up the primary signaling module, and (3) a beta chain, which acts as a signal amplifying component. Basophils and mast cells are unique in that they are the only two cell populations to express the beta chain. In humans, other cell populations, such as dendritic cells, express the alpha and gamma chains without the beta chain, although the exact function of this trimeric form of the receptor is still

being investigated (Shin & Greer, 2015). Both the gamma and beta chains have immunoreceptor tyrosine-based activation motifs (ITAMs) which contain specific tyrosines that can become phosphorylated and induce a downstream signaling cascade. Signaling is typically initiated when allergen crosslinks multiple IgE molecules, bound to FcεRI, on the cell surface. Crosslinking of IgE, and ultimately the receptors, results in transphosphorylation of the signaling chain ITAM, mediated by receptor associated Src family kinases, such as Lyn. The phosphorylated residues act as docking sites for SH2 domain-containing proteins, such as Syk kinase. The recruitment and activation of Syk is perhaps the most critical early kinase in the overall signaling pathway. Downstream of these initial signals, there is activation of phospholipase C and phosphoinositide 3-kinase (PI3K), MAP kinase signaling, and intracellular calcium release, ultimately resulting in degranulation, leukotriene synthesis, cytokine production, and more (Gilfillan & Beaven, 2011). Unfortunately, much of the information regarding this cascade comes from FcεRI-expressing cell lines and mast cells, and significant work is needed to clarify the importance of many of these pathways in primary basophils.

Numerous determinants affect outcome of the signaling cascade downstream of FcεRI. Upstream determinants associated with IgE and antigen crosslinking include the affinity between the antigen and IgE, the relative concentrations of antigen and IgE, antigen valency, and the spacing between IgE-binding epitopes on the antigen (Christensen, Holm, Lund, Riise, & Lund, 2008; Collins, Thelian, & Basil, 1995; Healicon & Foreman, 1986; Paar, Harris, Holowka, & Baird, 2002; Pruzansky & Patterson, 1988). The affinity of IgE has been of particular interest as merely having antigen-specific IgE does not necessarily correlate with downstream responses. One clinical report had shown that detection of antigen specific IgE was not an accurate predictor of allergen skin prick sensitivity, compared to the affinity of IgE for the allergen (Pierson-Mullany, Jackola, Blumenthal, & Rosenberg, 2002). One possible explanation for this phenomenon involves what has been termed, “kinetic proofreading.” Just prior to similar

investigations related to FcεRI, the concept of kinetic proofreading had been used to account for experimental observations related to T cell receptor (TCR) signaling (Lyons et al., 1996; McKeithan, 1995; Rabinowitz, Beeson, Lyons, Davis, & McConnell, 1996). Kinetic proofreading is a property of many biochemical pathways to ensure that a false signal does not propagate to completion (Goldstein, Coombs, Faeder, & Hlavacek, 2008). More specifically, kinetic proofreading is a consequence of signaling pathways by which irreversible secondary messenger amplification is first preceded by a series of reversible signaling steps that only stable receptor-ligand complexes (or crosslinking) are able to overcome. If the initial signaling complex is dissociated, early downstream phosphorylation events are quickly reversed, presumably through phosphatase activity. When high affinity antigen crosslinks IgE/FcεRI, the stability of the active receptor-ligand complex allows for signaling steps to propagate until an irreversible amplification step is reached. However, when low affinity antigen crosslinks IgE/FcεRI, the receptor-ligand complex may form transiently, but will quickly dissociate. In such a situation, downstream phosphorylation events will be quickly reversed prior to the irreversible signal amplifying step (Z. J. Liu, H. Haleem-Smith, H. X. Chen, & H. Metzger, 2001b; C. Torigoe, J. K. Inman, & H. Metzger, 1998). As stated by Goldstein et al., “this gives rise to a mechanism by which cells can discriminate among ligands that bind to the same receptor, but form different ligand-receptor complexes with different lifetimes (Goldstein et al., 2008).” Consequently, from an immunological standpoint, this mechanism may prevent effector cells from unintentionally initiating a potent inflammatory response to cross-reactive antigen and act as a filter to modulate the body’s response to antigen it perceives as more dangerous.

While certain functions downstream of IgE-antigen binding are constrained by this proofreading mechanism, other functions are not, suggesting that there is much complexity to the IgE/FcεRI signaling model. When FcεRI-expressing rat basophilic leukemia cells (RBL-2H3) were activated through the FcεRI/IgE complex by low or high affinity antigen, high affinity antigen led

to greater downstream phosphorylation of kinases, such as Syk and Erk, as well as degranulation. However, synthesis of the chemokine MCP-1 was similar between the two conditions, appearing to escape the “proofreading” mechanism (Hlavacek, Redondo, Metzger, Wofsy, & Goldstein, 2001; Z. J. Liu et al., 2001b; C. Torigoe et al., 1998). This finding suggested that a branched signaling pathway exists downstream of FcεRI whose intermediate signaling messengers quickly become independent of the need for a continued ligand-receptor complex (Gonzalez-Espinosa et al., 2003b). Interestingly, a more recent similar study on bone marrow-derived mast cells revealed that high affinity antigen led to enhanced degranulation, leukotriene B4 synthesis, TNF-alpha, IL-6, and IL-13 production. Conversely, lower affinity antigen led to greater production of the chemokines CCL2, CCL3, and CCL4. Moreover, activation of mast cells in vivo with high affinity antigen led to increased neutrophil recruitment, while activation with lower affinity antigen led to increased monocyte/macrophage accumulation. A mechanistic explanation for these differences in responses has been postulated as lower affinity antigen leading to enhanced activation of Fgr (a Src family kinase) and LAT2 (an adaptor protein), versus higher affinity antigen leading to better recruitment of Syk and LAT1 (R. Suzuki et al., 2014). Since the majority of these studies have been carried out in cell lines or mast cells, it is unclear whether these mechanisms behave similarly in primary basophils.

Aside from the high affinity IgE receptor, basophils can also be activated through numerous other mechanisms. Cytokines, in particular, are known to stimulate effector function and prime basophils to become more sensitive to FcεRI-mediated stimulation. As mentioned earlier, IL-3 is the most well-known cytokine for enhancing basophil responses. In the late 1980’s, IL-3 was observed to significantly enhance degranulation of basophils upon IgE/FcεRI-mediated activation (Hirai et al., 1988; MacDonald, Schleimer, Kagey-Sobotka, Gillis, & Lichtenstein, 1989; Schleimer et al., 1989). IL-3 alone can also induce the secretion of certain cytokines such as IL-4 and IL-13, or synergistically enhance secretion of these cytokines in the presence of

IgE/FcεRI stimulation (Gibbs et al., 1996; D. MacGlashan, Jr. et al., 1994; Sullivan et al., 2011). In this way, IL-3 secreted from Th2 cells in the inflamed tissue may not only enhance survival of basophils, but also enhance their effector function. In addition to IL-3, the cytokines IL-33, IL-25, and IL-18 are also able to stimulate basophils. IL-33 is an allergy-associated cytokine, normally localized within the nucleus of fibroblasts, epithelial cells, and endothelial cells. Upon cellular injury, it is released into the extracellular environment, with some reports proposing an active mechanism of release as well (Gordon et al., 2016; Roan, Obata-Ninomiya, & Ziegler, 2019). Like IL-3, IL-33 can potently enhance cytokine secretion and degranulation of basophils (Pecaric-Petkovic, Didichenko, Kaempfer, Spiegl, & Dahinden, 2009; Salter et al., 2016; Smithgall et al., 2008; Suzukawa et al., 2008). Similarly, IL-25 derived from specific epithelial cells, such as intestinal tuft cells, can induce cytokine secretion by basophils (Salter et al., 2016; von Moltke, Ji, Liang, & Locksley, 2016)., and bone marrow-derived basophil-like cells respond to IL-18 from activated monocytes by producing IL-4 and IL-13 (Kroeger, Sullivan, & Locksley, 2009; Rathinam, Vanaja, & Fitzgerald, 2012; Yoshimoto et al., 1999). However, purified human basophils appear unresponsive to IL-18 stimulation, despite normal expression of the IL-18 receptor, suggesting that certain preconditions may first need to be met in order for IL-18 to effectively stimulate basophil activity (Pecaric-Petkovic et al., 2009). While some cytokines help promote basophil effector function, others have a suppressive effect. Basophils taken from mice infected with *Litomosoides sigmodontis* were hyporesponsive to IgE-mediated activation. Infection correlated with increased IL-10 in the blood, and injection of IL-10 into naive mice could phenocopy the basophil hyporesponsiveness (Larson et al., 2012). Taken together, the cytokine milieu clearly has a drastic impact on regulating basophil effector function.

In addition to signals from cytokines, basophil activation and function is also modulated in response to a wide range of other stimuli. Basophils can respond to activated-complement (C3a and C5a) and proteases, which are commonly associated with allergens and helminths (Ali,

2010; Eglite et al., 2000; Phillips, Coward, Pritchard, & Hewitt, 2003; Rosenstein, Bezbradica, Yu, & Medzhitov, 2014; Webb et al., 2019). Basophils also express many pattern recognition receptors such as toll like receptors, NOD-like receptors, formyl peptide receptors, and C-type lectin receptors. In theory, basophils would be able respond to a wide range of pathogenic stimuli through these pattern recognition receptors (Lundberg et al., 2016; Steiner et al., 2016). Allergic inflammation often occurs at barrier sites where microbial colonization and entry commonly take place, such as the skin or mucosal tracts. Furthermore, many ecto-parasites (e.g. ticks) can be vectors for microbial pathogens, and some endoparasites (e.g. helminths) host bacteria as a form of symbiosis. Since basophils participate in allergic inflammation, including inflammation directed toward many parasites, many physiological opportunities exists for basophils to be stimulated by products not often thought to be involved in allergy. How basophils may actually protect against microbial pathogens is an area that still requires further investigation; one recent report showed a protective role for basophils in a model of sepsis, possibly through the secretion of TNF-alpha (Piliponsky et al., 2019). Cell-contact associated signals have also been implicated in enhancing the effector function of basophils. Co-culture of activated T cells has been demonstrated to induce IL-4 production in basophils. While supernatant of activated T cells induced IL-4 production in basophils through an IL-3-dependent mechanism, blocking IL-3 in co-culture of basophils and T cells was insufficient to prevent T cell-mediated production of IL-4 in basophils. When basophils and activated T cells were separated through a transwell insert, basophils produced significantly less IL-4 than when both cells were cultured together (Sullivan et al., 2011). In a separate study the cell contact-associated receptor, Notch-2, was implicated in mediating numerous transcriptional changes in basophils. IL-3 or IL-33 activated basophils and basophils derived from mice infected with *Trichuris muris* upregulated expression of Notch-2. Suppression of downstream Notch signaling reduced cytokine production in purified basophils after IL-3 or IL-33 stimulation. Though the data in this report suggests that Notch can act in a basophil intrinsic manner, other cells may provide

Notch-ligand signals to promote basophil function in a contact-mediated manner (Webb et al., 2019).

In addition to expressing the high affinity receptor for IgE, basophils also express both the activating and inhibitory receptors for IgG. Both mouse and human basophils express the low affinity, inhibitory IgG receptor, Fc gamma receptor 2B (FcγRIIB). Human basophils express the activating Fc gamma receptor 2A (FcγRIIA), while mice express Fc gamma receptor 3 (FcγRIII) (Cassard, Jonsson, Arnaud, & Daeron, 2012). IgG, most likely in the form of immune complexes (conglomerated form of IgG antibodies and antigen), would be able to bind to the Fcγ receptors on basophils, and potentially amplify or inhibit the downstream response. More specifically, the signaling chains of FcγRIIA and FcγRIII contain ITAM activating motifs, while the signaling chain of FcγRIIB contains ITIM inhibitory motifs. Activation of FcγRIIB leads to the phosphorylation of its ITIM regions, resulting in the recruitment of SH2 domain-containing 5-inositol phosphatases (SHIP1 and SHIP2) and/or SH2 domain containing tyrosine phosphatases (SHP1 and SHP2). By dephosphorylating inositol phosopholipids and tyrosyl-phosphorylated proteins, these phosphatases (primarily SHP-1/SHIP-1) effectively shut down IgE or IgG-mediated basophil activation when activated in parallel (Bruhns et al., 2000; D'Ambrosio, Fong, & Cambier, 1996; Famiglietti, Nakamura, & Cambier, 1999; Hof, Pluskey, Dhe-Paganon, Eck, & Shoelson, 1998; Kepley et al., 2000; T. Kimura et al., 1997; Leung & Bolland, 2007; Mahajan et al., 2014; Malbec et al., 2016; Waterman & Cambier, 2010). Taken together, basophils are capable of responding to a wide variety of signals, both activating and inhibitory, to respond with optimal effector capacity.

Techniques for Determining the Function of Basophils In Vivo

Despite extensive literature investigating basophil activation and mediators *in vitro*, only recently have techniques been developed to study the role of basophils *in vivo*. The initial and primary method for testing basophil function *in vivo* had been enabled through the development of monoclonal antibodies (mAbs) that could target basophils for depletion or isolation. However, interpretations from studies using these antibody-mediated techniques have since been called into question because of reliability concerns inherent to the monoclonal antibodies or antibody targets used. The most common monoclonal antibody used for basophil depletion and isolation is the clone MAR-1, which targets the high affinity IgE receptor surface chain FcεR1α on basophils and mast cells (Perrigoue et al., 2009; Sokol et al., 2008; Yoshimoto et al., 2009). Another commonly used mAb is the clone Ba103, which binds to the activating receptor CD200R3, also highly expressed on basophils (Kojima et al., 2007; Obata et al., 2007). However, we now know there are important caveats to *in vivo* mediated depletion and isolation of basophils using these monoclonal antibodies. First, both MAR-1 and Ba103 have the capacity to activate basophils and mast cells through receptor crosslinking (Hubner et al., 2011; Kojima et al., 2007; C. Ohnmacht et al., 2010; Pellefigues et al., 2019). This antibody-mediated activation of basophils and mast cells prior to the priming steps of the adaptive immune system may result in the unintended release of effector mediators or even unresponsiveness to future activation. Second, both MAR-1 and antibodies targeting CD200R3 may bind cell populations beyond basophils such as DCs, suggesting the possibility of bystander cell modulation or depletion (Duriancik & Hoag, 2014; Hammad et al., 2010; Sato et al., 2009). Our lab recently discovered that while MAR-1 was thought to be specific to the FcεR1α chain, it surprisingly bound DCs, monocytes, and macrophages even in FcεR1α-deficient mice, revealing non-specificity. We determined that MAR-1, in fact, also binds to both FcγRI and FcγRIV (X. Z. Tang, Jung, & Allen, 2019). Functionally, the injection of MAR-1 led to a far greater reduction in

the allergic response (eosinophil recruitment and Th2 cell numbers) compared to Ba103, despite similar levels of basophil depletion in a house dust mite-induced model of lung inflammation (Hammad et al., 2010). As will be discussed later, reports using genetic methods of basophil depletion also could not recapitulate many of the findings that antibody-depletion methods had previously revealed. Third, adoptive transfer experiments to test the sufficiency of basophils was accomplished through prior isolation of bone marrow-derived basophils using MAR-1 (and sometimes DX5, an anti-CD49b mAb) (Perrigoue et al., 2009; Sokol et al., 2008; Yoshimoto et al., 2009). It was later revealed that these basophil cultures derived from bone marrow incubated with IL-3 were highly contaminated with MAR-1$^+$ DCs (Hammad et al., 2010). Therefore antibody-mediated purification of basophils in these settings may have had a high propensity to be contaminated by DCs. Altogether, understanding the *in vivo* role of basophils using antibody-based methods has long been hampered by cross-reactive monoclonal antibodies and a lack of reliable basophil-specific surface markers.

More recently, genetic methods of basophil depletion (or functional manipulation) have been employed to more effectively test the function of basophils in vivo. One genetically modified mouse strain that has been employed to deplete basophils are the Bas-TRECK mice. These mice were generated by inserting DTR under the control of a 3' enhancer after the last exon of IL-4, which the authors believed to be specific to basophils (Sawaguchi et al., 2012). Aside from the Bas-TRECK mice, the more common genetic element used to deplete basophils has involved the mast cell protease 8 (*Mcpt8*) gene. Although termed "mast cell protease," *Mcpt8* is primarily expressed in mouse primary basophils (Lunderius & Hellman, 2001; Poorafshar, Helmby, Troye-Blomberg, & Hellman, 2000). Interestingly, three groups independently generated basophil-deficient mice which took advantage of the *Mcpt8* gene. The Karasuyama lab generated the *Mcpt8*-DTR mice, which express the human diphtheria toxin receptor (DTR) and GFP following an internal ribosome entry site (IRES) inserted into the 3' untranslated region

of *Mcpt8*, allowing for the specific ablation of basophils upon administration of diphtheria toxin (DT) (Wada et al., 2010).The Voehringer lab generated *Mcpt8*-Cre mice through bacteria artificial chromosome (BAC)-mediated genome integration of 5-7 copies of *Cre-recombinase* (Cre) under the expression of *Mcpt8* regulatory elements. In these mice, basophils are depleted through constitutive Cre overexpression which leads to non-specific recombination events of cryptic loxP sites and ultimately cell death (C. Ohnmacht et al., 2010). Finally, the Locksley lab generated Basoph8 mice, which was accomplished by inserting a reporter cassette sequence that included the yellow fluorescent protein (YFP), IRES, and Cre at the start site of the endogenous *Mcpt8* gene. Breeding Basoph8 mice to *Rosa-26*-Floxed-stop-DTA mice leads to the specific expression of Cre, excision of the stop codon upstream of the diphtheria toxin gene, the expression of diphtheria toxin A, and ultimately basophil cell death (Sullivan et al., 2011). It is also worth noting that the Karasuyama lab recently developed an additional mouse strain that appear similar to the Basoph8 mice, termed *Mcpt8*^{iCre}, that possess an improved Cre (iCre) in replace of the first exon of *Mcpt8*. These mice were bred to IL-4 floxed mice (Il4$^{fl/fl}$) to test the function of basophil-derived IL-4 by specifically removing IL-4 from basophils (Shibata et al., 2018b; Yamanishi et al., 2020).

Although the *Mcpt8*-mediated method of depletion has appeared much more specific than antibody mediated depletion, it has not been without its caveats. Certain progenitor populations also appear to express *Mcpt8* to a lesser extent than basophils (Hachem et al., 2018). This has complicated the use of *Mcpt8*-DTR mice, as injection of high dose of DT leads to depletion of these progenitor populations, including granulocyte-macrophage progenitors (GMPs), monocyte-dendritic cell progenitors (MDPs), and common dendritic cell progenitors (CDPs), leading to a subsequent reduction in granulocytes generated independently from basophils. Nonetheless, these mice were not entirely compromised as injection of lower doses of diphtheria toxin still led to effective basophil depletion without compromising progenitor cell

survival. *Mcpt8-Cre* mice were lineage traced by crossing to a Cre-inducible red fluorescent reporter leading to a majority of peritoneal mast cells and approximately 15% of peripheral T cells, DCs, NK cells, and eosinophils with fluorescent expression, suggesting transient expression at some progenitor stage (C. Ohnmacht et al., 2010). Nonetheless both *Mcpt8*-Cre and Basoph8 (with DT or DTR genes) mice have minimal changes in mast cells and other circulating leukocyte populations, presumably due to an insufficient expression of Cre recombinase, which is tied to *Mcpt8* expression (C. Ohnmacht et al., 2010; Pellefigues et al., 2019; Sullivan et al., 2011).

Controversy Regarding the Role of Basophils in Enhancing Adaptive Immunity

The critical mediators involved in priming an allergic adaptive immune response have long been of great interest. *In vitro*, Th2 cell differentiation can be induced by stimulating naïve T cells through CD3, CD28, and IL-4 whereas B cell activation and IgE class switch recombination can be achieved by stimulating naïve B cells through CD40 and IL-4. However, *in vivo*, the exact signals as well as the cells delivering them have been less clear. Historically, DC's have been considered the primary cells responsible for naïve T cell activation by inducing T cell receptor signaling through antigen presentation on major histocompatibility complex (MHC) and activation of CD28 through the expression of its ligands B7-1 and B7-2 (Lambrecht et al., 2000; van Rijt et al., 2005). Through this interaction, naïve T cells become activated and polarize toward effector subsets such as Th2 and Tfh cells. Tfh cells are thought to provide CD40L and cytokines such as IL-4 to B cells, inducing B cells to undergo class switch recombination. IL-4 has often been regarded as a critical cytokine *in vivo* for allergic adaptive responses. While the importance of IL-4 for IgE production *in vivo* has been consistently observed, conflicting evidence exists regarding the necessity of IL-4 on the generation of Th2 responses (Finkelman et al., 1988; Kim et al., 2013; Kopf et al., 1993; Kuhn, Rajewsky, & Muller, 1991; Liang et al.,

2011; Noben-Trauth et al., 1997; Robinson et al., 2017; Shimoda et al., 1996; van Panhuys et al., 2008; Voehringer et al., 2006). The idea that IL-4 might be critical for Th2 responses *in vivo* had in part been presumed because of the requirement for IL-4 in inducing Th2 cell differentiation *in vitro* (Le Gros, Ben-Sasson, Seder, Finkelman, & Paul, 1990; Shimoda et al., 1996; Swain, Weinberg, English, & Huston, 1990). Some studies had demonstrated a reduction of some Th2-associated cytokines in re-stimulated T cells from the draining lymph node of IL-4-deficient or IL-4R knockout mice infected with *N. brasiliensis* (Barner, Mohrs, Brombacher, & Kopf, 1998; Kopf et al., 1993; Noben-Trauth et al., 1997). Later reports would demonstrate a more complex role for IL-4 as it pertains to the development of Th2 responses (Forbes, van Panhuys, Min, & Le Gros, 2010; Jankovic et al., 2000; Liang et al., 2011; K. Mohrs et al., 2005; Prout et al., 2018; van Panhuys et al., 2008; Voehringer et al., 2006). In particular, immunized or parasite-infected mice deficient in IL-4, IL-4R, or Stat6 displayed no or only minor differences in IL-4$^+$ T cells in the lymph node (thought to primarily be Tfh) (Liang et al., 2011; Prout et al., 2018; van Panhuys et al., 2008; Voehringer et al., 2006). Additionally, Stat6 deficient and control mice also displayed similar numbers of IL-13 producing Th2 cells in the peripheral tissue (Liang et al., 2011). A reduction in the number of T cells expressing high GATA3 (master transcription factor for Th2 cells) or T cells reporting IL-4 transcript was observed in the peripheral tissue of some immunized Stat6 or IL4/13 deficient mice, suggesting that IL-4 or IL-13 may contribute toward Th2 cell maturation (Liang et al., 2011; M. Mohrs, Shinkai, Mohrs, & Locksley, 2001; Prout et al., 2018; Voehringer et al., 2006). Based on the controversial hypothesis that IL-4 is required for Th2 cell development, multiple studies sought to determine the cellular source of initial IL-4 production in lymph nodes. (Mowen & Glimcher, 2004).

In vivo and in vitro, basophils are known to be a major source of IL-4, which as previously mentioned, had been hypothesized to be critical for initiating allergic adaptive responses (Bensasson et al., 1990; Kuhn et al., 1991; Min et al., 2004). Furthermore, *in vitro* culture of

basophils with naïve T cells was found to stimulate Th2 differentiation (Oh, Shen, Le Gros, & Min, 2007). In several studies in 2008-2010 basophils were also reported to promote Th2 responses *in vivo*. The first seminal study revealed that exposure to certain stimuli such as the protease papain (from papaya) could lead to the recruitment of basophils into the draining lymph node. Of critical importance, injection of the mAb MAR-1 to deplete basophils was reported to cause loss of IL-4 mRNA expressing T cells (thought to be Th2 cells) in the lymph node after immunization using papain (Sokol et al., 2008). Further studies suggested that basophils were critical antigen presenting cells to T cells, while dendritic cells were not required. In these experiments, co-culture of bone marrow derived basophils with antigen and T cells promoted Th2 differentiation *in vitro*. Moreover, depletion of dendritic cells using CD11c-DTR bone marrow chimeras did not affect IL-4 production from restimulated T cells taken from draining lymph nodes of immunized mice (Sokol et al., 2009). Additional studies using mAbs for depletion and isolation of basophils further implicated these cells in promoting other Th2-associated responses, IgE and IgG1 production, B cell memory, plasma cell survival, protection against helminth infection, and even CD8 T cell priming (Charles, Hardwick, Daugas, Illei, & Rivera, 2010; Charles et al., 2009; Denzel et al., 2008; Kim, Shen, & Min, 2009; Perrigoue et al., 2009; Rodriguez Gomez et al., 2010; H. Tang et al., 2010; Torrero et al., 2010; Yoshimoto et al., 2009). Taken together, these studies provided exciting in vivo evidence that basophils could promote adaptive immunity.

More recent evidence suggests, however, that basophils are not critical initiators of allergic adaptive immune responses. This conclusion has arisen in part over the concerns about the reliability of methods used in many of the original articles suggesting basophil importance, as well as new techniques for basophil manipulation which have had contrasting results. First, as previously mentioned, the primary method of basophil depletion in these studies used mAbs which were later revealed to target and potentially deplete or activate cells other than basophils

(Duriancik & Hoag, 2014; Hammad et al., 2010; Hubner et al., 2011; Kojima et al., 2007; Sato et al., 2009; X. Z. Tang et al., 2019). Second, bone marrow-derived basophils were shown to be highly contaminated with differentiated dendritic cells, making interpretation of co-culture experiments or adoptive transfer of bone marrow-derived “basophils” inconclusive (Hammad et al., 2010). Third, follow-up studies testing the role of DC’s using alternative methods of depletion found them to in fact be necessary for Th2 responses. It was reasoned that the lack of effect from dendritic cell depletion in the previous studies may have been due to incomplete depletion of DCs (Hammad et al., 2010; C. Ohnmacht et al., 2010). Fourth, basophils within the lymph node showed little evidence that would functionally suggest their involvement in enabling naïve T cells to differentiate into Th2 cells. Specifically, basophils could not be found to produce IL-4 protein or meaningfully interact with T cells in the lymph node (Sullivan et al., 2011). Fifth, and most important, experiments using genetic methods of basophil depletion could not recapitulate any effects that basophils were previously demonstrated to have on primary adaptive immune responses. Th2 responses were not statistically different in mice genetically depleted of basophils compared to controls upon immunization using papain, *S. mansoni* eggs, *N. brasiliensis*, dead *N. brasiliensis* and alum, or ovalbumin and alum (Kim et al., 2013; C. Ohnmacht et al., 2010; Sullivan et al., 2011). Taken together, basophils seem unlikely to be critical for promoting allergic adaptive immunity.

It is less clear whether in certain circumstances, such as in the periphery or during a memory response when basophils can respond to antigen through IgE, if basophils could in fact enhance a T cell or B cell response. Indeed, one report using a genetic depletion of basophils had revealed that basophils could accelerate the Th2 response during a memory response to *H. polygyrus* (C. Schwartz, Turqueti-Neves, et al., 2014). In a memory setting, if basophils are recruited into the lymph node, these cells would have captured antigen-specific IgE and thus would be capable of activation through allergen re-exposure, thereby releasing IL-4 and other

potential mediators. Given that basophils have been found to enhance Th2 differentiation and IgE B cell differentiation in vitro, it seems plausible that under certain circumstances, basophils could similarly enhance T or B cell differentiation during a memory response in vivo (Gauchat et al., 1993; Oh et al., 2007). Some evidence also opens up the possibility that basophils may influence T and B cell responses within inflamed peripheral tissues. Using two-photon time lapse imaging, our lab had revealed that basophils could actually interact with T cells for relatively long durations in the lung after helminth infection (Sullivan et al., 2011). Interaction of T cells at these sites may be facilitated by the low level display of MHC II on the surface of basophils, which appears to arise through trogocytosis from nearby DCs rather than endogenous expression and antigen processing (Miyake et al., 2017). Ultimately though, whether basophils can directly accelerate memory responses in the lymph node or enhance T or B cell function in peripheral tissue has yet to be determined.

Alternative Role for Basophil Entry into the Lymph Node – Barrier Defense

While basophil entry into lymph nodes has been clearly observed in numerous conditions, the primary role of basophil recruitment into the lymph node has remained a mystery. Experimentally, a wide range of stimuli lead to the recruitment of basophils into the lymph node. These include the cysteine proteases papain (from papaya) and bromelain (from pineapple), allergic dermal irritation through calcipotriol (MC903), parasite infection with *N. brasiliensis*, *H. polygyrus*, and *Trichinella spiralis*, and exposure to other allergens such as house dust mite (Giacomin et al., 2012; Hammad et al., 2010; S. Kim et al., 2010; Leyva-Castillo, Hener, Michea, et al., 2013; C. Schwartz, Turqueti-Neves, et al., 2014; Sokol et al., 2008; Sullivan et al., 2011). The accumulation of basophils into lymph nodes has primarily been hypothesized to enhance the adaptive immune response. Nonetheless, numerous reports using genetic methods of basophil depletion have shown minimal impact on the adaptive immune response, despite the

accumulation of basophils into lymph nodes. While it remains plausible that basophils directly impact the adaptive immune response within lymph nodes under certain circumstances, as will be explored more in later chapters of this dissertation, we hypothesize that basophil accumulation is part of a coordinated defensive response to protect against and eliminate potential threats (i.e. parasites) that have entered into the lymph node.

Conceptually, lymph nodes are reasonable sites of immune defense. Interstitial fluid that is not reabsorbed at capillary beds is alternatively collected into a series of lymphatic channels. Fluid within these lymphatic channels must ultimately pass through multiple lymph nodes before collecting into the right lymphatic duct or thoracic duct (left lymphatic duct), which empty back into the blood through the subclavian veins (Choi, Lee, & Hong, 2012; Randolph, Ivanov, Zinselmeyer, & Scallan, 2017). Upon disruption of physical barriers, invasive pathogens that are not contained at the immediate site of entrance have the potential to drain into lymphatic channels, and ultimately gain entrance to the bloodstream, where they have greater motility and access to more nutrients. Therefore, prevention of pathogen access to the blood is crucial for preventing more serious infection as well as in preventing potentially fatal immunological syndromes such as in the case of sepsis. Lymph nodes are strategically interspersed along lymphatic channels, ensuring that virtually all material within the lymphatic fluid, including foreign antigen and pathogens, is processed. In this way, lymph nodes not only act as sites of antigen sampling, but potentially as barriers of defense against invading pathogens.

Numerous experimental reports have supported the notion that lymph nodes can act as additional sites of barrier defense. Aside from T cells and B cells, many innate cell populations also reside within the lymph node. The subcapsular and medullary sinuses are noticeably interspersed with macrophages that can capture a variety of particles that enter into the sinuses, including bacteria and viruses. In this way, these macrophages act as a sort of “flypaper”,

controlling the spread of many pathogens that might otherwise escape the lymph node (Bogoslowski & Kubes, 2018). In contrast to sinus-lining macrophages, many other cell populations reside directly below the sinuses and/or at interfollicular regions and are positioned to be able to respond rapidly to pathogens, foreign antigen, and/or inflammatory signals (often from the macrophages) that travel further into the lymph node parenchyma. Many of the cells that reside below the sinus have been identified to be innate-like lymphoid cells such as gamma delta T cells, innate-like CD8 T cells, and natural killer T cells (Kastenmuller, Torabi-Parizi, Subramanian, Lammermann, & Germain, 2012). Gamma delta T cells in particular have actually been reported to survey the sinus, entering and then exiting back into the parenchyma, which may allow greater opportunity to recognize potential threats (Y. Zhang et al., 2016). Coordinated mechanisms exist to effectively respond to various pathogens. For example, exposure of sinus macrophages to bacteria and viruses was shown to induce inflammasome activation and the secretion of the cytokines IL-1 beta and IL-18 (Kastenmuller et al., 2012). Underlying innate lymphoid cells subsequently responded to these inflammatory signals by producing key anti-viral or anti-bacterial cytokines such as interferon-gamma and IL-17 (Kastenmuller et al., 2012; Y. Zhang et al., 2016). Moreover, macrophage activation can also lead to the re-localization and/or recruitment of additional cells such as natural killer cells, neutrophils, and plasmacytoid dendritic cells (Iannacone et al., 2010; Kastenmuller et al., 2012). Perturbations of these defensive mechanisms, for example, by depletion of macrophages, NK cells, or neutrophils; or blockade of cytokines, such as interferon gamma and IL-18; result in more severe viral or bacterial infection. Uncontrolled infection then results in further spread of pathogens to the blood and other organs (Bogoslowski, Butcher, & Kubes, 2018; Farrell et al., 2016; Junt et al., 2007; Kastenmuller et al., 2012; Rosenheinrich, Heine, Schmuhl, Pisano, & Dersch, 2015). In the case of vesicular stomatitis virus (VSV), depletion of lymph node macrophages allows the virus to gain access to peripheral neurons in the lymph node, resulting in viral spread to the central nervous system (Iannacone et al., 2010). Numerous DC

populations also exist within the lymph node, some lining the sinuses and having direct exposure to lymph-borne pathogens. These sinus-associated DCs provide an avenue for antigen presentation leading to T cell priming and may also enable rapid activation of localized innate-like or memory T cell populations (Gerner, Torabi-Parizi, & Germain, 2015). It is clear that the lymph node is capable of much more than just the maturation of the adaptive immune response; it is an organ with powerful and local defensive capability as well.

Human and animal studies have also revealed lymph nodes to be common sites of entry and infection in many parasitic infections. Within the filarial family of parasitic roundworms are those that dwell within the lymphatic system. Infection by lymphatic filarial worms can lead to lymphatic filariasis, a disease which is estimated to affect 120 million people. This disease results from damage to the lymphatic system and is characterized by inflammation of the lymphatic channels and lymph nodes, lymphedema, and in more severe instances hydrocele and elephantiasis. The most epidemiologically significant of these parasitic worms include *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori,* which are all transmitted through mosquitos (Chandy, Thakur, Singh, & Manigauha, 2011; Jungmann, Figueredo-Silva, & Dreyer, 1991). Numerous reports have revealed the presence of these parasites within lymph nodes of infected hosts, often in association with profound eosinophilic infiltration (Baird & Neafie, 1988; Elenitoba-Johnson, Eberhard, Dauphinais, Lammie, & Khorsand, 1996; Figueredo-Silva & Dreyer, 2005; Jungmann et al., 1991; Mak, 2012). These observations reveal that the lymph node is a potential site of infection and that allergic inflammation is commonly mounted against such organisms. Mouse models have further demonstrated the importance of the immune system in protection against these parasites. Though wild-type mice are highly resistant to infection by *B. malayi* and *Brugia pahangi* (a similar species to *B. malayi*), immunodeficiency of T cells, B cells, eosinophils, macrophages, IL-4, IL-5, or IFN-γ results in worsened infection (Babu, Ganley, Klei, Shultz, & Rajan, 2000; Babu, Shultz, Klei, & Rajan, 1999; Morris, Evans,

Larsen, & Mitre, 2013; Nelson, Greiner, Shultz, & Rajan, 1991; Paciorkowski, Shultz, & Rajan, 2003; B. Rajan, Ramalingam, & Rajan, 2005; T. V. Rajan et al., 2002; Ramalingam, Ganley-Leal, Porte, & Rajan, 2003; Ramalingam, Porte, Lee, & Rajan, 2005; Spencer, Shultz, & Rajan, 2001, 2003; Suswillo RR 1981; Suswillo, Owen, & Denham, 1980; Turner et al., 2018). Despite only a select few species of helminths that dwell for long periods of time within the lymphatic system, many other species have nonetheless been observed to migrate through the lymphatics prior to reaching distal sites. One study investigated the migratory behavior of 6 different filarial parasites and found that all of them could be recoverable to some degree from lymph nodes (though also including associated vessels) a few days after infection, despite many species not primarily persisting in the lymphatics (Bain et al., 1994). Non-filarial parasites have also been found to infect or transit through lymph nodes. Infection of mice with cercariae from *S. mansoni*, a type of flatworm that ultimately reside within blood vessels, led to an inflammatory response within the draining lymph nodes that involved eosinophils and associated parasites (Lozzi, Machado, Gerken, & Mota-Santos, 1996). Moreover, another study that had euthanized macaques as part of a viral pathogenesis project found the presence of large *Baylisascaris procyonis* larvae within mesenteric and pancreatic lymph nodes in more than half of the animals studied. Rather than entering from the skin, these parasites are ingested and then undergo aggressive migration to various organs (Gozalo et al., 2008). Given the propensity for even helminths to reside in or pass through lymph nodes, it would be understandable for defensive measures to exist within the lymph node to respond to these pathogens.

To my knowledge, no animal studies have mechanistically investigated the protective response to helminths or allergic inflammation occurring from within the lymph node. Despite numerous reports in humans demonstrating the lymph node to be a potential site of allergic inflammation, recent mouse studies about the recruitment of allergy-associated cells, such as eosinophils and basophils, have primarily focused on the potential for these cells to promote the adaptive

immune response (H. Chen, Thompson, Aguilera, & Abbondanzo, 2004; Elenitoba-Johnson et al., 1996; Figueredo-Silva & Dreyer, 2005; Jungmann et al., 1991; Sokol et al., 2008; Svensson et al., 2011; Swanson, Manivel, Valen, & Mesa, 2017; van Rijt et al., 2003). Yet, very little evidence has been found to strongly support the hypothesis that these innate cells directly enhance T and B cell responses from within the lymph node. We alternatively hypothesize that the recruitment of innate allergy-associated cells into the lymph node is part of a coordinated response to protect the body from pathogens that traverse the lymphatic system. Later chapters of this dissertation will further expand on the potential for basophils to participate in this defensive response at the lymph node.

The Contribution of Basophils in Experimental Models of Allergic Inflammation

Basophil biology has been perhaps most well-studied in experimental models of allergic skin inflammation. In many ways, this ought not to be surprising since, historically, basophil recruitment has been highly associated with allergic skin inflammation. A seminal study by the laboratory of Dr. Karasuyama revealed basophils to be involved in a form of delayed skin inflammation that depends upon IgE. In this model, transgenic mice that express IgE specific for 2,4,6-trinitrophenol (TNP) and mice that had been passively sensitized with anti-TNP IgE were injected with ovalbumin haptenated to TNP. This resulted in three phases of skin inflammation: 1) an immediate inflammatory response occurring within 1 hour, 2) a late response occurring within 6-10 hours, and 3) a prolonged period of intense ear swelling that peaked around day 4. This final phase that lasted for days was termed “chronic allergic inflammation” (CAI), though this should not be conflated with chronic inflammation in patients that develops over time with repeated allergen exposure (Mukai et al., 2005). This experimentally induced CAI was associated with an increase in neutrophils, eosinophils, and basophils. T and B cells were dispensable for ear swelling and eosinophil recruitment in this model, but were critical for

generating IgE. Moreover, mast cells, though important for the early and late phase responses, were unnecessary for the third phase of intense and prolonged inflammation. Injection of $CD49b^+$ bone marrow cells, enriched for basophils, restored inflammation in mice deficient for FcεRI signaling (Fc receptor gamma knockout). Importantly, the depletion of basophils using the Ba103 (anti-CD200R3) antibody prevented the CAI response (Mukai et al., 2005; Obata et al., 2007). Since the publication of this important study, the dependency of CAI (and related studies) on basophils have been further confirmed using numerous genetic depletion methods (Cheng et al., 2015; Egawa et al., 2013; Mukai et al., 2012; C. Ohnmacht et al., 2010; Yamanishi et al., 2020).

Following the discovery that basophils contribute to allergic skin inflammation, the scientific community sought to determine the mechanisms by which basophil-derived products orchestrate this response. Mast cell protease-11 (MCPT-11) was found to contribute to basophil-dependent allergic skin inflammation, as MCPT-11 knockout mice had reduced skin thickening and inflammatory cell recruitment in response to antigen challenge (Iki et al., 2016). Injection of mast cell protease-8 (MCPT-8), which is very highly and specifically expressed in basophils, was observed to induce transient skin inflammation, suggesting MCPT-8 may also contribute to basophil-dependent inflammation (Tsutsui et al., 2016). In a model of chemically-induced skin inflammation and tick infection, basophil-derived histamine promoted epidermal hyperplasia and skin thickening, though whether this was as a result of a direct or indirect effect on the epithelium was unclear (Hayes et al., 2020; Tabakawa et al., 2018). In addition to proteases and histamine, interleukin-4 (IL-4) has also been investigated for its impact on allergic skin inflammation. The role of IL-4 has been especially intriguing since it seems to contribute to both pro-inflammatory and anti-inflammatory pathways. In IgE-dependent models of skin inflammation, IL-4 and downstream signaling through STAT6 were found to be required for eosinophil recruitment (Cheng et al., 2015; Hashimoto, Satoh, & Yokozeki, 2015). One study

found that, following IgE-induced activation, basophils adhered to the vascular endothelium and secreted IL-4. Upon stimulation by IL-4, the vascular endothelium upregulated VCAM-1, enabling eosinophil recruitment into the skin (Cheng et al., 2015). Additional studies have suggested that IL-4 may also induce the vascular endothelium or macrophages to produce eotaxin-2 (CCL24) to enhance extravasation of eosinophils into the tissue (Bitton et al., 2020; Diny et al., 2016; Eberle, Radtke, Nimmerjahn, & Voehringer, 2019; Lee et al., 2020; Turner et al., 2018). Furthermore, an *in vitro* study found that IL-4 could enhance the production of eotaxin-1 (CCL11) in fibroblasts, which might also promote eosinophil migration (Nakashima et al., 2014). Other than eosinophil recruitment, some reports have implicated basophil-derived IL-4 in polarizing macrophages to become alternatively activated. These polarized macrophages contribute to helminth defense and may also dampen excessive inflammation (Egawa et al., 2013; Obata-Ninomiya et al., 2013). One study suggested that basophil-derived IL-4 could induce an alternative, suppressive state in recruited inflammatory monocytes. In support of this, preventing inflammatory monocyte recruitment or IL-4 signaling in inflammatory monocytes enhanced skin inflammation (Egawa et al., 2013). Altogether, the skin has proved a useful site for unraveling basophil-dependent mechanisms.

Surprisingly, evidence for the role of basophils in modulating allergic inflammation in organs other than the skin is limited. As will be further discussed in the next section, basophils appear to contribute to helminth defense in the intestines. In addition, one food allergy model in the intestine had utilized Bas-TRECK mice to deplete basophils but the conclusions of this study have not been confirmed using a *Mcpt8*-associated depletion method. In this report, intestinal inflammation was induced by first sensitizing skin to allergen and then injecting allergen intragastrically (Noti et al., 2014). Compared to controls, Bas-TRECK mice displayed reduced intestinal symptoms of food allergy as well as a large reduction in intestinal mast cell numbers, Th2 cytokine expression, and IgE levels. Mice that were made genetically deficient for a 3’

proximal enhancer of IL-4 (the same element used in Bas-TRECK mice) to specifically silence IL-4 production in basophils demonstrated similarly reduced responses as Bas-TRECK mice (Hussain et al., 2017). Basophils have also been studied in allergic inflammation of the esophagus. An allergic condition in patients called eosinophilic esophagitis leads to the progressive destruction of the tissue, which is believed to be due to eosinophil-derived compounds. In a mouse model of this disease, depletion of basophils with Ba103 prior to allergen challenge dampened the resulting inflammatory response. Although Ba103 treatment can affect Th2 priming, this study depleted basophils with Ba103 at a time point beyond the initial priming phase, providing better evidence that basophils may in fact be involved in this disease (Noti et al., 2013). Though basophils are known to be recruited to the lung in human asthmatics and in allergic mouse models, thus far no study has clearly demonstrated a role for basophils in allergic lung inflammation. In both an ovalbumin/alum model of allergic lung inflammation and a model of parasitic infection of the lung by *N. brasiliensis* the primary immune response was unaltered by basophil-depletion. In the secondary response to *N. brasiliensis,* Th2 cell and eosinophil accumulation into the lung as well as serum IgE levels were unaltered in basophil depleted mice compared to controls; however, the number of worms found in the small intestine was increased in basophil depleted mice (C. Ohnmacht et al., 2010). The increased worm count of basophil deficient mice in the small intestine suggests the possibility that basophils may control parasite infection at the lung, but another report had demonstrated a protective role for basophils in the skin during a secondary infection (Obata-Ninomiya et al., 2013). It has yet to be shown whether basophils could impact other type-2 associated responses in the lung, such as the polarization of macrophages to become alternatively activated, particularly in the secondary response. Interestingly, basophils were found to be involved in a model of elastase-induced chronic obstructive pulmonary disease (COPD) (Shibata et al., 2018a). This disease is not normally thought to be associated with type 2 inflammation. Nonetheless, in the model tested, basophil-derived IL-4 was found to be important

for inducing recruited monocytes to secrete matrix metaoproteinase-12 (MMP12), leading to emphysema. Finally, basophils have long been suspected to be involved in anaphylaxis because of their production and release of histamine upon degranulation. Yet, studies of IgE-mediated anaphylaxis have failed to find any impact of basophil depletion (C. Ohnmacht et al., 2010; Tsujimura et al., 2008). Interestingly, one report showed an IgG-mediated form of anaphylaxis involved basophils; however, a later study could not validate that finding (C. Ohnmacht et al., 2010). Overall, our understanding of the impact of basophils on allergic inflammation is just beginning to emerge. New genetic tools will likely greatly expand upon this knowledge in the near future.

The Contribution of Basophils in Protection against Parasites

Basophils have been shown to be protective against a variety of parasites. More recently, basophil-mediated anti-tick responses have garnered attention, resulting in significant new mechanistic insights. Tick bites have long been associated with basophil accumulation, thus implicating basophils in protection against the parasite. One of the oldest studies testing the protective nature of basophils was done in guinea pigs. Guinea pigs were depleted of basophils using rabbit anti-serum derived against enriched guinea pig basophils. Guinea pigs were first infested with ticks, and then some animals were injected with the basophil-depleting serum. Basophil depletion increased guinea pig weight and the yield of ticks recovered, indicating a lesser protective immune response in guinea pigs without basophils (Brown, Galli, Gleich, & Askenase, 1982). More recently, using mice that were genetically depleted of basophils, the laboratory of Dr. Karasuyama reaffirmed the finding that basophils could protect against ticks during a memory response. Using a fluorescent reporter, basophils were clearly shown to densely accumulate around tick mouthpieces. Moreover, protection against ticks and basophil accumulation were found to be dependent on IL-3 derived from memory T cells. Fc receptor

signaling, presumably through FcεRI, and basophil-derived histamine, through the H1 receptor, were also required for tick protection. It was reasoned that in this context, basophil-derived histamine may be more important than mast cell-derived histamine since basophils can migrate and localize in much greater density around the tick mouthpart and thereby provide a much higher concentration of histamine to the target site. Interestingly, the injection of histamine at the tick infestation site during a primary response provided protection against the parasite which corresponded to a significant increase in epidermal hyperplasia compared to PBS injection at the tick site. This increase in epidermal thickening may provide a greater physical barrier against the tick mouthpiece, thereby preventing effective feeding. (Hayes et al., 2020; Ohta et al., 2017; Tabakawa et al., 2018; Wada et al., 2010).

Basophils have also been shown to be protective against a number of parasitic helminths. Though basophils provide less obvious benefit against a primary infection of *N. brasiliensis* or *H. polygyrus*, they enhance protection during memory responses (C. Ohnmacht et al., 2010; C. Schwartz, Turqueti-Neves, et al., 2014). Interestingly, in the primary response against *N. brasiliensis*, it was found that when IL-4 was deleted from both T cells and basophils protection was significantly worse than when IL-4 was only removed from T cells (Sullivan et al., 2011). This suggests a redundant, but potentially important contribution of basophils to IL-4 production. In the memory response against *H. polygyrus*, basophils were protective in a manner that depended upon IL-4 and IgE (C. Schwartz, Turqueti-Neves, et al., 2014). Additionally, basophils accelerated the Th2 response. One can imagine that against motile parasitic helminths, which often traverse through multiple tissue barriers, the speed of the immune response may be critical in effectively impacting the parasite prior to its migration and maturation. Indeed, in one study, basophils were found to be protective against *N. brasiliensis* in a memory response at the immediate site of infection, the skin. In this context, basophils were thought to quickly migrate to the skin and secrete IL-4, polarizing macrophages toward an anti-parasitic

phenotype. Killing of larvae at the skin thereby led to reduced lung migration of the parasite and reduced resulting lung damage. (Obata-Ninomiya et al., 2013). *Strongyloides ratti* and *Strongyloides venezuelensis* are helminths that penetrate the skin and ultimately make their way into the intestines. Basophil-deficiency resulted in worse intestinal infection during the primary response. During the secondary response, both control and basophil-deficient mice displayed near-complete protection from parasites infecting the intestines; however, whether basophils could play a greater role in the very early response at the initial skin infection site (as described above) was not investigated (Mukai, Karasuyama, Kabashima, Kubo, & Galli, 2017; Reitz et al., 2016; Reitz, Brunn, Voehringer, & Breloer, 2018). Finally, basophils were not observed to protect against *S. mansoni* or *Litomosoides sigmodontis* (Hartmann et al., 2018; C. Schwartz, Oeser, et al., 2014). However, in the *S. mansoni* study, basophils were not investigated for protection during the memory response. In the *L. sigmodontis* study, although the memory response was investigated, the analysis was performed a month after re-infection. At this time point, no parasites were recoverable, presumably because of killing at a much earlier time point. It is therefore unclear whether basophils were important for early killing upon reinfection. Overall, significant evidence suggests that basophils protect against parasitic helminths, particularly during memory responses, though this protective capacity may not apply to all parasites.

It is worth noting that basophils likely provide protection against more than just multicellular parasites. Given that basophils possess microbial recognition receptors such as toll-like receptors, it ought not to be surprising that basophils can provide some degree of protection against smaller pathogens, like bacteria. In a study by Dr. Galli's laboratory, basophils were found to be protective in a cecum-ligation and puncture model of sepsis. Basophils migrated into the peritoneum in surprisingly high numbers, led to reduced bacteria in the peritoneal cavity and blood, and protected against fatality. Interestingly, basophil-depletion resulted in much lower

peritoneal concentrations of TNF-alpha, a cytokine important for bacterial clearance, thereby implicating basophil-derived TNF-alpha in anti-bacterial protection (Piliponsky et al., 2019).

Summary

Overall, significant progress has been made in understanding basophil biology and the contribution of basophils to allergic inflammation. Of particular importance, basophils have been revealed as a primary effector cell for IgE/FcεRI-mediated responses. Exposure of basophils to allergen results in the crosslinking of allergen-specific IgE/FcεRI on the cell surface that induce signaling events which promote a wide variety of responses such as degranulation, cytokine production, and adhesion. The characteristics of the IgE-antigen interactions that promote these disparate responses in basophils need further investigation. Basophils have also been demonstrated to enhance allergic inflammation through IgE/FcεRI by recruiting inflammatory cells and polarizing macrophages to become alternatively activated. Through IgE/FcεRI, basophils can also contribute to early protection against parasitic organisms upon re-exposure to the host. Intriguingly, basophils have been observed to enter into lymph nodes in response to certain stimuli, but the functional impact of basophil accumulation in the lymph node remains unknown. These findings have advanced the field and provoked further investigation. The research contained in this dissertation builds upon these earlier findings and specifically seeks to answer three important questions:

1.) How crucial is the affinity of IgE and cognate antigen to the activation of basophils?

2.) How do basophils promote an inflammatory response on a spatial-temporal level?

3.) What role do basophils have after recruitment into the lymph node?

Chapter 2: The Affinity of IgE for Antigen is a Primary Determinant of Basophil Degranulation

Abstract

IgE may induce potent inflammatory responses in allergic diseases and parasitic infections through the high affinity receptor FcεRI expressed on mast cells and basophils. However, the characteristics of the molecular interactions between antigen and IgE that trigger cellular activation remain incompletely defined. Here we considered the impact of the affinity of the binding interaction between IgE and cognate antigen on the responses of murine basophils. We observed that high affinity antigens promoted enhanced basophil degranulation and IL-4 production compared to low affinity antigens, even when the relative antigen concentrations were adjusted to achieve equivalent equilibrium binding. We reveal that despite differential activation based on affinity, basophils in mouse lymph nodes readily captured both low and high affinity antigens and remained motile, suggesting a potential antigen transport role for basophils independent of degranulation and IL-4 production. The local administration of a high affinity antigen, but not a low affinity antigen, promoted systemic basophil activation at distal sites and increased susceptibility to systemic anaphylaxis. We propose that the affinity of IgE for antigen is a primary determinant of basophil activation and is a crucial factor in susceptibility to severe IgE-mediated responses such as anaphylaxis.

Introduction

The production of IgE specific for allergens in atopic individuals may result in hypersensitivity reactions upon allergen exposure and is thought to be a major driver of allergic diseases. These reactions are elicited by the IgE-mediated activation of mast cells and basophils, which results in the release of granules containing potent inflammatory mediators, such as histamine, as well

as the secretion of several cytokines and chemokines (Kraft & Kinet, 2007a). While allergen-specific IgE can be detected in the serum of atopic patients, indicating that sensitization has occurred, this does not always predict the extent of clinical reactions to allergens (Sampson & Ho, 1997). In addition to the specificity and quantity of IgE, the characteristics of the molecular interactions between IgE and allergen may determine whether functional activation of mast cells and basophils occurs in vivo.

The IgE-mediated activation of mast cells and basophils occurs through the Fc receptor FcεRI (Kraft & Kinet, 2007a). This high affinity receptor binds free secreted IgE molecules, such that allergen-specific IgE molecules are pre-bound to mast cells and basophils prior to allergen exposure. Extensive studies of IgE-mediated responses and FcεRI over the past few decades have provided deep insights into how this receptor is activated and the roles of numerous downstream signaling components (Kraft & Kinet, 2007a; Suzuki, Scheffel, & Rivera, 2015). Biological experiments coupled with detailed mathematical modeling have revealed that the characteristics of the interaction of antigen with cognate antigen-specific IgE and FcεRI are major determinants of the types of responses that ensue. In particular, the fraction of FcεRI molecules occupied by IgE, the clonality of the IgE antibodies, the valency of the antigen, the spacing of the epitopes, the affinity of the binding between the antigen and IgE, and ultimately the degree and kinetics of cross-linking and aggregating FcεRI are all thought to be important characteristics that continue to be actively investigated (Hlavacek, Redondo, Wofsy, & Goldstein, 2002; Holowka, Sil, Torigoe, & Baird, 2007; Kagey-Sobotka, MacGlashan, & Lichtenstein, 1982; Suzuki et al., 2015; Wilson, Oliver, & Lidke, 2011) .

The affinity of the binding between antigen and IgE has been reported to affect both the sensitivity to the amount of antigen or IgE as well as the nature of the cellular responses downstream of FcεRI. Some responses, including degranulation and the production of some

cytokines, are favored in the context of high affinity binding, consistent with the kinetic proofreading model, in which a receptor must stay clustered with ligand for a sufficient period of time to induce downstream signaling (Goldstein et al., 2008). However, exceptions to this model have also been noted, particularly with regard to the production of some chemokines and cytokines, which can occur with relatively low concentrations of antigen or lower affinity binding of antigen to IgE (Gonzalez-Espinosa et al., 2003a; Z. J. Liu, H. Haleem-Smith, H. Chen, & H. Metzger, 2001a). A recent elegant study of mast cells revealed that in a system in which the antigen-IgE binding affinity differed over three orders of magnitude, the activation of distinct downstream signaling molecules provided a basis for these different cellular responses (Ryo Suzuki et al., 2014). Sophisticated quantitative imaging studies and perturbations of the proximal kinase Syk have given new insights into the mechanism by which IgE affinity for antigen may result in differential signaling through FcεRI and distinct functional outcomes (S. L. Schwartz et al., 2017). Suggesting that IgE affinity is relevant to in vivo FcεRI-mediated responses in humans, the affinity of IgE for antigen was reported to correlate better with skin prick test responses than the amount of antigen-specific IgE (Pierson-Mullany et al., 2002).

The affinity of the interaction between antigen and IgE determines the equilibrium binding, such that in a lower affinity interaction, a greater concentration of antigen or IgE is needed to achieve the same equilibrium binding as a higher affinity interaction. Several studies have adjusted antigen and IgE concentrations and measured the degree of FcεRI phosphorylation (Z. J. Liu et al., 2001a; Ryo Suzuki et al., 2014; Chikako Torigoe, John K. Inman, & Henry Metzger, 1998), yet the relationship between equilibrium binding and cellular activation has not been directly assessed. Equilibrium binding may be relevant to consider in light of allergic responses. Typically, upon allergen exposure at specific body sites, mast cells and basophils will be activated in these regions and trigger local inflammatory responses, which is thought to have evolved as a protective mechanism against parasites and insects. However, in some

individuals, even with minute amounts of local allergen exposure, systemic activation of mast cells and/or basophils occurs, resulting in life-threatening anaphylaxis. The ability for a small amount of locally-administered antigen to elicit systemic responses suggests a high affinity interaction with IgE, yet this has not been tested.

Here we directly measured equilibrium binding in the context of an examination of the impact of the affinity of antigen-IgE interaction on the cellular responses of mouse basophils. Despite extensive studies of basophils from human patients and in mouse models, the functions of this cell type have been controversial and remain incompletely understood (Voehringer, 2013). We reveal that both basophil degranulation and the production of IL-4 are highly dependent on the affinity of the antigen-IgE interaction when we control for equivalent equilibrium binding. In the context of lower affinity interactions, basophils captured and transported antigen in vivo without degranulating. Conversely, higher affinity interactions led to basophil degranulation even at distal sites when antigen was administered locally. We further demonstrate that in the context of a high-affinity interaction, the administration of antigen to a local site was sufficient to induce systemic anaphylaxis.

Results

To test the role of the affinity of the antigen-IgE interaction on basophil responses, we loaded mouse basophils in vivo with an extensively studied IgE monoclonal antibody, SPE-7, that has 100-500-fold higher affinity for the hapten DNP compared with NP (James & Tawfik, 2003). In order to directly measure equilibrium binding of antigen to IgE, we chemically conjugated DNP or NP to the fluorescent protein allophycocyanin (APC) and measured binding by flow cytometry. As the extent of oligomerization of an antigen is also known to be an important determinant of responses mediated by FcεRI, we generated conjugates with different hapten densities, designated as “lo”, “med”, or “hi”. Of note, $DNP_{hi}APC$ exhibited low fluorescence,

presumably due to fluorescence quenching by the DNP groups, and thus was not further characterized. By adjusting the relative concentrations of these reagents, we found that we could achieve comparable equilibrium binding to basophils loaded with anti-DNP IgE, for example as shown with DNP_{med}APC and NP_{med}APC (Fig. 2.1A). Strikingly, however, at equivalent equilibrium binding, the higher affinity ligand DNP_{med}APC led to much higher basophil degranulation compared with NP_{med}APC, as revealed by flow cytometric evaluation of CD63 translocation to the cell surface (Fig. 2.1B). Over an extensive titration to assess antigen equilibrium binding (Fig. 2.1C) and degranulation (Fig. 2.1D), we were able to establish a comparison of degranulation vs antigen binding (Fig. 2.1E). At a comparable range of equilibrium binding over several independent experiments (Fig. 2.1F), DNP_{med}APC led to much more extensive basophil degranulation compared with NP_{med}APC. We excluded the possibility that this difference was due to the conjugation efficiency and oligomeric state of the reagents, as DNP_{lo}APC gave similar responses as DNP_{med}APC, and NP_{hi}APC gave similar responses to NP_{med}APC. We confirmed that binding of NP_{med}APC and NP_{hi}APC to basophils was completely dependent on the presence of anti-DNP IgE, even at a higher antigen concentration of 10,000 ng/ml (Fig. 2.2A). The low valency reagent NP_{lo}APC exhibited relatively poor binding to anti-DNP IgE-loaded basophils at 10,000 ng/mL (Fig. 2.2B) and also induced minimal basophil degranulation compared with NP_{med}APC (Fig. 2.2C). A nearly 100,000 fold higher concentration of NP_{lo}APC compared to DNP_{lo}APC was required to achieve equivalent equilibrium binding to anti-DNP IgE-loaded basophils (Fig. 2.2D), yet even at this extremely high concentration of 100,000 ng/mL, NP_{lo}APC still poorly induced basophil degranulation (Fig. 2.2E). Taken together, these data indicate that in the context of multivalent antigen, the affinity for IgE, rather than the avidity and overall density of epitopes, is the primary determinant of whether basophil activation occurs. We confirmed that similar results could be observed for mast cells (Fig. 2.3), although a more pronounced ‘bell-shaped’ curve was evident for the degranulation response of mast cells (Fig. 2.3E), similar to previous reports.

We sought to confirm these findings were due to affinity rather than other potential explanations, such as the different chemical structures of NP and DNP or particular characteristics of the SPE-7 monoclonal antibody (Bax et al., 2017)}. A well-characterized IgG antibody to NP, termed B1-8, is known to bind with moderate affinity and a single mutation of Trp33 to Leu confers 10-fold higher affinity to NP (D. Allen, Simon, Sablitzky, Rajewsky, & Cumano, 1988). We generated a secreted IgE version of the B1-8 antibody with the germline variable region versus with the high affinity Trp33->Leu mutation, allowing us to study responses of basophils loaded with IgE molecules of different affinity but using the same antigen, NP_{med}APC. A measurement of equilibrium antigen binding compared with a titration of antigen concentration confirmed an approximately 10-fold difference in affinity of the two different IgE variants for this antigen (Fig. 2.1G). When we considered degranulation over this titration range (Fig. 2.1H), we again observed that at equivalent equilibrium binding (Fig. 2.1I and 1J), the higher affinity IgE led to substantially greater basophil degranulation compared with the lower affinity IgE. Thus, a 10-fold difference in the affinity of the antigen binding to IgE resulted in distinct basophil degranulation responses when we controlled for equilibrium binding of the antigen. These results also further confirm that the NP_{med}APC reagent is capable of eliciting robust degranulation in the context of a high affinity IgE interaction, further validating the distinct results observed with DNP_{med}APC and NP_{med}APC in the above experiments with SPE-7.

We next considered the role of equilibrium binding and antigen affinity for IgE in the production of IL-4 by basophils. We evaluated IL-4 production using a human CD2 (hCD2) surface reporter that has been extensively characterized. A previous study of bone marrow-derived mast cells had shown that IL-4 production could be elicited at lower antigen concentrations and reduced receptor occupancy compared with degranulation (Gonzalez-Espinosa et al., 2003b), suggesting that IL-4 production may represent an exception to the kinetic proofreading model. A

discordance between the signaling requirements for IL-4 secretion versus degranulation was also noted in a study of a mast cell line with mutant Syk (S. L. Schwartz et al., 2017). Studies of human basophils had shown some difference in the optimal concentrations of anti-IgE to elicit IL-4 versus histamine release, although it was suggested that this might be due to desensitization having a greater effect on the IL-4 response (D. MacGlashan, Jr. et al., 1994; Schroeder, MacGlashan, Kagey-Sobotka, White, & Lichtenstein, 1994). Similar to our degranulation results in Fig. 2.1, we found with anti-DNP IgE loaded murine basophils that with antigen concentrations adjusted for equivalent equilibrium binding (Fig. 2.4A), the high affinity antigen DNP_{med}APC led to much greater basophil expression of the IL-4 reporter than the low affinity antigen NP_{med}APC (Fig. 2.4B). Of note, although SPE-7 is known to be a highly cytokinergic antibody (Bax et al., 2017; Kawakami & Kitaura, 2005), at the doses of SPE-7 administered there was little background basophil IL-4 production in the absence of antigen (Fig. 2.4B), thus this was not a concern for the interpretation of our studies. Again, over an extensive antigen titration range in which we measured equilibrium antigen binding (Fig. 2.4C) and expression of the IL-4 reporter (Fig. 2.4D), we found that at equivalent equilibrium binding (Fig. 2.4E), the high affinity antigen DNP_{med}APC led to substantially greater IL-4 expression than the low affinity antigen NP_{med}APC (Fig. 2.4F). Similar results were again obtained comparing DNP_{lo}APC and NP_{hi}APC (Fig. 2.4C-E), confirming that antigen affinity for IgE rather than the density of epitopes were the primary determinants of IL-4 production.

Based on these results with primary murine basophils in cell culture, we next considered how the affinity of IgE for antigen would affect basophil responses in mice. Basophils have been reported to be recruited to lymph nodes in mice in the context of certain stimuli, such as cysteine proteases, the topical vitamin D analog calcipotriol (MC903), and helminth parasite infection, yet the function of basophils in the lymph nodes in these settings remains unclear (Sohee Kim et al., 2010; Leyva-Castillo, Hener, Michea, et al., 2013; Caspar Ohnmacht et al.,

2010; Perrigoue et al., 2009; Sokol et al., 2008; Sullivan et al., 2011). To recruit basophils to the facial lymph nodes, we immunized mice by subcutaneous injection of the cysteine protease papain together with topical application of calcipotriol to the ears. To test the impact of IgE-mediated binding to high and low affinity ligands, we loaded the basophils with the anti-DNP IgE antibody intravenously, and then administered the high affinity antigen DNP_{med}APC or the low affinity antigen NP_{med}APC subcutaneously in one ear for drainage to the facial lymph node on that side, but not on the contralateral side which served as a control.

We found that adjusting to a 5-fold higher concentration of NP_{med}APC than DNP_{med}APC led to equivalent equilibrium binding on basophils in the draining lymph node (Fig. 2.5A). Similar to our results in cell culture, we found that at equivalent equilibrium binding, the high affinity ligand DNP_{med}APC resulted in substantially greater basophil degranulation compared with the lower affinity ligand NP_{med}APC in the draining lymph node (Fig. 2.5B). We also considered the dynamics of basophils labeled with DNP_{med}APC vs NP_{med}APC within the draining lymph node. Time-lapse imaging by two-photon microscopy revealed similar aggregates of DNP_{med}APC and NP_{med}APC were captured by the basophils, which remained motile and transported the antigen (Fig. 2.5C). These data suggest that in vivo, basophils readily capture and transport both high and low affinity antigens, but preferentially degranulate in response to high affinity antigens.

Although the antigen was administered locally, basophils in the spleen were labeled with the high affinity antigen DNP_{med}APC (Fig. 2.5A), and accordingly, basophils degranulated in the spleen in response to DNP_{med}APC but not NP_{med}APC (Fig. 2.5B). A small amount of antigen reached the contralateral non-draining lymph node, where we again observed that basophil degranulation was greater with DNP_{med}APC than NP_{med}APC (Fig. 2.6). These data suggest that high affinity IgE-antigen interactions may lead to basophil degranulation at distal sites even when antigen is administered locally.

Based on the known affinities of SPE-7 anti-DNP IgE for DNP vs NP, and our own antigen titration data in cell culture (Fig. 2.1), we would have anticipated the need to inject much larger amounts of NP_{med}APC than DNP_{med}APC to achieve equivalent equilibrium binding on anti-IgE DNP-loaded basophils. When we administered a 100-fold higher concentration of NP_{med}APC than DNP_{med}APC, this local subcutaneous injection unexpectedly led to much greater NP_{med}APC binding than DNP_{med}APC binding on basophils in the draining lymph node, but as expected resulted in comparable antigen binding in a distal systemic site, the spleen (Fig. 2.5D). These results would be consistent with reduced lymphatic drainage to the draining lymph node in the context of exposure to DNP_{med}APC, presumably by the activation of mast cells in the ear. Nevertheless, the injection of 100-fold more NP_{med}APC than DNP_{med}APC allowed us to achieve equivalent systemic distribution and equilibrium binding of antigen to basophils in the spleen. Similar to our results in Fig. 2.5B in draining lymph nodes, we found that when equivalent amounts of antigen were bound by basophils in the spleen, the high affinity ligand DNP_{med}APC induced substantially greater degranulation compared with the lower affinity ligand NP_{med}APC (Fig. 2.5E).

Our findings that the local administration of limiting doses of a high affinity antigen could result in degranulation at distal sites led us to consider whether systemic anaphylaxis could be induced in this context. Previous functional studies of IgE affinity for antigen in vivo have focused on local passive cutaneous anaphylaxis responses or the systemic administration of antigen. As an indicator of systemic anaphylactic responses, we measured the rectal temperature of mice in the context of the subcutaneous injection model described above in which we achieved an equivalent systemic equilibrium binding of the lower affinity antigen NP_{med}APC compared with the high affinity antigen DNP_{med}APC in IgE-sensitized mice. We indeed observed that a temperature drop was induced by the local administration of the high

affinity ligand $DNP_{med}APC$, but not by the local administration of a 100-fold higher concentration of the lower affinity ligand $NP_{med}APC$ (Fig. 2.5F). These data indicate that a high affinity ligand for IgE, administered to a local tissue site, may induce systemic anaphylaxis.

Concluding Remarks

Taken together, our data indicate that the affinity of the binding of antigen to IgE is a major determinant of basophil activation. Here we directly measured the binding of fluorescently-labeled antigen enabling us to achieve equivalent equilibrium binding. Cell culture studies revealed that in the context of equivalent equilibrium binding, both basophil degranulation and IL-4 production were preferentially induced by a high affinity antigen-IgE binding. In vivo, basophil degranulation was also dependent on the affinity of the antigen-IgE binding interaction. However, basophils similarly captured and transported antigen in the draining lymph node regardless of affinity when we adjusted for equivalent equilibrium binding. This finding suggests that in some physiologic settings in which antigens are of lower affinity, basophils will capture and transport these antigens without undergoing degranulation. This antigen transport may reflect a potential functional role of basophil recruitment to local tissues such as the lymph node that has not yet been investigated.

Interestingly, even in the context of local administration of limiting doses of a high affinity antigen, small amounts of antigen that reached the systemic circulation were sufficient to elicit basophil degranulation at distal sites. Consistent with this finding, indications of systemic anaphylaxis were observed when a small amount of high affinity antigen was locally administered. In this experiment, presumably both mast cells and basophils may contribute to the systemic response. Our data, building on results from previous studies, indicates that the sensitivity of both mast cells and basophils to degranulate depends on the affinity of IgE for antigen even when equivalent equilibrium binding is achieved. Relatively low concentrations of a

high affinity antigen were able to elicit robust degranulation responses. Our findings provide experimental evidence that the affinity of IgE for allergens may be a major determinant of whether anaphylaxis occurs in response to small amounts of local allergen exposure. We therefore propose that assessing the affinity of IgE for allergens could help to predict whether systemic anaphylaxis will occur in individuals with allergic sensitization.

Figures

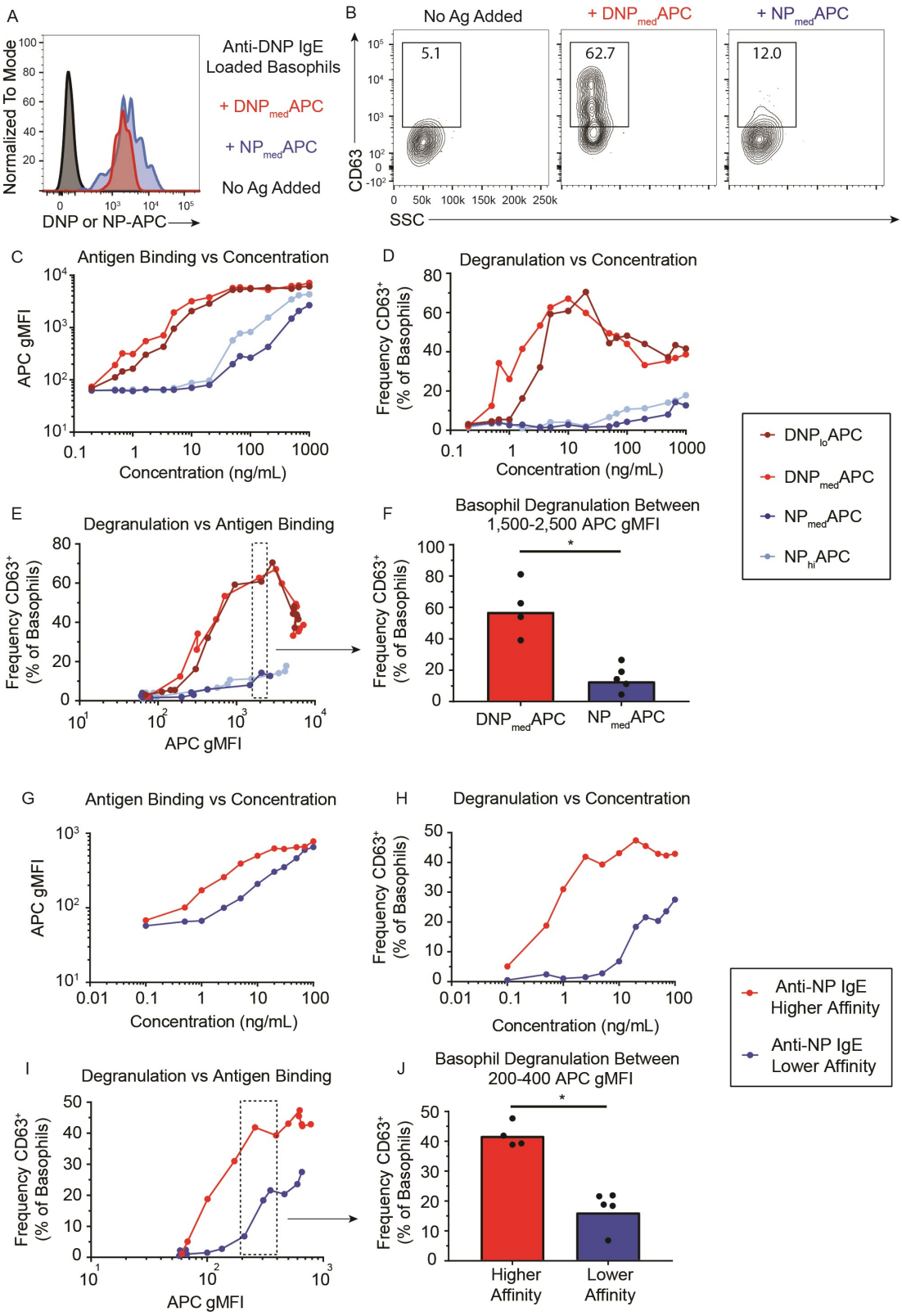
A
Normalized To Mode
DNP or NP-APC
Anti-DNP IgE Loaded Basophils
+ DNP$_{med}$APC
+ NP$_{med}$APC
No Ag Added
B
No Ag Added
+ DNP$_{med}$APC
+ NP$_{med}$APC
5.1
62.7
12.0
CD63
SSC
C
Antigen Binding vs Concentration
APC gMFI
Concentration (ng/mL)
D
Degranulation vs Concentration
Frequency CD63$^+$ (% of Basophils)
Concentration (ng/mL)
DNP$_{lo}$APC
DNP$_{med}$APC
NP$_{med}$APC
NP$_{hi}$APC
E
Degranulation vs Antigen Binding
Frequency CD63$^+$ (% of Basophils)
APC gMFI
F
Basophil Degranulation Between 1,500-2,500 APC gMFI
Frequency CD63$^+$ (% of Basophils)
DNP$_{med}$APC
NP$_{med}$APC
G
Antigen Binding vs Concentration
APC gMFI
Concentration (ng/mL)
H
Degranulation vs Concentration
Frequency CD63$^+$ (% of Basophils)
Concentration (ng/mL)
Anti-NP IgE Higher Affinity
Anti-NP IgE Lower Affinity
I
Degranulation vs Antigen Binding
Frequency CD63$^+$ (% of Basophils)
APC gMFI
J
Basophil Degranulation Between 200-400 APC gMFI
Frequency CD63$^+$ (% of Basophils)
Higher Affinity
Lower Affinity

Figure 2.1: Basophil degranulation is enhanced by higher affinity IgE-antigen interactions independent of equilibrium binding. (A-E) Basophils were first loaded with anti-DNP IgE in vivo and then splenic basophils were activated in vitro by culturing cells with high (DNP-APC) or low (NP-APC) affinity antigen for 30 min. (A) Example of equivalent equilibrium binding of 5 ng/mL DNP_{med}APC and 1000 ng/mL NP_{med}APC to basophils as assessed by flow cytometry. (B) Representative flow cytometry plots comparing basophil degranulation ($CD63^+$) among samples with equivalent DNP-APC and NP-APC binding. (C-E) The concentration of antigen was titrated over several orders of magnitude and the relationship of antigen concentration to antigen binding (C) and degranulation (D) are shown. These data were combined in (E) to show the relationship of antigen binding to degranulation. (F) Quantification of basophil degranulation at equivalent equilibrium binding of DNP-APC and NP-APC. Samples that had antigen binding resulting in 1,500-2,500 APC gMFI were compared as shown in the box in (E). (G-J) Basophils were first loaded with anti-NP IgE of higher vs lower affinity and then activated in vitro by culturing cells with NP-APC. (G) The concentration of antigen was titrated over several orders of magnitude and the relationship of antigen concentration to antigen binding (G) and degranulation (H) are shown. (J) Quantification of basophil degranulation at equivalent equilibrium binding of NP-APC. Samples that had antigen binding resulting in 200-400 APC gMFI were compared as shown in the box in (I). gMFI, geometric mean fluorescence intensity

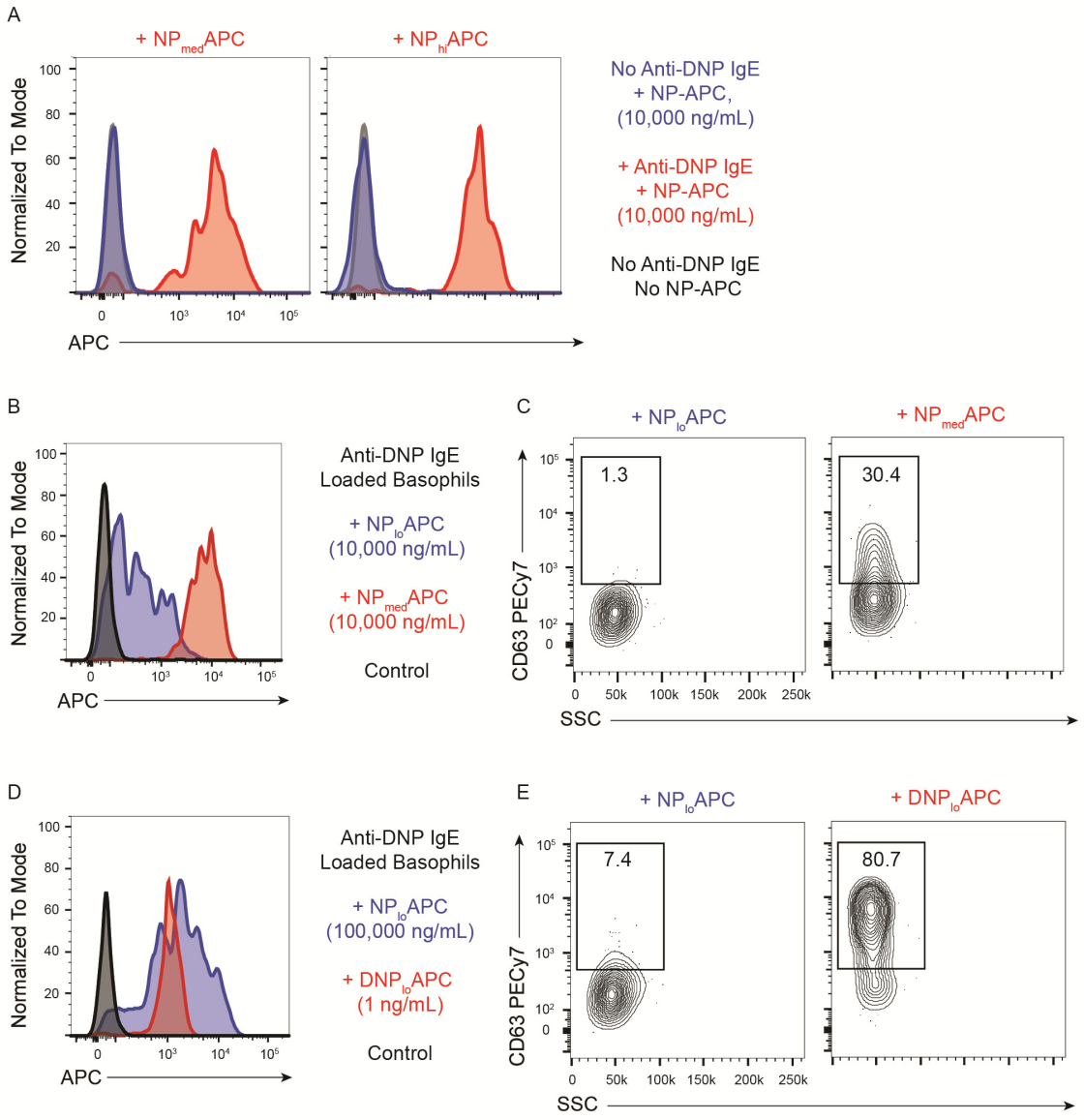

Figure 2.2: Basophil degranulation through IgE is enhanced by higher affinity antigen even when lower affinity antigen binding is comparable. Basophils were first loaded with anti-DNP IgE in vivo and then splenic basophils were activated in vitro by culturing cells with the indicated concentrations of antigens for 30 min, and then analyzed by flow cytometry for antigen binding (APC fluorescence) and degranulation (CD63$^+$). (A) Comparison of binding of NP-APC to basophils in the presence versus the absence of anti-DNP IgE, at a high concentration of $NP_{med}APC$ or $NP_{hi}APC$. (B-C) Comparison of antigen binding to basophils (B) and degranulation of basophils (C) after culturing cells with $NP_{lo}APC$ versus $NP_{med}APC$ at a high concentration. (D-E) Comparison of antigen binding to basophils (D) and degranulation of basophils (E) after culturing cells with a very high concentration of $NP_{lo}APC$ versus a low concentration of $DNP_{lo}APC$, with amounts adjusted to achieve equivalent equilibrium binding of antigen.

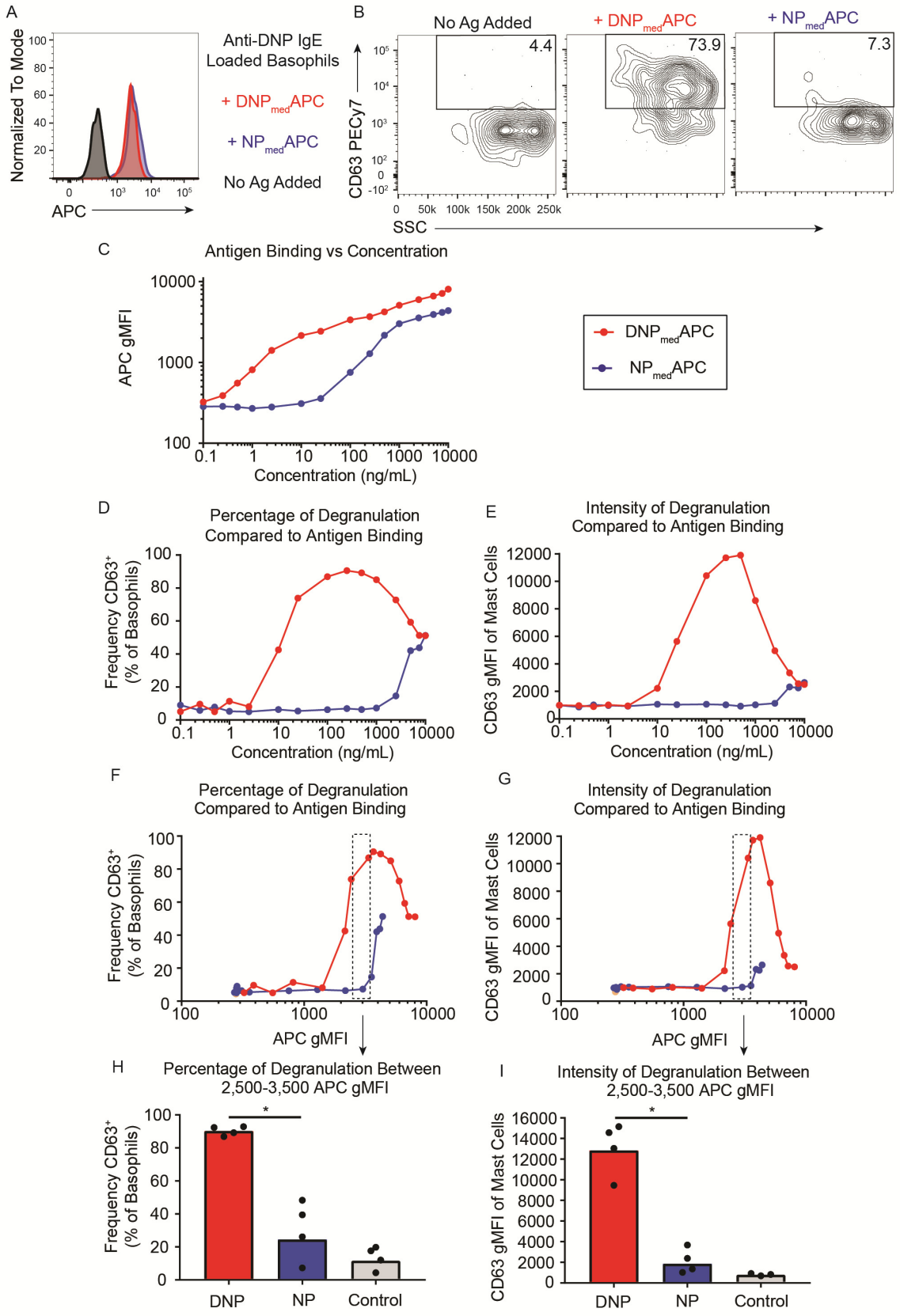
A
Normalized To Mode
APC
Anti-DNP IgE
Loaded Basophils
+ DNP med APC
+ NP med APC
No Ag Added
B
No Ag Added
+ DNP med APC
+ NP med APC
4.4
73.9
7.3
CD63 PECy7
SSC
C
Antigen Binding vs Concentration
APC gMFI
Concentration (ng/mL)
DNP med APC
NP med APC
D
Percentage of Degranulation
Compared to Antigen Binding
Frequency CD63+
(% of Basophils)
Concentration (ng/mL)
E
Intensity of Degranulation
Compared to Antigen Binding
CD63 gMFI of Mast Cells
Concentration (ng/mL)
F
Percentage of Degranulation
Compared to Antigen Binding
Frequency CD63+
(% of Basophils)
APC gMFI
G
Intensity of Degranulation
Compared to Antigen Binding
CD63 gMFI of Mast Cells
APC gMFI
H
Percentage of Degranulation Between
2,500-3,500 APC gMFI
Frequency CD63+
(% of Basophils)
DNP
NP
Control
I
Intensity of Degranulation Between
2,500-3,500 APC gMFI
CD63 gMFI of Mast Cells
DNP
NP
Control

Figure 2.3: Mast cell degranulation through IgE is enhanced by higher affinity antigen even when lower affinity antigen binding is comparable. Peritoneal mast cells were first loaded with anti-DNP IgE in vivo and then activated in vitro by culturing cells with high (DNP-APC) versus low (NP-APC) affinity antigen. (A) Example of equivalent equilibrium binding of 25 ng/mL DNP_{med}APC and 1000 ng/mL NP_{med}APC to mast cells as assessed by flow cytometry. (B) Representative flow cytometry plots comparing mast cell degranulation ($CD63^+$) among samples with equivalent DNP-APC and NP-APC binding. (C-E) The concentration of antigen was titrated over several orders of magnitude and the relationship of antigen concentration to antigen binding (C), the frequency of degranulated (CD63+) mast cells (D), and the extent (CD63 gMFI) of mast cell degranulation (E) are shown. These data were combined to show the relationship of antigen binding to the frequency (F) and extent (G) of mast cell degranulation. (H-I) Quantification of the frequency (H) and extent (I) of mast cell degranulation at equivalent equilibrium binding of DNP-APC and NP-APC. Samples that had antigen binding resulting in 2,500-3,500 APC gMFI were compared as shown in the boxes in (F,G).

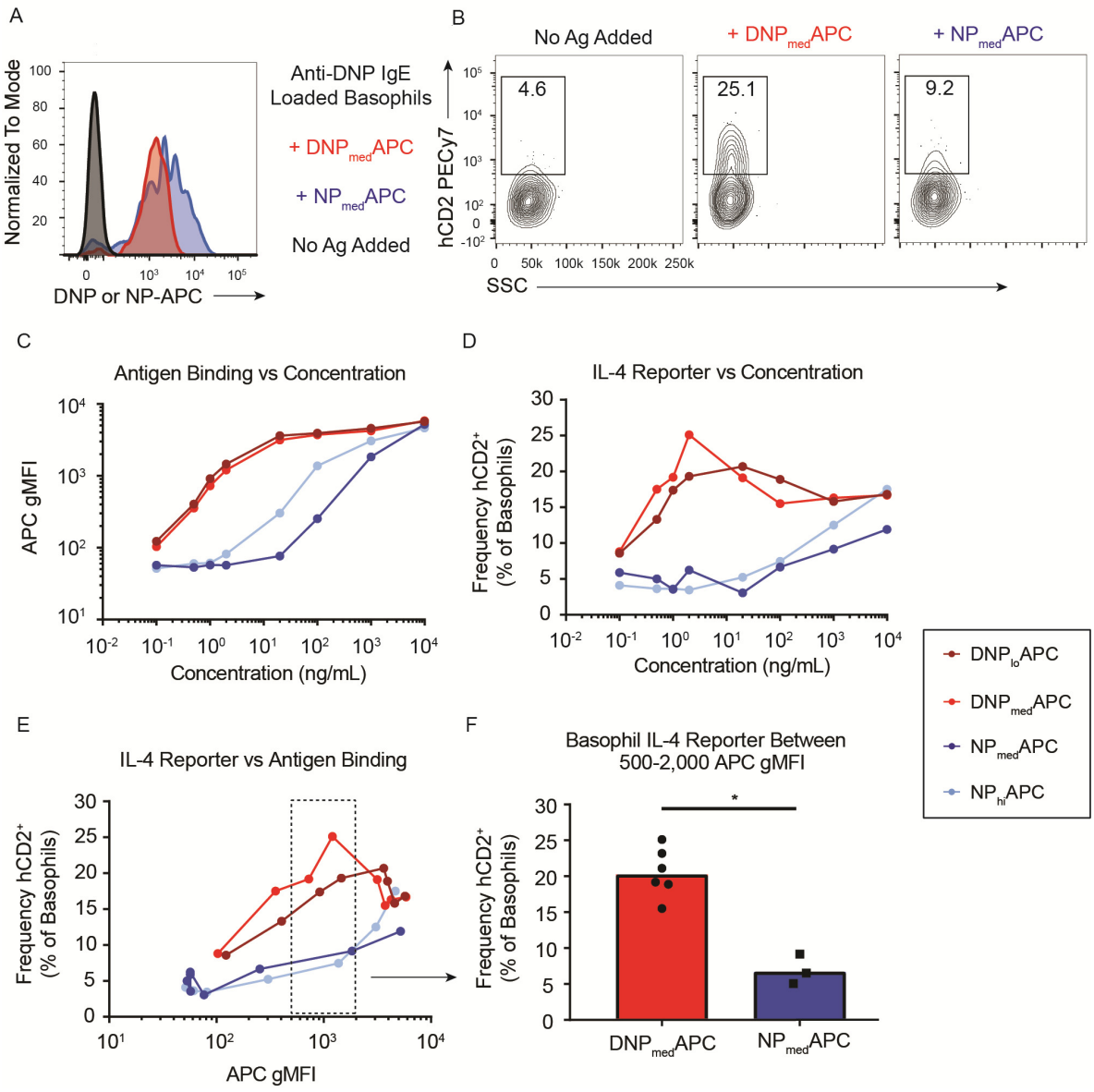

Figure 2.4: Basophil production of IL-4 is enhanced by higher affinity IgE-antigen interactions independent of equilibrium binding. Basophils from KN2 IL-4 reporter mice were first loaded with anti-DNP IgE in vivo and then splenic basophils were activated in vitro by culturing cells with high (DNP-APC) or low (NP-APC) affinity antigen for 4 h. (A) Example of equivalent equilibrium binding of 2 ng/mL $DNP_{med}APC$ and 1000 ng/mL $NP_{med}APC$ to basophils as assessed by flow cytometry. (B) Representative flow cytometry plots comparing basophil IL-4 reporter expression ($huCD2^+$) among samples with equivalent DNP-APC and NP-APC binding. (C-E) The concentration of antigen was titrated over several orders of magnitude and the relationship of antigen concentration to antigen binding (C) and IL-4 reporter expression (D) are shown. These data were combined in (E) to show the relationship of antigen binding to IL-4 reporter expression. (F) Quantification of basophil IL-4 reporter expression at equivalent equilibrium binding of DNP-APC and NP-APC. Samples that had antigen binding resulting in 500-2,000 APC gMFI were compared as shown in the box in (E).

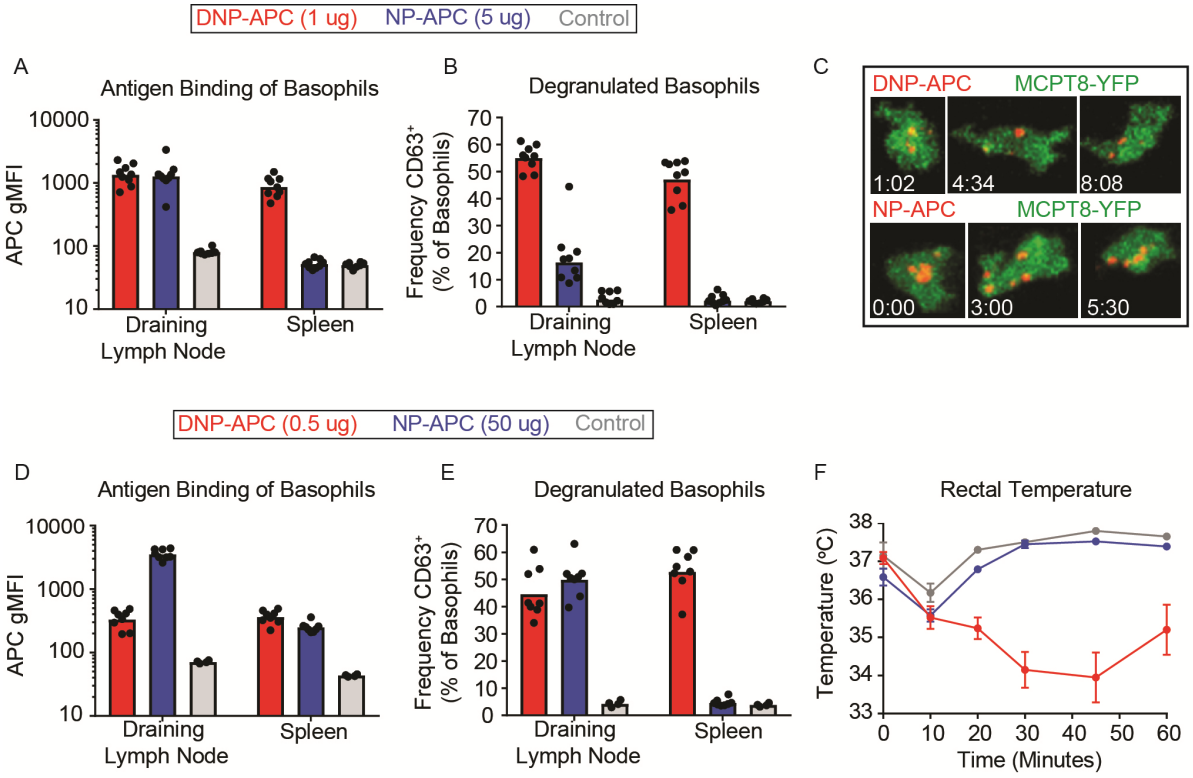

Figure 2.5: IgE affinity for antigen is a primary determinant of basophil degranulation in vivo and systemic anaphylaxis. Mice were passively sensitized with anti-DNP IgE, then ears were treated with papain and calcipotriol to recruit basophils into the draining facial lymph nodes. Three days later, one ear was injected with high ($DNP_{med}APC$) or low ($NP_{med}APC$) affinity antigen. (A) Equivalent equilibrium binding of high (DNP-APC) and low (NP-APC) affinity antigen to basophils in draining lymph nodes after the injection of 1 ug $DNP_{med}APC$ versus 5 ug $NP_{med}APC$. Binding at a distal site, the spleen, is shown for comparison (B) Comparison of basophil degranulation in tissues of mice that received 1ug DNP-APC versus 5 ug NP-APC. Controls in (A,B) are non-draining contralateral lymph nodes. (C) Time-lapse imaging by two photon microscopy showing a basophil (MCPT8-YFP+) that has captured DNP-APC (red) versus a basophil that has captured NP-APC (red) within explanted draining lymph nodes. (A) Equivalent equilibrium binding of high (DNP-APC) and low (NP-APC) affinity antigen to basophils in the spleen after the injection of 0.5 ug $DNP_{med}APC$ versus 50 ug $NP_{med}APC$. (B) Comparison of basophil degranulation in tissues of mice that received 0.5 ug DNP-APC versus 50 ug NP-APC. Controls in (D,E) are non-draining contralateral lymph nodes. (F) Systemic anaphylaxis, measured by rectal temperature over time, after the injection of 0.5 ug $DNP_{med}APC$ or 50 ug $NP_{med}APC$ injection.

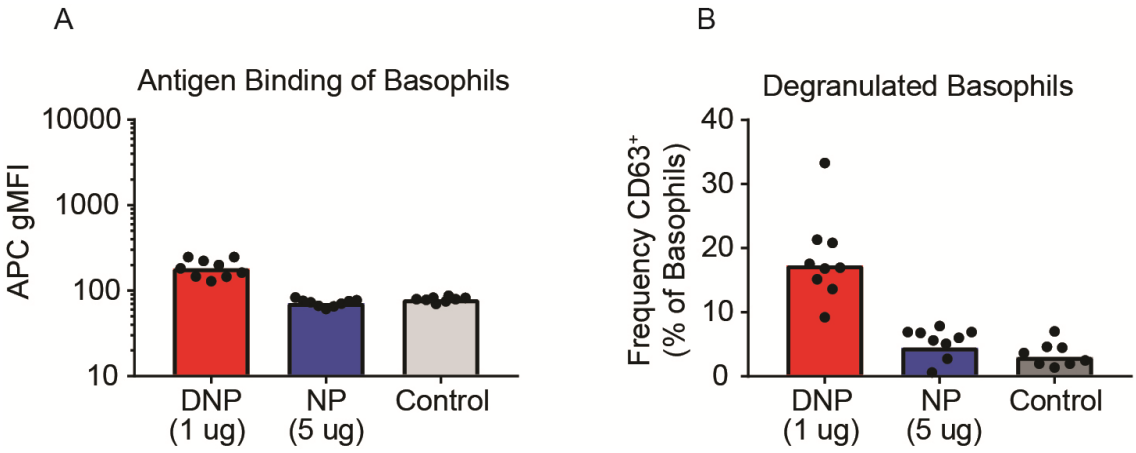

Figure 2.6: IgE affinity for antigen regulates basophil degranulation in the non-draining lymph node. In the experiment shown in Fig. 2.5A-B, the non-draining contralateral lymph node from each mouse was assessed by flow cytometry for (A) antigen binding to basophils and (B) degranulation of basophils. Data were pooled from two independent experiments.

Methods

Mice

C57BL/6J (B6, 000664) and Boy/J (B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ, 002014) mice were originally obtained from the Jackson Laboratory (Bar Harbor, Me) and bred on-site. C57BL/6NCr mice were purchased from Charles River (National Cancer Institute Model 556). C57BL/6J, C57BL/6NCr, and Boy/J were used as wild type mice for experiments that did not require the use of fluorescent reporters. Basoph8 and KN2 (K. Mohrs et al., 2005; Sullivan et al., 2011) founder mice were originally provided by the laboratory of Dr. Richard Locksley and were maintained on the B6 background. All mice were maintained in specific pathogen-free facilities, and protocols were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco.

IgE Antibodies and Antigen Haptenation

Mouse anti-dinitrophenyl (DNP) IgE monoclonal antibody (SPE-7) was purchased from Sigma Aldrich. Anti-NP monoclonal IgE was derived by cloning the heavy chain VDJ region of the NP-specific B-cell receptor in B1-8i mice (Sonoda et al., 1997). A point mutation was introduced in Trp33->Leu for the higher affinity variant. The cloned heavy chain VDJ region was then combined with the mouse secreted IgE constant region into the retroviral vector pQEF-Ceru-T2A, packaged in Phoenix-Eco cells, and then transduced into J558L cells, which express the λ1 light chain, as described (Z. Yang & Allen, 2018). The transduced cells were cultured in Corning Celline Disposable Bioreactor flasks (Fisher Scientific) and the concentrated supernatant containing the antibody was harvested. The amount of NP-specific IgE was determined by ELISA.

To activate cells loaded with DNP or NP-binding IgE, we conjugated APC (Prozyme) to the NP or DNP haptens as previously described (Z. Yang et al., 2016), based on an original method

from Michael McHeyzer-Williams (Louise J. McHeyzer-Williams & Michael G. McHeyzer-Williams, 2004). In brief, prior to the reaction, APC was first dialyzed in sodium bicarbonate (3% $NaHCO_3$ dissolved in H_2O) while the succinimidyl esters of NP (NP-Osu; Biosearch Technologies) and DNP (DNP-ε-aminocaproyl-OSu: Biosearch technologies) were dissolved in dimethylformamide. The reactive haptens were added to APC and incubated with rolling and tilting for 1-2 hours. The mixture was then passed through a Biospin 30 column (Biorad Laboratories) to perform buffer exchange into phosphate buffered saline and to remove excess, free hapten.

By calculating the moles of hapten to APC used and assuming a 20-25% reaction efficiency, we separated our reagents into three categories: hapten low (lo), medium (med), and high (hi). $DNP_{lo}APC$ and $NP_{lo}APC$ were estimated to have a hapten ratio around 1.6. $DNP_{med}APC$ and $NP_{med}APC$ were estimated to have a hapten ratio around 4.7 and 6.4 respectively. $NP_{hi}APC$ was estimated to have a hapten ratio around 12.7. We report a medium DNP hapten ratio that is slightly lower than NP because at higher ratios, DNP led to quenching of APC fluorescence.

Basophil Degranulation and IL-4 Production Assay

Splenic basophils from Basoph8 mice were loaded with antigen-specific IgE (10 µg SPE-7 or 20 µg anti-NP IgE antibodies) by retro-orbital injection. One day later, mice were sacrificed, and spleens removed. Spleens were placed in RPMI-1640 media supplemented with antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin; Gibco, Carlsbad, Calif) and L-glutamine (Gibco). Spleens were then mashed and the cell suspensions were passed through 70 micron nylon cell strainers (Falcon). Three million splenocytes in 200 uL RPMI media were aliquoted into PCR tubes (8-strip PCR Tubes, Genesee Scientific) and antigen was added. Cells were placed in a 37°C water bath for 30 minutes, washed with FACS Buffer consisting of PBS, 1 mmol/L EDTA (Gibco), and 2% FBS (Gibco), and stained with an anti-CD63 fluorescent antibody (NVG-2,

Biolegend) to measure degranulation. Flow cytometry was used to detect basophils through YFP expression and to measure CD63 and APC fluorescent intensities.

For assessments of IL-4 production, Basoph8 crossed to KN2 (IL-4 reporter) mice were used. The same procedure was used as in the basophil degranulation assay to load basophils with IgE and to isolate splenocytes. Splenocytes were aliquoted in 96 well U-bottom plates (Falcon). After adding antigen, cells were placed in a 5% CO2 incubator at 37^{o}C for 4 hours, then washed with FACS Buffer and stained with anti-human CD2 fluorescent antibody (RPA-2.10, Biolegend) for analysis by flow cytometry.

Mast Cell Degranulation Assay

Peritoneal mast cells from wild type mice were loaded with 10 ug anti-DNP IgE (SPE-7) by intraperitoneal injection. One day later, mice were sacrificed and the peritoneal space was lavaged with 5 mL PBS to extract cells in the peritoneum. The peritoneal cells were centrifuged and resuspended in 1 mL PBS. 75,000 cells in 200 µL RPMI per stimulating condition were aliquoted into PCR tubes (described above). After antigen addition, the cells were placed in a 37^{o}C water bath for 30 minutes, washed with FACS buffer, and analyzed by flow cytometry. In parallel to the stimulation of the cells, another comparable set was incubated with the same DNP or NP-APC reagents, but in FACS buffer and on ice for 30 minutes. This was to allow detection of antigen binding (APC fluorescence intensity) on mast cells since we had observed a significant loss of APC signal after normal stimulation – likely due to internalization and degradation of the antigen.

In Vivo Challenge and Anaphylaxis

Mice were immunized by subcutaneous injection of 10 ug papain (EMD Chemicals) in 20 µL PBS and the dermal application of 0.2 mM calcipotriol (Fisher Scientific) in 10 µL ethanol per

ear. Mice were also passively sensitized with 10 ug of Spe-7 IgE through retro-orbital injection. Three days after immunization, mice were challenged with a subcutaneous injection of NP_{med}APC (5ug or 50 ug) or DNP_{med}APC (0.5ug or 1ug) into one ear. To measure anaphylaxis, a rectal probe (Braintree Scientific) lubricated with Vaseline was inserted at specific time intervals to measure changes in body temperature over the course of an hour. One hour after antigen challenge, mice were euthanized and spleens, draining lymph nodes, and contralateral non-draining lymph nodes were collected for analysis. Some mice that were not used to measure anaphylaxis were given isoproterenol prior to antigen challenge for flow cytometric assessment.

Two-Photon Microscopy

Mice were immunized as described above but challenged with 10 ug of DNP_{med}APC or NP_{med}APC. After one hour, mice were sacrificed, and the draining lymph nodes removed. For imaging, the lymph nodes were placed in a heated, diffusion chamber containing oxygenated, flowing RPMI and imaged using a Zeiss LSM 7 MP indimo two-photon microscope as described in Sullivan et al., 2011.

Flow Cytometry

Three million cells were plated in 96 well U-bottom plates for flow cytometric staining. Cells were stained with antibodies for analysis by flow cytometry, as described (Z. Yang, Sullivan, & Allen, 2012). Antibodies used are listed in Table 1. Data were collected on an LSRFortessa (BD, San Jose, Calif) with FACSDiva software (BD) and analyzed with FlowJo software (TreeStar, Ashland, Ore).

Table 2.1: Antibodies for Flow Cytometry

Target	Conjugate	Clone	Vendor	Dilution Factor
CD2 (Human)	PE-Cy7	RPA-2.10	Biolegend	1:100
CD11b	BV785	M1/70	Biolegend	1:400
CD16/32 (Fc Block, TruStain FcX)	Purified	93	Biolegend	1:600
CD45	A700	30-F11	Biolegend	1:100
CD63	PECy7	NVG-2	Biolegend	1:200 (Basophils) 1:600 (Mast Cells)
CD127 (c-Kit)	PE	2B8	Biolegend	1:200
CD200R3	PE	Ba13	Biolegend	1:100
IgE	FITC	RME-1	Biolegend	1:100
Ly-6G	BV510	1A8	Biolegend	1:100
Siglec-F	BV421	E50-2440	BD Biosciences	1:100
Viability Dye	DAPI		Sigma Aldrich	1:3500
Viability Dye	eFluor780		eBioscience	1:600

*APC, allophycocyanin; FITC, fluorescein isothiocynate; PE, phycoerythrin.

Chapter 3: Basophils Promote Allergic Lymphadenitis and the Organization of Inflammatory Cell Clusters

Abstract

Exposure to a variety of stimuli leads to the accumulation of basophils in the draining lymph node. Although basophils in lymph nodes were initially proposed to act as critical initiators of type 2 adaptive immune responses, more recent reports have challenged this hypothesis. Since then, a clear role for basophils in entering the lymph node has remained elusive. Here, we investigated an alternative function for basophil accumulation into the lymph node: to orchestrate a local allergic inflammatory response. We found that immunization with chicken IgY and calcipotriol led to the accumulation of large numbers of basophils in lymph nodes. Through immunofluorescence microscopy, these basophils were observed to closely associate with dense inflammatory cells clusters comprised of eosinophils, alternatively activated macrophages, multinucleated giant cells, and a reorganized stromal cell population. The abundance of these cell clusters increased upon antigen rechallenge. Analysis of basophil-deficient mice revealed that basophils were important contributors in both inflammatory cell recruitment and cell cluster formation. Moreover, IgE-mediated activation of basophils was sufficient to promote the allergic inflammatory response in the lymph node. Basophils were observed to respond to incoming antigen by degranulating, producing IL-4, relocalizing closer to sites of antigen entry, and engaging in extended interactions with macrophages, suggesting important contributions of basophils to the generation of inflammatory cell clusters. Similar to the IgY and calcipotriol model, we observed the formation of allergic inflammatory cell clusters containing basophils in lymph nodes infiltrated by larvae of the filarial helminth, *Brugia pahangi*, revealing a physiological role for inflammatory cell cluster formation in lymph nodes in immune defense. Altogether, we propose that a primary function for basophil entry into the lymph node is

to participate in and promote a local allergic inflammatory response to protect against a perceived threat.

Introduction

In the past decade, significant progress has been made in revealing the contribution of basophils to type 2 immunity. In particular, numerous mouse models have demonstrated the in vivo capacity of basophils, as IgE effector cells and producers of cytokines, to promote a wide range of immune responses, as well as to provide defense against some parasitic organisms. In the skin, IgE-activated basophils promoted tissue swelling, epidermal hyperplasia, and leukocyte infiltration from the blood, potentially through histamine, MCPT-8 and/or MCPT-11 (Hayes et al., 2020; Iki et al., 2016; Mukai et al., 2005; Obata et al., 2007; Tabakawa et al., 2018; Tsutsui et al., 2016). Basophil-derived interleukin-4 (IL-4) has been reported to promote the recruitment of eosinophils into peripheral tissues through the upregulation of VCAM-1 on vascular endothelium or the secretion of eotaxin-2 from fibroblasts (Cheng et al., 2015; Eberle et al., 2019). IL-4 produced from basophils has also been strongly implicated in the alternative activation of monocytes or macrophages, which dampens excessive inflammation and was reported to provide early protection against larvae of *Nippostrongylus brasiliensis* (Egawa et al., 2013; Obata-Ninomiya et al., 2013). Other reports have demonstrated a role for basophils in immune responses to other helminths *Heligmosomoides polygyrus*, *Strongyloides ratti*, and *Strongyloides venezuelensis*; and to the tick, *Haemaphysalis longicornis* (Mukai et al., 2017; C. Ohnmacht et al., 2010; Reitz et al., 2016; Reitz et al., 2018; C. Schwartz, Turqueti-Neves, et al., 2014; Sullivan et al., 2011; Wada et al., 2010).

Although the functions of basophils were initially thought to largely overlap with mast cells, a series of studies emerged suggesting that basophils were critical for adaptive immune

responses in lymph nodes. Cysteine proteases, such as papain, have been shown to promote basophil recruitment into draining lymph nodes in the context of type 2 immune responses (Sokol et al., 2008). It was reasoned that in the lymph node basophils may act as critical early producers of IL-4 as a possible mechanism of inducing Th2 cell differentiation. The injection of the anti-FcεRI monoclonal antibody, MAR-1, to deplete basophils reportedly led to the reduced generation of IL-4 expressing T cells in lymph nodes, thereby suggesting an important role for basophils in promoting adaptive immunity (Sokol et al., 2008). Subsequent studies proposed that basophils, rather than dendritic cells, were the crucial antigen presenting cells for the initial generation of Th2 cells (Kim et al., 2009; Perrigoue et al., 2009; Sokol et al., 2009; Yoshimoto et al., 2009). A series of studies based on antibody-mediated depletion strategies implicated basophils as critical cells for Th2 differentiation, B cell responses, downstream inflammation, and protection against helminths (Perrigoue et al., 2009; Rodriguez Gomez et al., 2010; Sokol et al., 2008; Sokol et al., 2009; H. Tang et al., 2010; Torrero et al., 2010; Yoshimoto et al., 2009).

Though these studies suggested an intriguing role for basophils in adaptive type 2 immune responses, other studies using alternative approaches have challenged this conclusion. In IL-3 deficient mice, basophils were not recruited into lymph nodes after *N. brasiliensis* infection; however, the Th2 response was comparable to wild-type control mice (S. Kim et al., 2010). Moreover, efficient depletion of basophils using the monoclonal antibody Ba103, which binds to CD200R3, led to only a partial reduction in the Th2 response, whereas much more profound effects were observed after injection of MAR-1 (Hammad et al., 2010). Finally, studies of mice with genetic deficiency in basophils, achieved by overexpression of Cre recombinase or selective expression of diphtheria toxin in cells expressing the basophil-specific *Mcpt8* gene, also could not demonstrate an essential function for basophils in promoting adaptive immunity (C. Ohnmacht et al., 2010; Sullivan et al., 2011). Further characterization of the MAR-1 antibody

used in numerous basophil studies has revealed the potential for off-target effects, as this antibody was found to bind to inflammatory dendritic cells (Hammad et al., 2010) as well as monocytes and tissue macrophages due to cross-reactivity with FcγRI and/or FcγRIV (X. Z. Tang et al., 2019). Therefore, current evidence suggests that basophils are not critical promoters of adaptive immune responses; however, the function of basophils in lymph nodes remains unclear.

While lymph nodes provide a crucial function in promoting adaptive immune responses to foreign antigens, a growing body of research has highlighted the potential for lymph nodes to also act as sites of barrier defense against invading pathogens. Since lymphatic fluid ultimately drains into the bloodstream through lymphatic vessels, invading pathogens have the precarious potential to access this pathway and gain entrance into the blood or invade other vulnerable organs. However, prior to entrance into the bloodstream, lymphatic fluid and associated material must first pass through lymph nodes, which sample all incoming foreign material. At the lymph node, several immunological mechanisms have been described that may provide surveillance and protection against lymph-borne bacteria and viruses (Bogoslowski et al., 2018; Farrell et al., 2016; Iannacone et al., 2010; Junt et al., 2007; Kastenmuller et al., 2012; Rosenheinrich et al., 2015; Y. Zhang et al., 2016). For example, subcapsular sinus macrophages that are directly exposed to incoming lymph can both capture pathogens and respond through inflammasome activation and secretion of the cytokines IL-1 beta and IL-18 (Kastenmuller et al., 2012). Beneath the subcapsular sinus, innate lymphoid cells can subsequently respond to these inflammatory signals by producing key anti-viral or anti-bacterial cytokines such as interferon-gamma and IL-17 (Kastenmuller et al., 2012; Y. Zhang et al., 2016). Macrophage activation can also lead to the relocalization and/or recruitment of additional cells such as natural killer cells, neutrophils, and plasmacytoid dendritic cells (Iannacone et al., 2010; Kastenmuller et al., 2012). While many reports have detailed the local type 1 inflammatory response in the lymph node,

little information exists mechanistically describing a type 2 response. Nonetheless, parasitic helminths, which typically induce type 2 inflammation, have been associated with eosinophilic lymphadenitis or been observed to infiltrate lymph nodes prior to their establishment at distal organs, suggesting a potential protective benefit for type 2 inflammation at the lymph node (Bain et al., 1994; Figueredo-Silva & Dreyer, 2005; Jungmann et al., 1991).

In this report, we describe a novel role for basophils recruited to the lymph nodes: to locally promote allergic inflammation. We found that immunization of mice with a combination of chicken IgY and calcipotriol (MC903) induced a profound accumulation of basophils in the draining lymph node. In the lymph node, basophils heavily associated with eosinophils below the subcapsular sinus, with both cells occasionally forming densely organized clusters. Antigen rechallenge promoted the formation of these cell clusters that were also found to contain alternatively activated macrophages and multinucleated giant cells, while surrounded by reorganized stromal cells. Importantly, basophils and IgE were shown to be crucial components of the allergic inflammatory response upon antigen rechallenge. Moreover, IgE-mediated activation was found to be sufficient to promote an inflammatory response that was basophil-dependent. Injection of larvae from the lymphatic-dwelling helminth, *Brugia pahangi*, resulted in the infiltration of larvae at the lymph node in association with a massive local allergic inflammatory response. Notably, allergic inflammatory cell clusters, with basophils, were found to form around infiltrated larvae. Altogether, we propose that a primary function for basophil recruitment into the lymph node is to participate in and promote allergic inflammation as part of a local protective response against a perceived threat.

Results

Massive basophil accumulation into lymph nodes is associated with eosinophilic lymphadenitis

To study the functional contributions of basophils, we first tested different stimuli that could recruit basophils into the lymph node. It had previously been demonstrated that both papain and calcipotriol could induce the recruitment of basophils into the lymph node (Leyva-Castillo, Hener, Michea, et al., 2013; Sokol et al., 2008). To study the nitrophenyl (NP)-specific response after basophil accumulation into the lymph node, we combined the dermal application of calcipotriol with the subcutaneous injection of NP-conjugated chicken gamma-globulin (NP-CGG). Unexpectedly, the combination of calcipotriol and NP-CGG led to a sustained and profound accumulation of basophils compared to papain or calcipotriol alone (Figure 3.1A). We next clarified whether the enhanced basophil response was a result of the hapten, NP, or the carrier protein. We found that, when combined with calcipotriol, only CGG or purified chicken IgY, the primary protein component of the CGG serum fraction, promoted a prolonged and substantial basophil response as previously observed. Other proteins, even when conjugated with NP, were insufficient for the enhanced basophil response (Figure 3.1B). In the context of these immunizations, we also observed the highest eosinophil recruitment with NP-CGG or IgY with calcipotriol, similar to the basophil response (Figure 3.1C). To determine whether the response to NP-CGG was dependent upon calcipotriol as an adjuvant, we also tested NP-CGG in combination with papain, alum, or no adjuvant at all. Only in combination with calcipotriol, NP-CGG induced massive basophil recruitment into the lymph node (Figure 3.1D). Once again, the eosinophil response reflected the basophil response in the context of these immunizations with different adjuvants (Figure 3.1E). Taken together, we had discovered a means to recruit significant numbers of basophils and eosinophils into lymph nodes, dependent upon both calcipotriol and chicken IgY.

We next sought to better understand the cellular mechanism by which the combination of IgY and calcipotriol induced recruitment of basophils. Numerous reports have revealed the cytokine IL-3 to be an important factor for basophil recruitment into lymph nodes (Kim et al., 2013; S. Kim et al., 2010; Leyva-Castillo, Hener, Michea, et al., 2013; Ohta et al., 2017). We therefore assessed the frequency of T cells capable of IL-3 production in the IgY and calcipotriol model compared to other models. Restimulation of T cells from lymph nodes revealed drastically more IL-3-capable T cells after immunization with IgY and calcipotriol compared to IgY and alum or goat IgG and calcipotriol (Figure 3.1F,G). To test whether IL-3 was required for basophil recruitment, we used A/J mice, which are known to have a disruptive mutation in the IL-3 receptor (CD123). After exposure to IgY and calcipotriol, significantly less basophils were recruited into the draining lymph nodes of A/J mice compared to C57B/J and BALB/c mice, suggesting that IL-3 is a primary mediator of the enhanced basophil response (Figure 3.2A). In addition to IL-3, we also tested the requirement for TSLP, as calcipotriol is known to induce the release of TSLP from keratinocytes in mice (M. Li et al., 2006). We observed a substantially reduced accumulation of basophils in the lymph nodes of TSLP receptor (TSLPR)-deficient mice compared to control mice, confirming that TSLP is an important component of this response (Figure 3.2B). The cell adhesion molecule CD62L (L-selectin) is known to be important for most leukocytes to enter lymph nodes, and, as reported in the papain model (Sokol et al., 2008), we confirmed that antibody-mediated blockade of CD62L led to reduced numbers of basophils in lymph nodes in the IgY and calcipotriol model (Figure 3.2C). Finally, we found that conditional deletion of the genes encoding IL-4 and IL-13 in T cells led to a marked decrease in the abundance of basophils in lymph nodes in the IgY and calcipotriol model, suggesting that IL-4/13 from T cells significantly contributes to basophil recruitment (Figure 3.2D). In summary, we determined that the mechanism for profound basophil accumulation in the IgY and calcipotriol model involved IL-3, TSLP, CD62L, and T cell derived IL-4/13.

Based on our observations above that immunization with IgY and calcipotriol promoted the recruitment of large numbers of basophils and eosinophils into draining lymph nodes, we further characterized this response by immunofluorescence microscopy. Using an advanced slide scanner with tiling and stitching, we collected images of numerous whole lymph node cryostat sections. Basophils were visualized in *Mctp8*-YFP-IRES-Cre (Basoph8) mice, in which YFP is expressed under the control of the Mcpt8 gene which is highly expressed in basophils, followed by amplification of the signal with an anti-GFP antibody. Most strikingly, this analysis revealed the occasional presence of dense and organized clusters of eosinophils, intermixed with basophils. These clusters were primarily found protruding into B cell follicles from the subcapsular sinus and/or in interfollicular regions. Less dense associations of eosinophils and basophils were also commonly observed near the subcapsular sinus or in interfollicular regions (Figure 3.1H). The overall enlargement and accumulation of cells in lymph nodes was also most pronounced in IgY and calcipotriol treated mice compared to other treatment conditions (Figure 3.3A,B). Taken together, these characteristics resemble a clinical condition referred to as lymphadenitis involving eosinophilic infiltration (H. Chen et al., 2004; Jungmann et al., 1991; Kaplinsky et al., 1988).

Basophils are associated with increased formation of inflammatory cell clusters in the lymph node after antigen rechallenge

Previous work has demonstrated that basophils can respond to antigens recognized via IgE/FcεRI and promote the recruitment of inflammatory cells, polarize macrophages to become alternatively activated, and provide protection against parasitic helminths and ticks (Cheng et al., 2015; Egawa et al., 2013; Mukai et al., 2005; Obata-Ninomiya et al., 2013; Obata et al., 2007; C. Schwartz, Turqueti-Neves, et al., 2014; Wada et al., 2010). Therefore, we reasoned that in our IgY and calcipotriol model, re-exposure to antigen in the draining lymph node at the

peak of the response, after antigen-specific IgY antibody had been generated, would stimulate basophils to promote an even greater inflammatory response. We re-injected mice with IgY 8 days after the primary immunization, and one day later we observed a clear and significant increase in the formation of organized clusters of inflammatory cells that were primary localized near sinuses and interfollicular regions (Figure 3.4A-C). In addition to basophils and eosinophils, we observed the differentiation of alternatively activated macrophages (AAMs) in these clusters, using mice expressing a YFP-reporter for Arginase-1 (YARG) that was detected by anti-GFP antibody staining. In these YARG mice, basophils were detected by staining for the surface marker CD200R3. The relative proportions of these cell types varied in different inflammatory foci (Figure 3.4A). We also observed that many clusters were surrounded by cells with high expression of GP-38 and the adhesion molecule, vascular cell adhesion protein 1 (VCAM-1), likely corresponding to a subset of stromal fibroblastic reticular cells (FRCs) (Figure 3.4D). Antigen rechallenge increased the number of FRCs that displayed very high expression of both GP-38 and VCAM-1 (Figure 3.4E). In the inflammatory cell clusters we also observed the presence of multinucleated giant cells, which are derived through the fusion of multiple monocytes or macrophages and are commonly involved in granulomas and foreign body reactions (Brodbeck & Anderson, 2009). Most multinucleated giant cells in our model expressed arginase-1 and displayed high surface staining for CD11c. These cells primarily associated with the inflammatory cell clusters and increased in number upon antigen rechallenge (Figure 3.4F,G). Taken together, antigen rechallenge promoted the recruitment of eosinophils, the differentiation of AAMs including multinucleated giant cells, and the organization of immune and stromal cell populations into heterogeneous and complex clusters.

Basophils contribute to the formation of allergic inflammatory cell clusters

Based on our observations of the formation of allergic inflammatory cell clusters in rechallenged mice, we next tested the direct contribution of basophils to this response in basophil-deficient mice. Specifically, we combined the Basoph8 allele, in which Cre is expressed in the *Mcpt8* locus, with a *Rosa26* allele containing a loxP-flanked STOP cassette and the DTa gene (*Rosa26*-LSL-DTA), such that DTa would only be expressed in *Mcpt8*+ cells during basophil development, resulting in specific depletion of basophils, as validated in previous studies (Cheng et al., 2015; Sullivan et al., 2011). In order to detect AAMs, we bred mice carrying these alleles to YARG mice. We compared basophil-deficient (Basoph8 *Rosa26*-LSL-DTA) YARG mice to control mice expressing the YARG allele but lacking Basoph8, so that we could specifically detect YFP+ AAMs. Fewer eosinophil and AAM-rich inflammatory clusters were observed in lymph nodes from basophil-deficient YARG mice compared with control YARG mice after antigen rechallenge (Figure 3.5A,B). The clusters that did form in basophil-deficient YARG mice showed lower expression of the YARG reporter for arginase-1, suggesting reduced AAM differentiation (Figure 3.5C). To confirm these findings, we also examined individual cell populations by flow cytometry. As expected, basophil counts were negligible in lymph nodes from basophil-deficient Basoph8 *Rosa26*-LSL-DTA mice (Figure 3.5D). We confirmed that the numbers of both eosinophils and AAMs were reduced in basophil-deficient mice after antigen rechallenge, consistent with our histological observations (Figure 3.5E-G). While the total population of recoverable monocyte-macrophages were increased after antigen rechallenge, we observed that a smaller fraction of these cells expressed the YARG reporter in basophil-deficient mice (Figure 3.5H). Neutrophil counts also variably increased one day after antigen rechallenge, and we observed a statistically significant reduction in neutrophils in basophil-deficient mice (Figure 3.5I). Finally, we also observed a decrease in the number of $GP38^{hi}VCAM\text{-}1^{hi}$ FRC in basophil-deficient mice, corresponding to the population we had

observed surrounding the inflammatory cell clusters, although this was close to our threshold for statistical significance (Figure 3.5J). Altogether, these findings reveal that basophils contribute to the accumulation of numerous leukocytes and the organization of allergic inflammatory cell clusters in the lymph node.

To gain insight into the early stages of inflammatory foci development, we performed histological and flow cytometric analysis of lymph nodes six hours after antigen rechallenge. At this early time point, cell clusters increased and were heavily comprised of eosinophils and neutrophils in variable proportions, but few alternatively activated macrophages were observed (Figure 3.6A-E). Basophils in the lymph node were highly associated with these clusters (Figure 3.6A). We next tested the involvement of basophils in early cell cluster formation in basophil-deficient mice. Basophil-deficiency resulted in a severe reduction in frequency and size of clusters compared to controls (Figure 3.7A-C). Flow cytometry confirmed the genetic depletion of basophils and reduction of eosinophils and neutrophils in basophil-deficient mice (Figure 3.7D-F). The frequency of neutrophils six hours after antigen rechallenge was higher and more consistent than when examined one day after rechallenge, perhaps owing to significant apoptosis or egress by the later time point (Figure 3.7F, Figure 3.5I). Inflammatory monocytes also increased after rechallenge at the early time point, but their numbers were not clearly affected by a deficiency in basophils (Figure 3.7G). These results reveal that basophils are critically involved in the early recruitment of eosinophils and neutrophils as well as their initial clustering into dense foci prior to the polarization of alternatively activated macrophages.

Basophils capture antigen, degranulate, and relocalize after rechallenge

To understand how basophils are involved in the initial formation of inflammatory clusters, we examined the behavior of basophils within two hours after antigen rechallenge. Two-photon

laser scanning microscopy revealed that basophils captured and carried antigen in the lymph node (Figure 3.8A). The capture of fluorescent antigen by basophils could also be detected by flow cytometry (Figure 3.8B). After antigen rechallenge, basophils showed evidence of activation through increased degranulation, measured by CD63 surface expression (Figure 3.8C). Since large molecular weight antigens such as IgY are thought to enter the lymph node parenchyma from the subcapsular sinus, we next asked whether newly-arrived IgY could induce basophils to relocalize closer to the sinus. Indeed, immunofluorescence microscopy analysis revealed that total basophils localized closer to the subcapsular sinus two hours after antigen rechallenge. (Figure 3.8D-F). Taken together, we found that basophils could respond to antigen re-exposure in the lymph node by capturing antigen, degranulating, and relocalizing in closer proximity to the site of antigen entry.

Basophils interact extensively with macrophages and produce IL-4

Since basophils were found to localize close to the subcapsular sinus, where many macrophages reside, we examined the potential for basophils to directly affect macrophage responses in the lymph node. Previous reports have implicated basophils and basophil-derived IL-4 in the polarization of macrophages or monocytes to become alternatively activated (Cohen et al., 2018; Egawa et al., 2013; Obata-Ninomiya et al., 2013). A recent study using single cell RNA sequencing had also highlighted the potential for basophil-derived mediators to promote macrophage development in the lung (Cohen et al., 2018). Immunofluorescence microscopy analysis showed that many lymph node basophils highly associated with $CD169^+$ macrophages near the subcapsular sinus (Figure 3.9A). Approximately 40% of basophils were in direct contact with $CD169^+$ macrophages by day 8 after immunization, and after antigen rechallenge the frequency of basophils in direct contact with CD169+ macrophages increased to over 60% (Figure 3.9B). Time-lapse imaging revealed extensive interactions of basophils with

macrophages that frequently lasted beyond 30 minutes, the typical duration of our recordings (Figure 3.9C). Basophils are known to be a major in vivo source of the cytokine IL-4 (Min et al., 2004; Sullivan et al., 2011; Voehringer et al., 2004). IL-4 is involved in the polarization of macrophages to become alternatively activated and the fusion of macrophages to become multinucleated giant cells (Martinez, Helming, & Gordon, 2009). To determine whether lymph node basophils generated IL-4 in vivo in this model, we used KN2 mice which report the production of IL-4 protein (K. Mohrs et al., 2005). After antigen rechallenge, we observed an increase in both the frequency of IL-4 producing basophils and the amount of IL-4 produced per cell (Figure 3.9D-F). Taken together, these observations reveal that basophils can directly influence macrophages through extended interactions and the secretion of IL-4, which may act to promote the differentiation of AAMs and the formation or maturation of inflammatory cell clusters.

Basophils organize allergic inflammatory cell clusters through IgE

Basophils are one of the primary effector cells for IgE. Antigen binds to IgE on the surface of basophils, leading to the clustering of FcεRI and downstream signaling (Kraft & Kinet, 2007b). We therefore investigated the role of IgE and FcεRI signaling on basophil-enhanced inflammation of the lymph node in our model using IgY and calcipotriol. Immunofluorescence microscopy examination revealed that the formation of early inflammatory cell clusters, which arise six hours after antigen rechallenge, depended upon FcεRIα, the IgE surface binding domain of FcεRI. Knockout of FcεRIα led to significantly fewer cell clusters compared to controls upon antigen rechallenge (Figure 3.10A). Similarly, early cell cluster formation was also drastically reduced in mice deficient for FcRγc, the FcεRI signaling chain which is also shared by FcγRI, FcγRIII, and FcγRIV (Bruhns, 2012) (Figure 3.10A). Flow cytometry revealed that FcRγc was important for eosinophil accumulation in the lymph node at the six hour time point.

Unexpectedly, although FcεRIα-deficiency prevented the formation of inflammatory cell clusters (Fig. 6A), FcεRIα-deficiency did not have a statistically significant effect on the total accumulation of eosinophils in the lymph nodes as measured by flow cytometry (Figure 3.10B). FcRγc was crucial for neutrophil accumulation and clustering, but the FcεRIα KO also led to only a partial reduction in neutrophil accumulation and clustering (Figure 3.10C, Figure 3.11A). Lastly, the accumulation of inflammatory monocytes remained unchanged between both knockouts and control mice (Figure 3.11B). These findings are consistent with our above data demonstrating a minimal impact of basophil deficiency on monocyte recruitment after antigen rechallenge (Figure 3.7G). Altogether, these results reveal that IgE and FcεRI signaling is important for the early clustering of inflammatory cells upon antigen rechallenge in our IgY and calcipotriol model, although the more pronounced effects of deficiency in FcRγc suggests the possibility of some compensation by IgG/FcγRIII in the absence of FcεRI.

We next asked whether IgE-mediated basophil activation was sufficient to drive the entire inflammatory process that we had observed in lymph nodes. In our previous experiments, we immunized mice with IgY and calcipotriol, then rechallenged with IgY to induce an inflammatory response in the lymph node. Since the same protein was re-administered, T cells may have been re-stimulated and therefore may have contributed to the inflammatory response. To bypass T cell re-stimulation, we injected NP-specific IgE intravenously to prime basophils in the lymph node, then challenged with NP-APC instead of IgY (Figure 3.10D). NP-APC rechallenge resulted in allergic inflammation of the lymph node characterized by the formation of eosinophil and AAM-rich cell clusters, similar to our IgY rechallenge condition (Figure 3.10E-G). The frequency of eosinophils, neutrophils, and inflammatory monocytes were also increased in the lymph nodes of mice administered NP-specific IgE and NP-APC compared to control mice that were only given NP-APC (Figure 3.10H-J). Importantly, the enhanced cluster formation and cell recruitment were all completely dependent upon basophils, since the responses of basophil-

deficient mice were indistinguishable from control mice that were not given NP-specific IgE (Figure 3.10E, G-L). In summary, IgE-mediated basophil activation is sufficient to drive both inflammatory cell recruitment and organized cell clustering in the lymph node.

Basophils associate with an allergic inflammatory response against a lymphatic-dwelling helminth

We hypothesized that the primary physiological function for basophil-enhanced inflammation of the lymph node would be to defend against pathogens that enter into lymph nodes through draining lymphatic vessels. Allergic inflammation is a common immunological response to parasitic helminths. Moreover, some filarial helminths which dwell long-term or transiently traverse through the lymphatic system have been observed to enter into lymph nodes (Bain et al., 1994; Jungmann et al., 1991). To determine whether basophils are involved in an allergic inflammatory response against parasites in the lymph node, we examined the infection of mice with the lymphatic-dwelling filarial helminth, *Brugia pahangi*. After subcutaneous injection of *B. pahangi* into the scruff of the neck, draining lymph nodes were examined for signs of infection. Seven days post-infection, the facial, superficial cervical, brachial, and axillary lymph nodes were dramatically enlarged compared to other immunizing agents that we have tested (Figure 3.12A). Time-lapse imaging of the lymph nodes one day after infection revealed the presence of live larvae and basophil infiltration (Figure 3.12B). We next performed histology to compare the resulting inflammatory response to *B. pahangi* with our model using IgY and calcipotriol. By day 7 of the primary response, many lymph nodes were massively infiltrated by eosinophils (Figure 3.13). We reasoned that basophils might be more involved in an early inflammatory response during a secondary infection in which parasite-specific IgE is present. Therefore, we first primed mice with larvae homogenate then infected with *B. pahangi* two to four weeks later. In these mice, lymph nodes were collected 4 days post-infection. Although significant variability was

observed, some lymph nodes contained significant numbers of basophils that were associated with dense clusters of CD11b+ cells surrounded by GP-38+ stromal cells, as we had observed in mice treated with IgY and calcipotriol (Figure 3.12C,D). Further examination revealed that these clusters were highly enriched for eosinophils and AAMs, similar to what we had observed in our IgY and calcipotriol model (Figure 3.12E,F). Larval fragments were also detected in some inflammatory clusters, suggesting that the organization of clusters occurred at sites where the larvae had infiltrated (Figure 3.12D,F). Overall, these experiments reveal that the infection of filarial helminths in the lymph node can induce an allergic inflammatory response that involves basophils, suggesting that the entry of basophils into lymph nodes is part of a larger defense mechanism for protection against lymphatic dwelling parasites and that basophils may help to enhance this protective response.

Discussion

Our work reveals the capacity for basophils to orchestrate allergic inflammation within the lymph node. In our model using IgY and calcipotriol, basophils associated with organized cell clusters comprised of eosinophils, alternatively activated macrophages, multinucleated giant cells, and reorganized stromal cells. By testing basophil-deficient mice, we could demonstrate that formation of these clusters was dependent upon basophils and that the activation of basophils through IgE was sufficient to promote inflammatory cell recruitment and cell cluster formation. Prior to the formation of cell clusters, basophil activation through antigen challenge led to their relocalization to the subscapsular sinus, where we observed frequent, sustained interactions of basophils with macrophages by two-photon microscopy. To explore a potential role for basophils and allergic inflammation in the lymph node in a more physiological setting, we infected mice with the lymphatic-dwelling parasite, *Brugia pahangi*. Upon infection, *B. pahangi* larvae were found to enter into lymph nodes, followed by intense allergic inflammation of similar composition to our IgY model. Inflammatory foci containing basophils were observed to

surround infective larvae. These findings support the notion that the recruitment of basophils, and many other leukocyte populations, into the lymph node is part of an orchestrated immunological response to defend the body against lymphatic-traversing parasitic organisms.

To our knowledge, this study is the first in mice to mechanistically explore the process of allergic inflammation within the lymph node. Numerous reports have demonstrated that allergic inflammation of the lymph node occurs in humans, though it is more often termed eosinophilic lymphadenitis because of the noticeable infiltration of eosinophils into the affected tissue. Some disorders associated with this condition include hyper-IgE syndrome, dermatitis, Churg-Straus syndrome (involving blood vessel inflammation), drug-induced hypersensitivity, and Kimura's disease (involving head and neck tissues) (H. Chen et al., 2004; Churg, Brallas, Cronin, & Churg, 1995; Garcia Carretero, Romero Brugera, Rebollo-Aparicio, & Vazquez-Gomez, 2016; Gowani, Gehrs, & Scordino, 2018; Kaplinsky et al., 1988; Makis, Hickeson, & Blumenkrantz, 2010; Nonaka et al., 2017; Swanson et al., 2017; Winter, Spiegel, & King, 2007). Eosinophilic lymphadenitis is also associated with infection by some lymphatic-dwelling filarial helminths such as *Wuchereria bancrofti* and those of the Brugia genus (e.g. *Brugia malayi*) (Baird & Neafie, 1988; Chandy et al., 2011; Elenitoba-Johnson et al., 1996; Figueredo-Silva & Dreyer, 2005; Jungmann et al., 1991). In our study, basophils and IgE were particularly important for enhanced allergic inflammation in response to antigen rechallenge. On the other hand, T-cell derived IL-4/13 and IL-3 were required for the massive basophil accumulation into the lymph node. Though we did not extensively test the role of T cells in rechallenge-induced inflammation, we observed some residual inflammation in basophil-deficient mice, suggesting that both Th2 cells and basophils can contribute to this process. Nonetheless, the early response, six hours after rechallenge, appeared particularly dependent upon basophils, indicating that basophils both promote and accelerate the formation of inflammatory cell clusters. Additionally, basophils could promote the same type of inflammatory response through

passive immunization and challenge with a previously unimmunized antigen, which suggests that basophil activation is sufficient, independent of T cell reactivation, to promote allergic inflammation, consistent with a previous skin inflammation model (Mukai et al., 2005).

Our study also provides greater evidence for the notion that the lymph node is a potential site of defense against lymphatic-traversing parasites and that basophils are involved in the protective response. Experimental infection of rodents had previously suggested that many parasites traverse through lymph nodes, even those that ultimately migrate to non-lymphatic, distal organs (Bain et al., 1994; Lozzi et al., 1996). In our study using the filarial helminth, *B. pahangi*, we found that numerous larvae could be detected within lymph nodes one day after infection by subcutaneous injection. Even at this early time point, some basophils had already accumulated in the lymph node as well, suggesting the potential for basophil-mediated responses to the larvae through innate signals or IgE-mediated pathways. We predict that basophils contribute to the efficient formation of inflammatory cell clusters in a secondary response to larvae within lymph nodes, as we demonstrated in the IgY and calcipotriol model. One report had revealed that IL-4, macrophages, and eosinophils were important in protection against a similar helminth, *Brugia malayi*, within the peritoneum (Turner et al., 2018). Given that basophils are a major source of IL-4, we speculate that basophil-derived IL-4 may contribute to protection from these *Brugia* species. Although basophils were not found to be critical for the overall immune response to the filarial parasite *Litomosoides sigmodontis* (Hartmann et al., 2018; Torrero et al., 2010), these studies did not specifically investigate the inflammatory response and remodeling within lymph nodes, where we would predict basophils may contribute to protection and accelerate secondary responses. Indeed, previous reports testing *H. polygyrus* and *N. brasiliensis* have demonstrated that basophils could provide an accelerated protection in peripheral tissues against some organisms during a memory response (Obata-Ninomiya et al., 2013; C. Schwartz, Turqueti-Neves, et al., 2014). Overall, we anticipate that basophils and Th2

cells may both contribute to immune responses to helminth larvae within lymph nodes, in which basophils may accelerate and promote the formation of organized inflammatory cell clusters after helminth re-exposure.

Our study builds off of previous literature in providing a much greater characterization of how basophils contribute to allergic inflammation in a spatial-temporal manner. Previous reports studying the contribution of basophils on inflammation have primary focused on parameters such as differences in the number of tissue-recruited inflammatory cells, tissue swelling, or the efficiency of parasite infection (Egawa et al., 2013; Mukai et al., 2005; Obata-Ninomiya et al., 2013; Obata et al., 2007; C. Ohnmacht et al., 2010; C. Schwartz, Turqueti-Neves, et al., 2014; Sullivan et al., 2011; Wada et al., 2010). In this report, we focused extensively on microscopy to characterize basophil behavior and downstream inflammatory processes at an anatomical level. Based upon our data, we propose a multistep process by which basophils in the lymph node promote inflammation. First, basophils respond to foreign antigen through IgE, promoting the secretion and increased production of various inflammatory mediators. Associated with this antigen recognition, basophils relocalize toward sites of greater antigen (or pathogen) exposure through direct release of chemotactic mediators or indirectly through the production of chemokines by resident cells in proximity to activated basophils. Activated basophils that have relocalized then increase interactions with $CD169^{+}$ macrophages. Macrophages are stimulated by basophil-derived IL-4, or other mediators, to release additional cytokines which promote the recruitment and/or re-localization of other cells such as eosinophils, monocytes, neutrophils, macrophages, and fibroblasts into inflammatory cell clusters. IL-4 later leads to the further polarization of macrophages and monocytes to become alternatively activated and in some cases multinucleated giant cells. In this model, basophils may protect against an incoming threat at the lymph node (and likely other sites) by coordinating a multistage and multicellular immunological response.

Figures

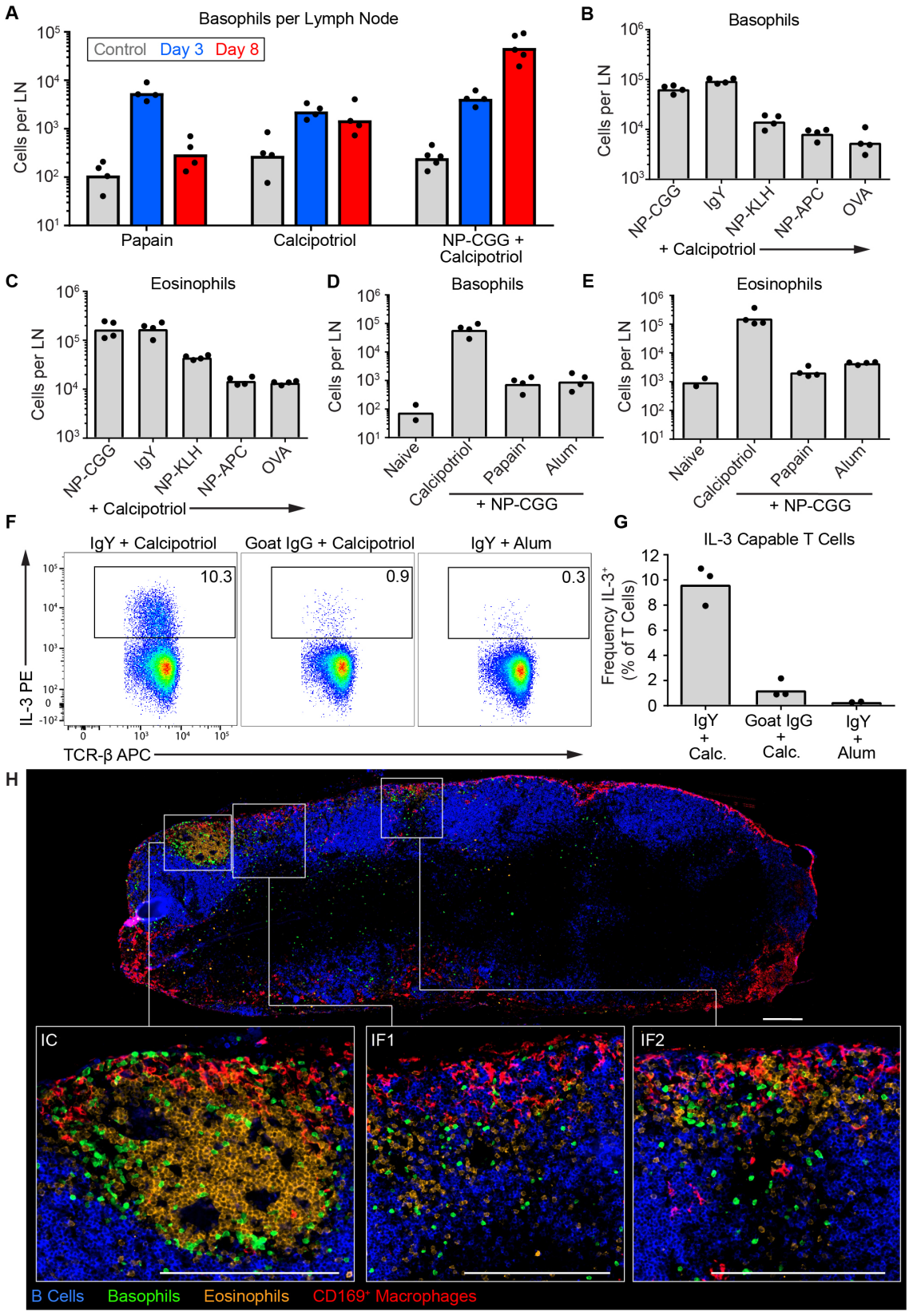
A
Basophils per Lymph Node
Control Day 3 Day 8
Cells per LN
Papain
Calcipotriol
NP-CGG + Calcipotriol
B
Basophils
Cells per LN
NP-CGG
IgY
NP-KLH
NP-APC
OVA
+ Calcipotriol
C
Eosinophils
Cells per LN
NP-CGG
IgY
NP-KLH
NP-APC
OVA
+ Calcipotriol
D
Basophils
Cells per LN
Naive
Calcipotriol
Papain
Alum
+ NP-CGG
E
Eosinophils
Cells per LN
Naive
Calcipotriol
Papain
Alum
+ NP-CGG
F
IgY + Calcipotriol
Goat IgG + Calcipotriol
IgY + Alum
10.3
0.9
0.3
IL-3 PE
TCR-β APC
G
IL-3 Capable T Cells
Frequency IL-3+ (% of T Cells)
IgY + Calc.
Goat IgG + Calc.
IgY + Alum
H
IC
IF1
IF2
B Cells
Basophils
Eosinophils
CD169+ Macrophages

Figure 3.1: The accumulation and localization of basophils in the lymph node after IgY and calcipotriol immunization. (A) One ear of each mouse was treated with papain, calcipotriol, or NP-CGG and calcipotriol to recruit basophils into the draining facial lymph node. Basophils in draining lymph nodes were enumerated by flow cytometry on day 3 or day 8 after treatment. The contralateral, non-draining facial lymph node of each mouse was used as a control. (B-E) Ears of mice were treated with different combinations of antigens and adjuvants as indicated. Draining facial lymph nodes were analyzed by flow cytometry on day 8 to enumerate basophils and eosinophils. (F-G) 7 days after ears were treated with the indicated combinations of antigens and adjuvants, T cells from draining facial lymph nodes were restimulated with PMA and ionomycin, then intracellularly stained for IL-3. Representative flow cytometry plots (F) and quantification (G) of the number of IL-3-capable T cells are shown. (H) Whole lymph node immunofluorescence microscopy from a Basoph8 mouse treated with IgY and NP-CGG and examined on day 8. Cells were identified as B cells (IgD^+), basophils (YFP^+), eosinophils (Siglec-F^+), and $CD169^+$ macrophages (CD169). IC, inflammatory cluster; IF, interfollicular regions; scale bar, 100 μm.

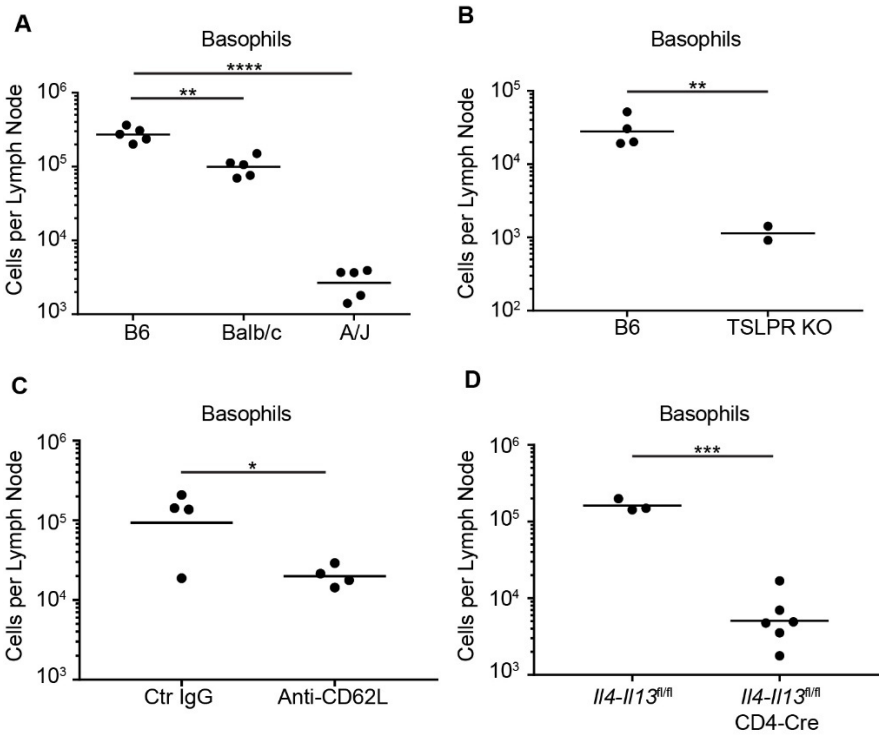

Figure 3.2: Requirements for basophil accumulation in lymph nodes.
Mice were treated with IgY and calcipotriol and lymph nodes were analyzed by flow cytometry on day 8. Basophils were enumerated in mice from C57BL/6, BALB/c, or A/J backgrounds (A); control C57BL/6 versus TSLPR KO mice (B); mice treated with control (Ctr) IgG or anti-CD62L antibody on day 5 (C); or (D) *Il4-Il13*$^{flox/flox}$ versus *Il4-Il13*$^{flox/flox}$, CD4-Cre mice. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001 (unpaired t test on log-transformed data).

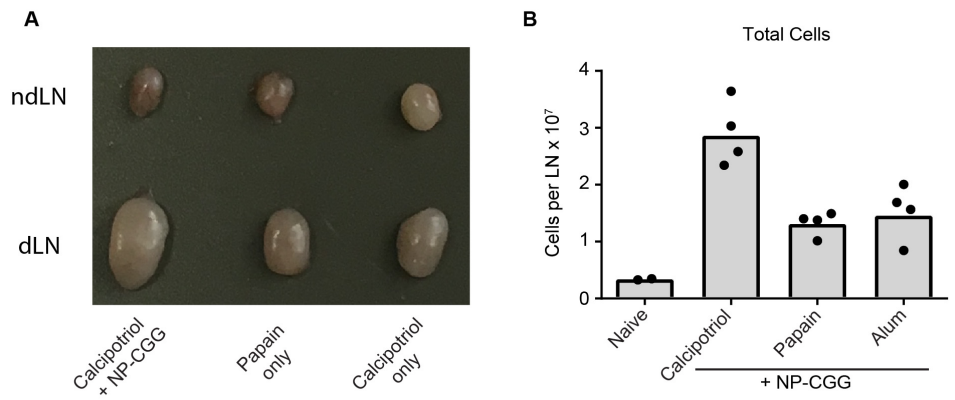

Figure 3.3: IgY and calcipotriol treatment induces exaggerated lymph node enlargement. One (A) or both (B) ears of mice were treated with calcipotriol and NP-CGG, papain, or calcipotriol only and lymph nodes were excised 8 days after treatment. (A) Draining and contralateral non-draining facial lymph nodes were imaged by stereomicroscopy. (B) The total number of draining lymph node cells were counted on a Coulter Z2 particle counter.

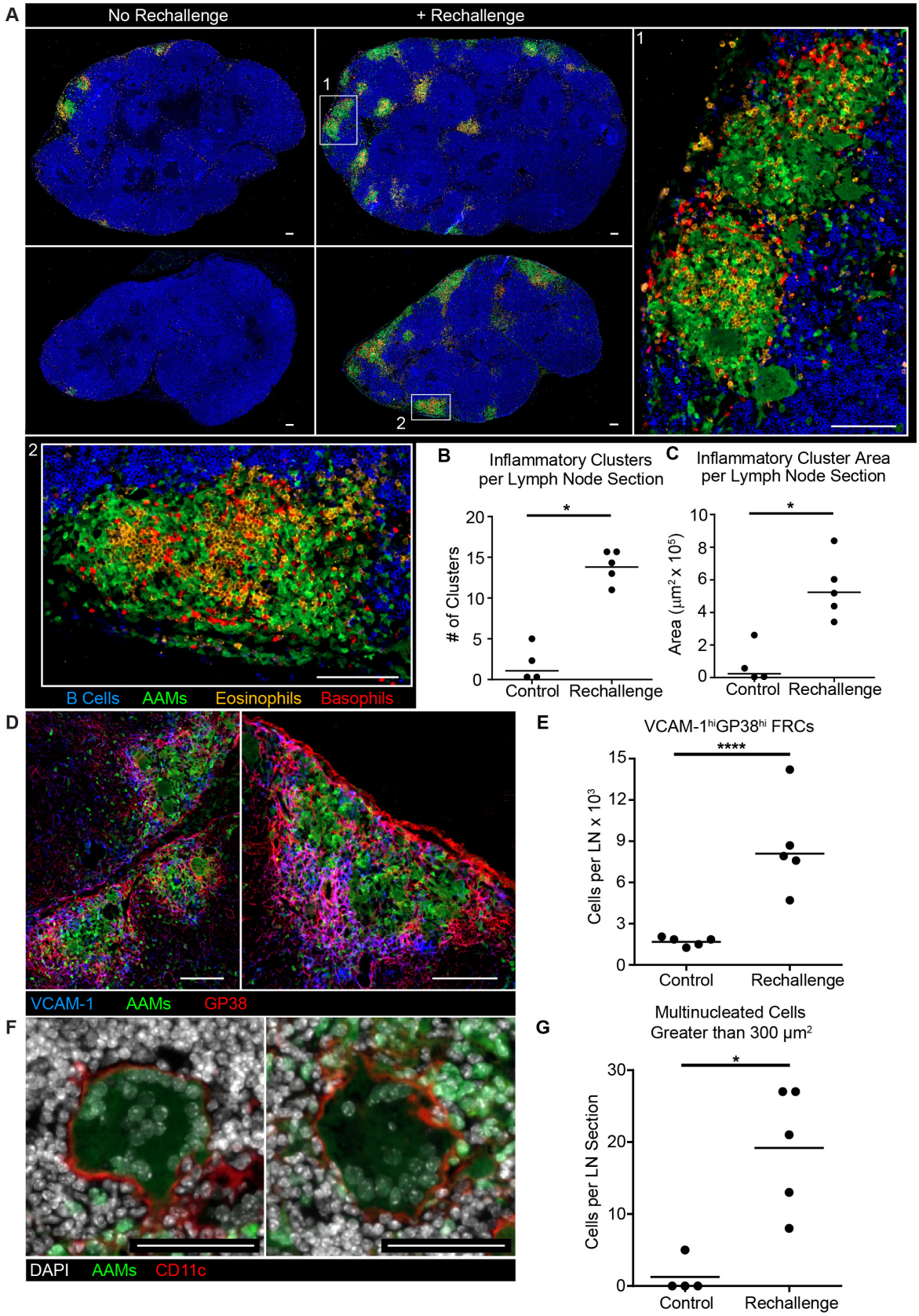
A
No Rechallenge
+ Rechallenge
1
2
B Cells AAMs Eosinophils Basophils
B
Inflammatory Clusters per Lymph Node Section
# of Clusters
Control Rechallenge
C
Inflammatory Cluster Area per Lymph Node Section
Area (μm² x 10⁵)
Control Rechallenge
D
VCAM-1 AAMs GP38
E
VCAM-1hiGP38hi FRCs
Cells per LN x 10³
Control Rechallenge
F
DAPI AAMs CD11c
G
Multinucleated Cells Greater than 300 μm²
Cells per LN Section
Control Rechallenge

Figure 3.4: The increased formation of inflammatory cell clusters containing basophils in the lymph node after antigen rechallenge. Ears of mice were treated with IgY and calcipotriol and rechallenged with IgY on day 8. Draining facial lymph nodes were analyzed one day after IgY rechallenge. All experiments were performed on YARG mice, except for (E), which was done using C57BL/6J mice. (A) Two representative whole lymph node immunofluorescence microscopy images with separate magnified views of inflammatory clusters are shown. Cells were identified as B cells (IgD^+), AAMs (YFP^+), eosinophils (Siglec-F^+), and basophils ($CD200R3^+$). (B-C) Quantification of the number (B) and size (C) of the inflammatory cell clusters in immunofluorescence microscopy images. (D) Representative immunofluorescence microscopy images showing $GP38^{hi}VCAM\text{-}1^{hi}$ stromal cells around inflammatory clusters. (E) Quantification of $GP38^{hi}VCAM\text{-}1^{hi}$ fibroblastic reticular cells (FRCs) by flow cytometry. (F) Representative immunofluorescence microscopy images of multinucleated giant cells. (G) Quantification of the number of multinucleated cells with a greater area than 300 μm^2 in tissue sections. Scale bars, 100 μm (A,D) and 50 μm (F). *, $p<0.05$; ****, $p<0.0001$ (Mann-Whitney test [B,C,G], unpaired t-test on log transformed data [E]).

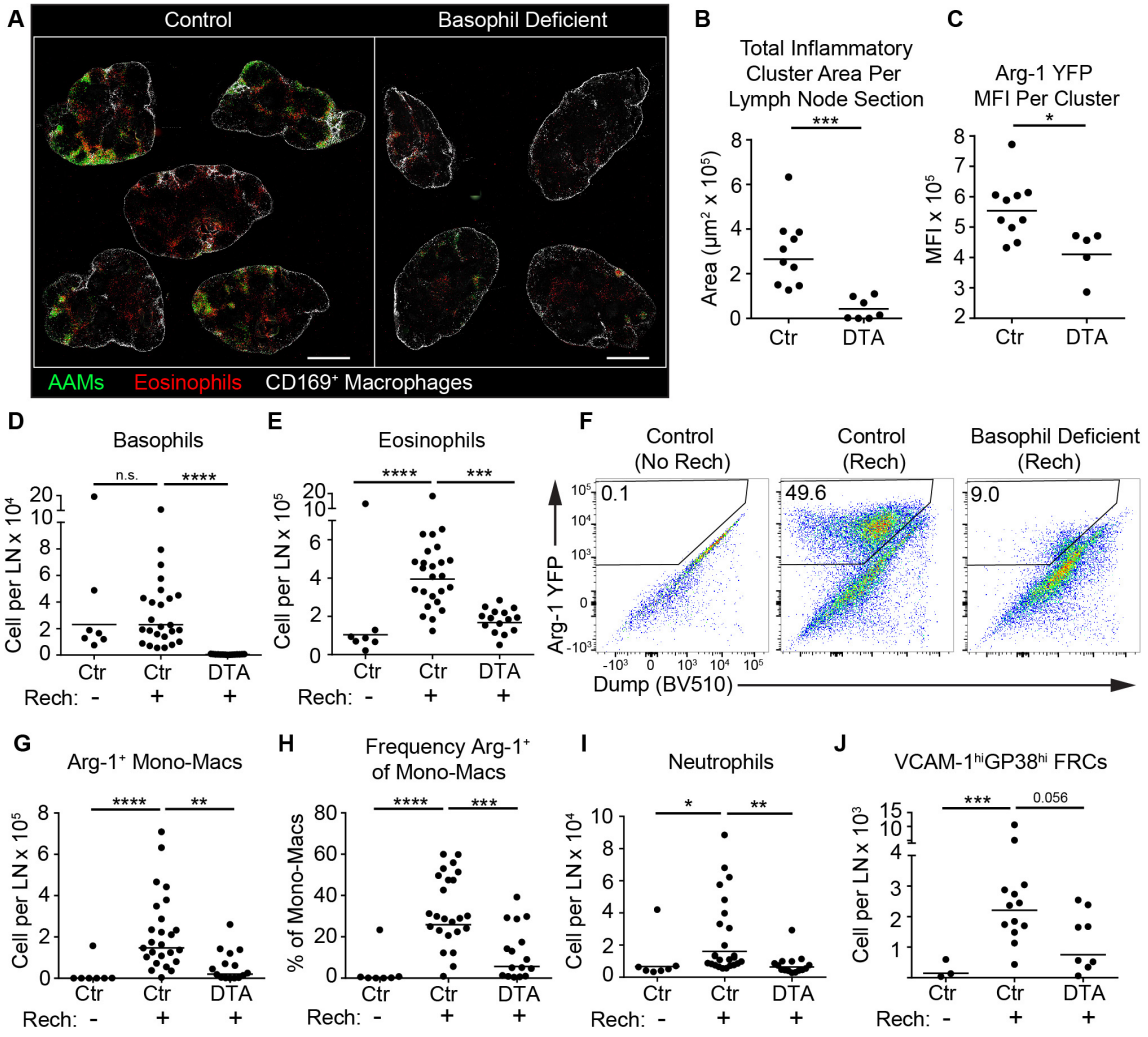

Figure 3.5: Basophils contribute to inflammatory cluster formation after antigen rechallenge.

Control and basophil deficient mice were treated with the antigens NP-CGG (A-C) or IgY (D-J) and the adjuvant calcipotriol. Mice were rechallenged (Rech) with the same antigen 8 days later and draining lymph nodes were analyzed one day after rechallenge. (A) Representative immunofluorescence microscopy images of whole lymph nodes showing inflammatory clusters in control (YARG) versus basophil-deficient mice (Basoph8, Rosa26-LSL-DTA, YARG) after antigen rechallenge. (B-C) Quantification of the inflammatory cluster size (B) and mean fluorescence intensity (MFI) of Arg-1 expression (C) per lymph node. (D-J) Analysis and quantification of the indicated cell populations by flow cytometry in control (YARG or Basoph8, YARG) versus basophil-deficient (Basoph8, Rosa26-LSL-DTA, YARG) mice after antigen rechallenge. Arg1+ monocytes (mono)/macrophages (macs) were gated based on YARG reporter expression compared to autofluorescence (Dump) as shown in (F). Scale bars, 1 mm. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001 (Mann-Whitney test [B,C], unpaired one-way ANOVA on log transformed data [D,E,G-J]). Ctr, control

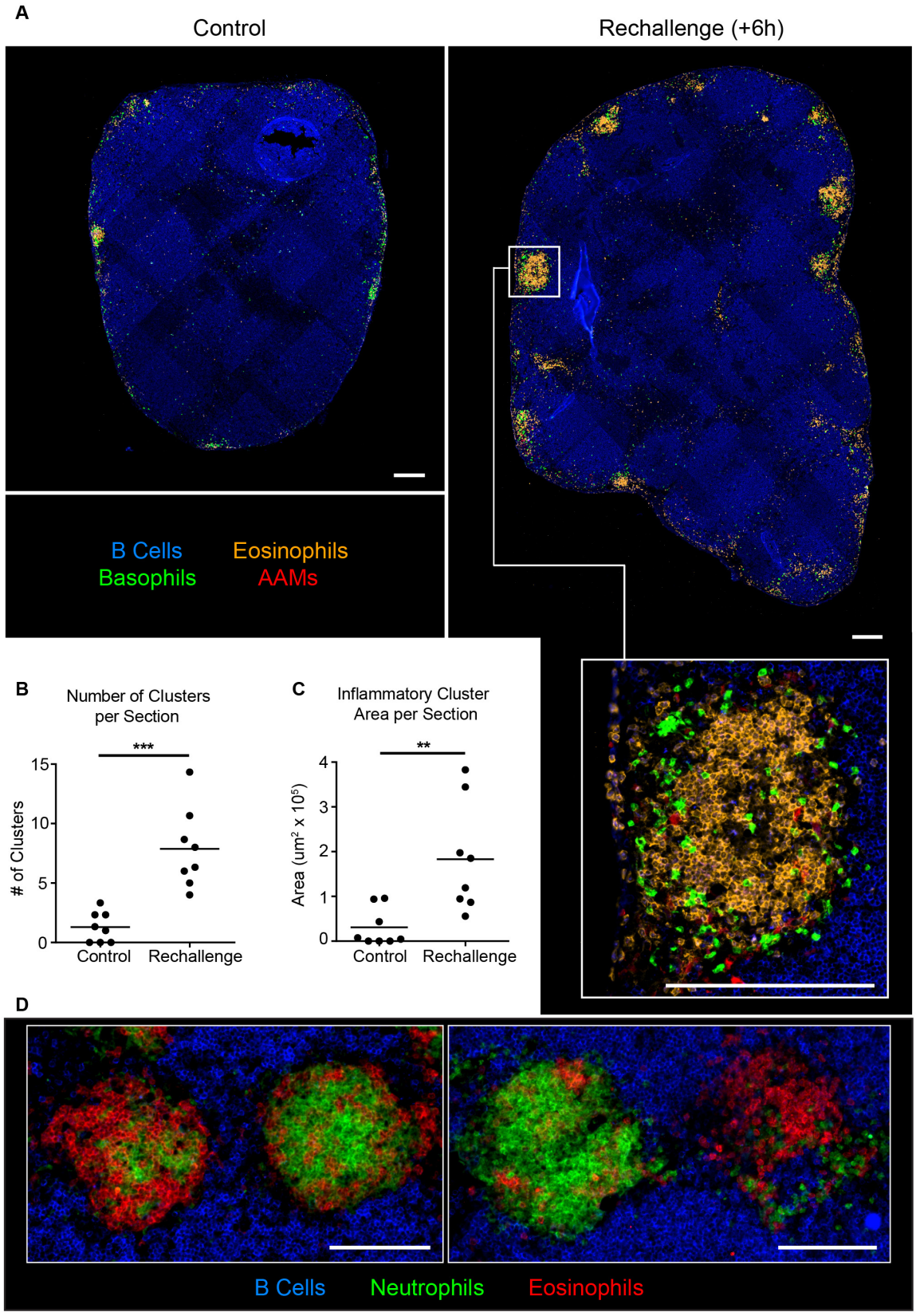
A
Control
Rechallenge (+6h)
B Cells
Basophils
Eosinophils
AAMs
B
Number of Clusters per Section
***
# of Clusters
15
10
5
0
Control
Rechallenge
C
Inflammatory Cluster Area per Section
**
Area (um² x 10⁵)
4
3
2
1
0
Control
Rechallenge
D
B Cells
Neutrophils
Eosinophils

Figure 3.6: Characteristics of the early response to allergen rechallenge.
Mice were treated with IgY and calcipotriol and then some mice were rechallenged with IgY 8 days later. Draining lymph nodes were analyzed 6 hours after rechallenge. (A) Representative immunofluorescence microscopy analysis of lymph nodes from YARG mice. Cells were identified as B cells (IgD^+), basophils ($CD200R3^+$), eosinophils ($Siglec\text{-}F^+$), and AAMs (YFP^+). (B-C) Quantification of number (B) and size (C) of inflammatory clusters from lymph node sections. (D) Examples of inflammatory clusters containing variable proportions of eosinophils and neutrophils. Cells were identified as B cells (IgD^+), neutrophils ($Ly\text{-}6G^+$), and eosinophils ($Siglec\text{-}F^+$). Scale bars, 200 μm. **, $p<0.01$; ***, $p<0.001$ (Mann-Whitney test).

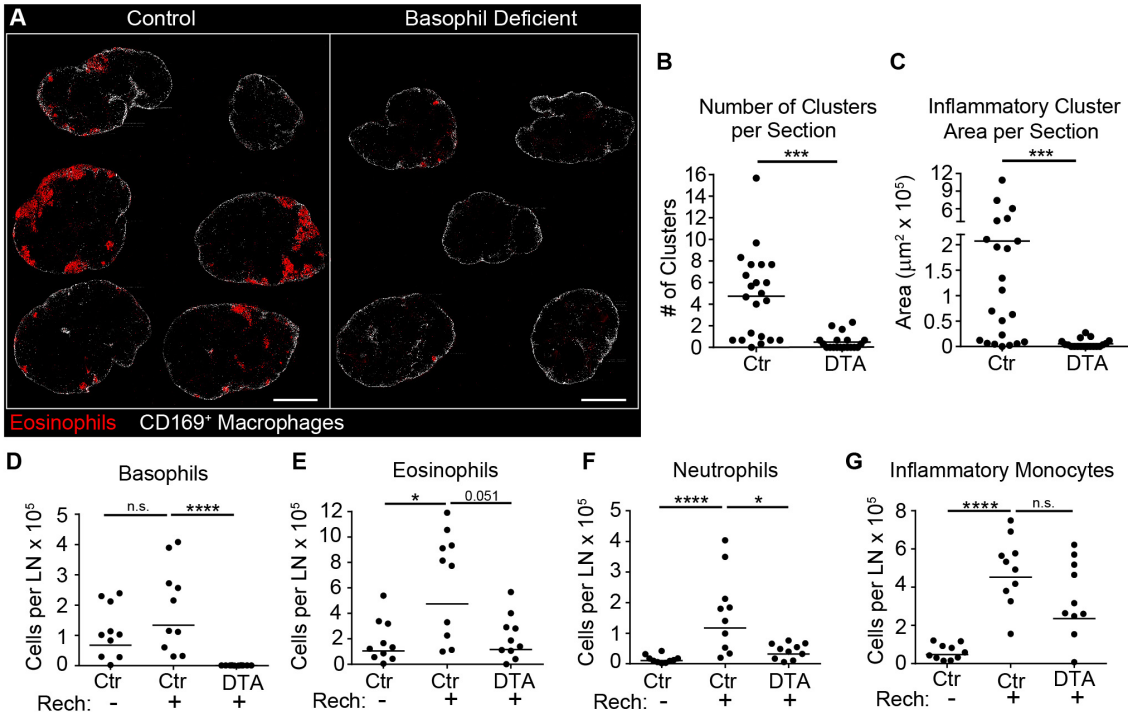

Figure 3.7: Basophils contribute to early inflammatory cluster formation.
Control (Basoph8) and basophil deficient (Basoph8, Rosa26-LSL-DTA) mice were treated with the antigens IgY and adjuvant calcipotriol. Mice were rechallenged (Rech) with IgY 8 days later and draining lymph nodes were analyzed 6 hours after rechallenge. (A) Representative immunofluorescence microscopy images of whole lymph nodes showing inflammatory clusters in control versus basophil-deficient mice after antigen rechallenge. (B-C) Quantification of inflammatory cluster number (B) and size (C) per lymph node. (D-G) Analysis and quantification of the indicated cell populations by flow cytometry in control versus basophil-deficient mice after antigen rechallenge. Scale bars, 1 mm. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001 (Mann-Whitney test [B,C], unpaired one-way ANOVA on log transformed data [D-G]). Ctr, control

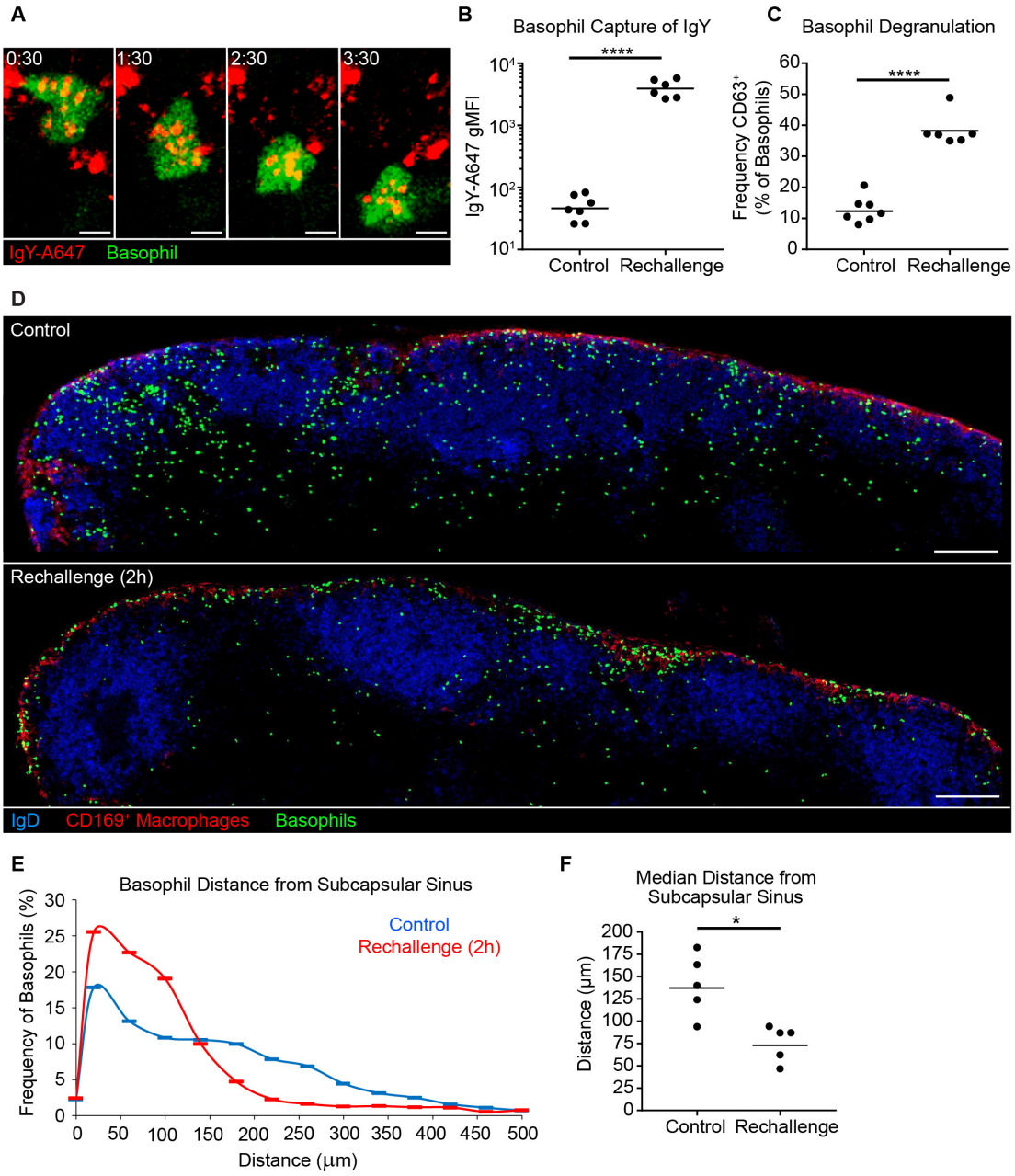

Figure 3.8: Characterization of early basophil responses to antigen rechallenge.
(A-C) Mice were treated with IgY and calcipotriol and then 8 days later, some mice were rechallenged with IgY-A647. (A) Representative time-lapse imaging by two-photon microscopy, showing a basophil that has captured fluorescent antigen within an explanted lymph node 6 hours after rechallenge. (B-C) Flow cytometric quantification of antigen capture (B) and degranulation (C) of basophils within the draining lymph node. The indicated mice had been rechallenged one hour earlier with IgY-A647. (D-F) Immunofluorescence microscopy analysis of draining lymph nodes from Basoph8 mice. The indicated mice had been rechallenged 2 hours earlier with IgY. (D) Representative images of basophil localization. (E) Histogram showing basophil distances from the subcapsular sinus. (F) Quantification of median distance of basophils from the subcapsular sinus in each image. Scale bars, 200 µm. *, $p<0.05$; ****, $p<0.0001$ (unpaired one-way ANOVA on log transformed data [B,C], Mann-Whitney test [F]).

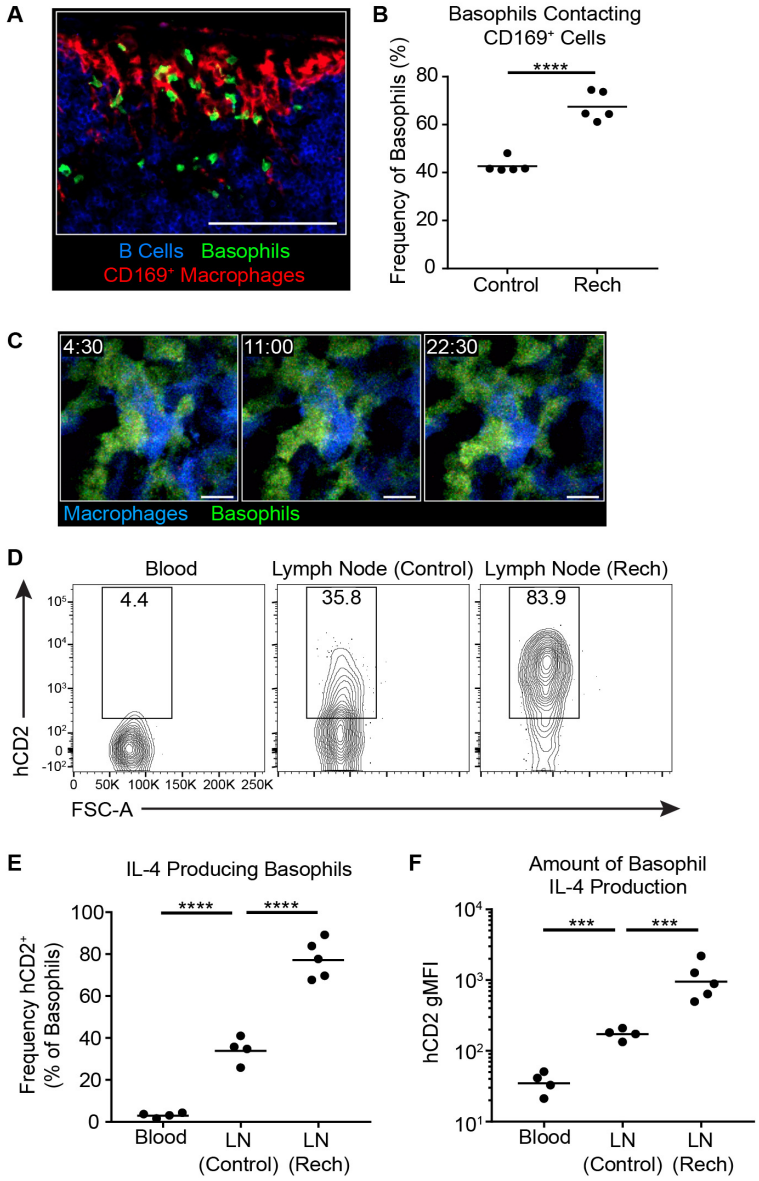

Figure 3.9: Basophils undergo long interactions with macrophages and produce IL-4. Mice were treated with IgY and calcipotriol and then some mice were rechallenged (Rech) with IgY 8 days later. (A-C) Lymph nodes from Basoph8 mice were analyzed 2 hours after antigen rechallenge. (A) Representative immunofluorescence microscopy image of basophils in contact with CD169+ macrophages after antigen rechallenge. (B) Quantification of the proportion of basophils in contact with CD169+ cells. (C) Representative time-lapse images acquired by two-photon microscopy of explanted lymph nodes from Basoph8, CX3CR1-GFP mice after antigen rechallenge. Basophils were visualized as YFP+ cells (green) and macrophages were visualized as CX3CR1-GFP+ cells (blue). (D-F) Basophils in the blood versus the draining lymph nodes were analyzed by flow cytometry in Basoph8, KN2 mice, 6 hours after antigen rechallenge. (D-F) Representative gating (D) and quantification of the frequency (E) and amount (F) of hCD2 surface expression as a reporter for IL-4 production. ***, p<0.001; ****, p<0.0001 (unpaired t test [B] or ordinary one-way ANOVA [E-F] on log-transformed data). gMFI, geometric mean fluorescence intensity.

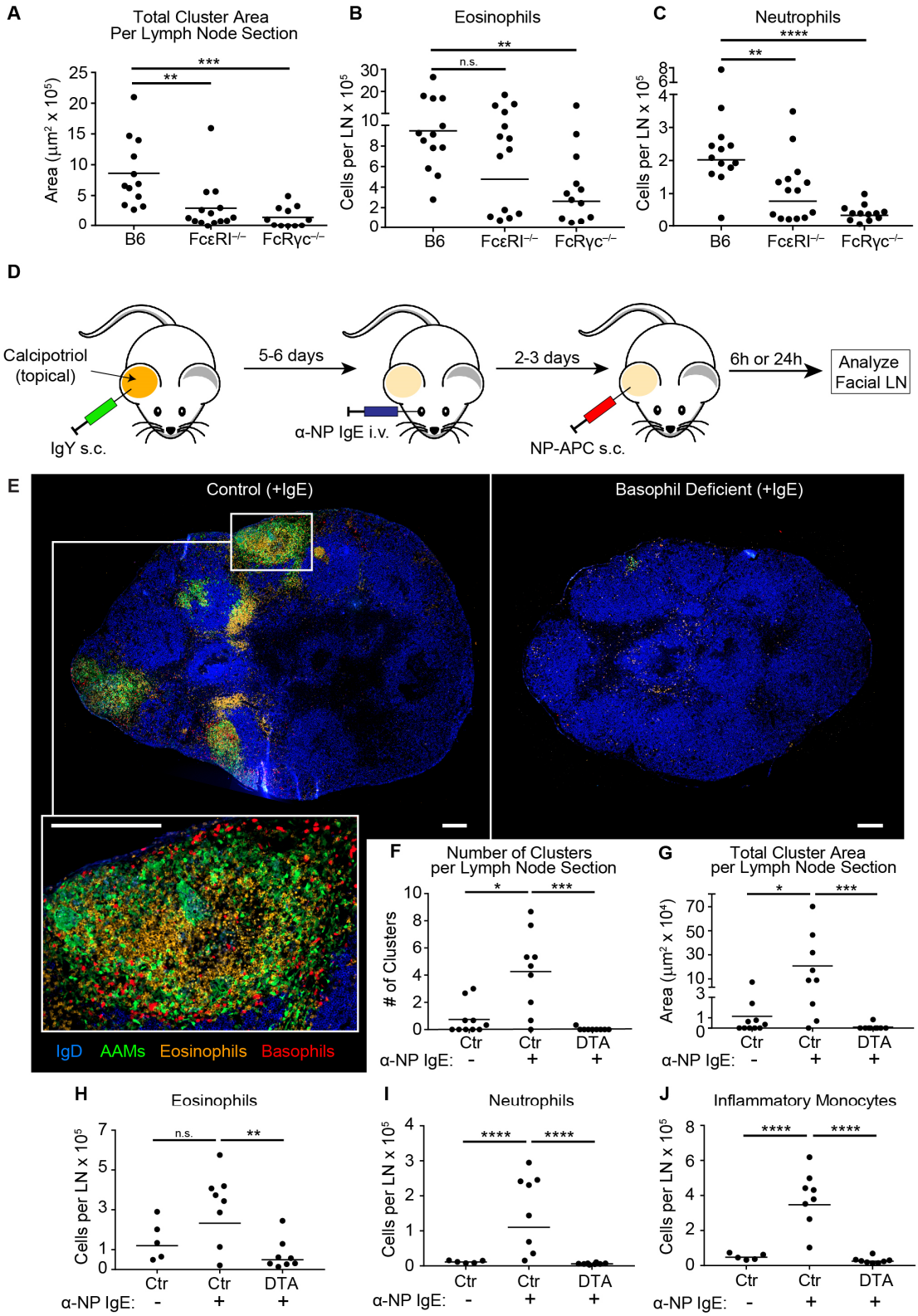
A
Total Cluster Area
Per Lymph Node Section
Area (μm² x 10⁵)
B6
FcεRI⁻/⁻
FcRγc⁻/⁻
B
Eosinophils
Cells per LN x 10⁵
n.s.
C
Neutrophils
D
Calcipotriol
(topical)
IgY s.c.
5-6 days
α-NP IgE i.v.
2-3 days
NP-APC s.c.
6h or 24h
Analyze
Facial LN
E
Control (+IgE)
Basophil Deficient (+IgE)
IgD AAMs Eosinophils Basophils
F
Number of Clusters
per Lymph Node Section
# of Clusters
Ctr
DTA
α-NP IgE:
G
Total Cluster Area
per Lymph Node Section
Area (μm² x 10⁴)
H
Eosinophils
I
Neutrophils
J
Inflammatory Monocytes

Figure 3.10: IgE signaling contributes to the early inflammatory response and is sufficient to promote cluster formation that is basophil-dependent.
(A-C) C57BL/6J (B6), FcεRI$^{-/-}$, or FcRγc$^{-/-}$ mice were treated with IgY and calcipotriol then rechallenged with IgY. Draining lymph nodes were analyzed 6 hours after rechallenge. (A) Quantification of inflammatory clusters in immunofluorescence microscopy images of tissue sections. (B-C) Quantification of the abundance of eosinophils (B) and neutrophils (C) by flow cytometry. (D) Diagram of the experimental design for (E-J). Mice were treated with IgY and calcipotriol on the ear, were passively sensitized with anti-NP IgE intravenously (i.v.) on day 5-6, and then challenged with NP-APC subcutaneously (s.c.) in the ear 2-3 days later. Draining facial lymph nodes were analyzed 24 (F-G) or 6 (H-J) hours later. (F-G) Quantification of number (F) and size (G) of inflammatory clusters in immunofluorescence microscopy images of tissue sections of the lymph nodes from control (YARG) versus basophil-deficient (Basoph8, Rosa26-LSL-DTA, YARG) mice. (H-J) Quantification of the number of the indicated cell types by flow cytometry in control versus basophil-deficient (DTA) mice. Scale bars, 200 μm. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$ (Mann-Whitney test [A,F,G)] and one-way ANOVA on log-transformed data [B,C, H-J]). Ctr, control

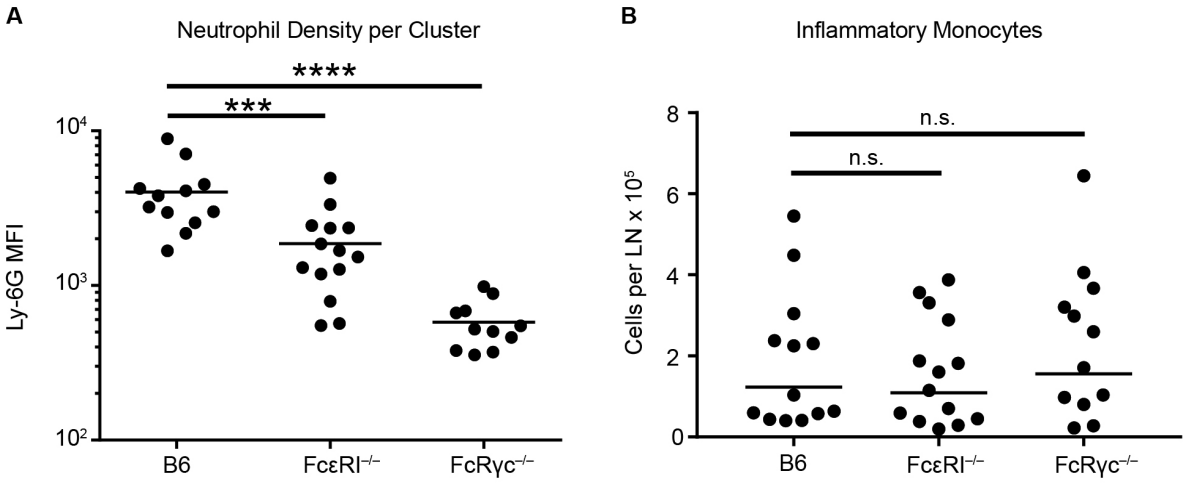

Figure 3.11: Neutrophil clustering and monocyte recruitment in FcR-deficient mice. C57BL/6J (B6), FcεRI$^{-/-}$, or FcRγc$^{-/-}$ mice were treated with IgY and calcipotriol then rechallenged with IgY 8 days later. Draining lymph nodes were analyzed 6 hours after rechallenge. (A) Quantification of neutrophil density by measuring Ly-6G mean fluorescence intensity in immunofluorescence microscopy images of tissue sections. (B) Quantification of the numbers of inflammatory monocytes in the draining lymph nodes by flow cytometry. ***, $p<0.001$; ****, $p<0.0001$ (one-way ANOVA on log-transformed data).

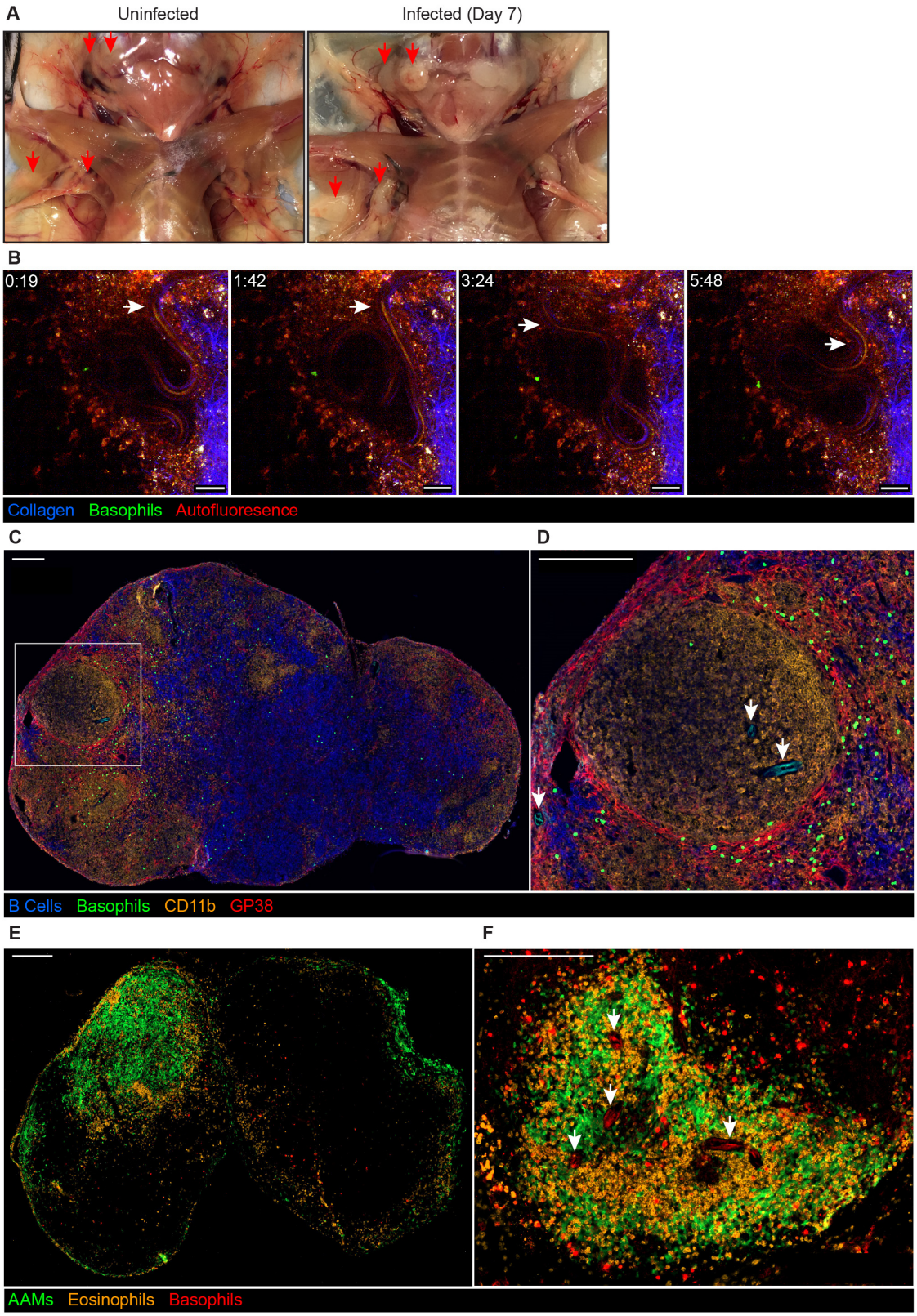
A
Uninfected
Infected (Day 7)
B
0:19
1:42
3:24
5:48
Collagen Basophils Autofluoresence
C
D
B Cells Basophils CD11b GP38
E
F
AAMs Eosinophils Basophils

Figure 3.12: Infection of lymph nodes with filarial larvae leads to the formation of inflammatory clusters containing basophils.
(A-B) Mice were infected with *B. pahangi* L3 larvae by subcutaneous injection into the scruff of the neck. (A) Representative stereomicroscopy images of superficial cervical, facial, brachial, and axillary lymph nodes in uninfected or infected mice (red arrows, lymph nodes). (B) Time lapse images acquired by two-photon microscopy of explanted lymph nodes from Basoph8 mice one day after infection. Collagen (second harmonic generation, blue), Basophils (YFP^+, green) and autofluorescence (red) were visualized. (C-F) Mice were first immunized with freeze-killed *B. pahangi* L3 larvae i.p., then infected with live larvae 2-3 weeks later subcutaneously in the scruff of the neck. Superficial cervical lymph nodes were removed for immunofluorescence microscopy analysis 4 days after infection of Basoph8 (C,D) or YARG (E-F) mice. Cells in (C,D) were identified as B cells (IgD^+), basophils (YFP^+), $CD11b^+$ cells, and FRCs ($GP38^+$), or in (E,F) as AAMs (YFP^+), eosinophils ($Siglec\text{-}F^+$), and basophils ($CD200R3^+$). Scale bars, 50 μm (B), 200 μm (C-F).

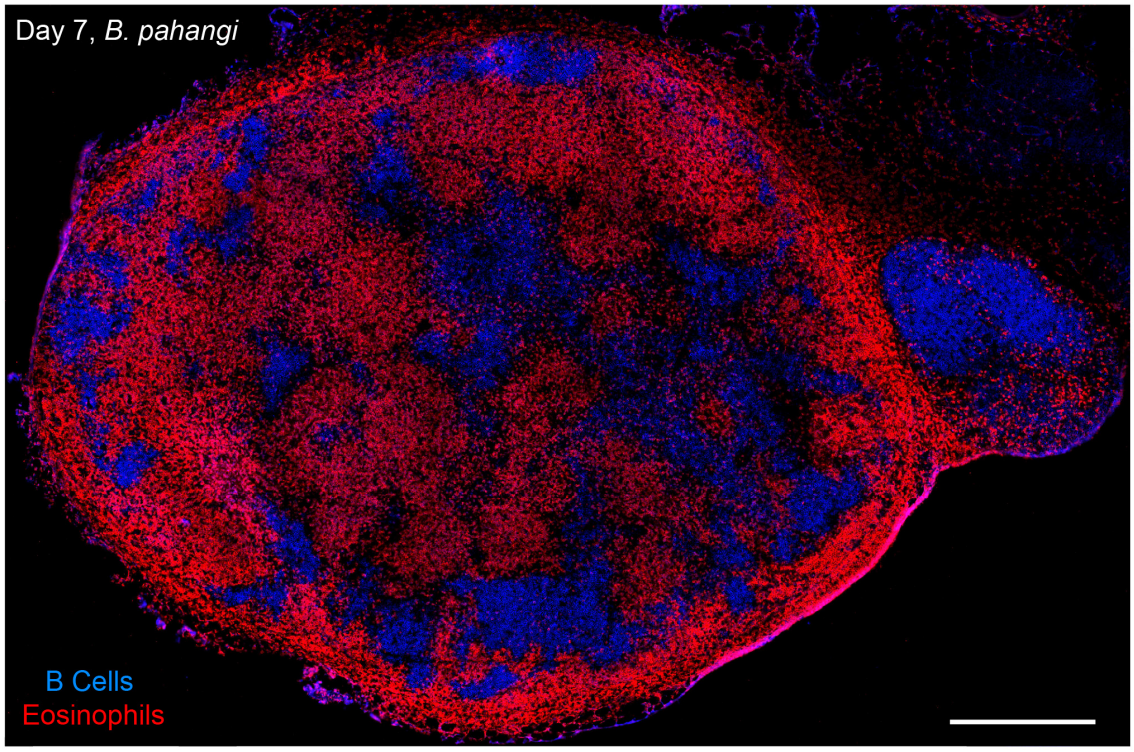

Figure 3.13: Lymph node response to B. pahangi 7 days after infection.
Mice were infected with *B. pahangi* L3 larvae by subcutaneous injection into the scruff of the neck. Representative immunofluorescence microscopy image of a tissue section from a whole superficial cervical lymph node. Cells were identified as B cells (IgD^+) and eosinophils (Siglec-F^+). Scale bar, 500 μm.

Methods

Mice

C57BL/6J (B6, 000664), BALB/cJ (000651), and A/J (000646) mice originated from the Jackson Laboratory. All other mice were maintained on a C57BL/6 background. Basoph8, KN2, YARG, TSLPR KO, and CD4cre-IL4/13flox founder mice were originally provided by the laboratory of Dr. Richard Locksley. To achieve basophil deficiency, Basoph8 mice were bred to Rosa26-stopflox-DTA mouse strains that were derived from either the Locksley lab or purchased from Jackson (010527). Similar levels of basophil ablation were achieved when using either DTA mouse strain. CX3CR1-GFP (B6.129P2(Cg)-*Cx3cr1*^{tm1Litt}/J) mice were purchased from Jackson (005582) and maintained. FcεRIα KO mice (Fcer1a^{tm1Knt}) and FcRγc KO mice (Fcer1g^{tm1Rav}) were also maintained on a B6 background (Dombrowicz, Flamand, Brigman, Koller, & Kinet, 1993; Takai, Li, Sylvestre, Clynes, & Ravetch, 1994). All mice were housed in UCSF specific-pathogen-free facilities and protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco.

IgE Antibody and Antigen Haptenation

The heavy chain VDJ segment encoding a BCR specific for NP was cloned from B1-8i mice and fused to an IgE constant region. An additional point mutation of Trp33->Leu ("B1-8hi") was induced in the antigen binding region to increase the affinity by approximately 10-fold. This B1-8hi IgE construct was transduced and expressed in J558L cells, which express the λ1 light chain. Concentrated NP-specific IgE was harvested using a Corning CELLine Disposable Bioreactor flask (Fisher Scientific, Cat#10-126-4). The concentration of NP-specific IgE in the concentrated supernatant was determined by ELISA. The equivalent of 20ug of NP-specific IgE was injected retro-orbitally into mice.

NP-conjugated allophycocyanin (NP-APC) was synthesized as described, based on the original protocol of Michael Mckeyzer Williams (L. J. McHeyzer-Williams & M. G. McHeyzer-Williams, 2004). Briefly, activated NP-OSU (Biosearch technologies Inc. Cat#N-1010-100) was reacted with allophycocyanin (Prozyme, Cat#PB20) at a ratio of 80 ug NP-OSU per 1 mg of allophycocyanin for 2 hours in 3% sodium bicarbonate buffer. Buffer exchange into PBS was performed using a Bio-Spin 30 column (Bio-rad laboratories, Cat#732-6231).

In Vivo Immunization and Challenge

To induce basophil recruitment into draining lymph nodes, mice were topically treated on the ears with calcipotriol (also referred to as MC903; 20uL of 0.2mM per ear) (Fisher Scientific, Cat#10009599) and subcutaneously injected with various antigen (10ug in 20uL per ear). Antigen included: NP-CGG (conjugation ratio 30-39, Biosearch Technologies, Cat# N-5055D-5), ChromPure Chicken IgY (Jackson Immunoresearch, Cat#003-000-003), NP-KLH (conjugation ratio 32, Biosearch Technologies, N-5060-5), NP-APC (described above), EndoGrade Ovalbumin (Biovendor R&D, Cat#321001), and Chrompure Goat IgG (Jackson Immunoresearch, Cat# 005-000-003). For some experiments, rather than calcipotriol, papain (10ug in 20uL per ear) (EMD Chemicals, Cat#5125-50GM) or alum (Diluted 1:1 with antigen solution) (Alhydrogel^{tm}, Fisher Scientific, Cat# NC9024010) were injected subcutaneously along with the antigen. The draining facial lymph node was subsequently removed for analysis. For rechallenge experiments, mice were reinjected subcutaneously with NP-CGG or IgY 8 days after the first immunization and lymph nodes analyzed according to time points indicated in the Results. To test the sufficiency of IgE activation on the inflammatory response, anti-NP IgE (described above) was retro-orbitally injected into mice on day 5 or 6. Mice were subsequently challenged with NP-APC on day 8 and lymph nodes analyzed according to time points indicated. Prior to dissection, mice were euthanized using a lethal exposure of isoflurane in a sealed glass container.

Infection of Brugia pahangi

Live *Brugia pahangi* L3 larvae were obtained from the laboratory of Dr. Brenda Beerntsen (Veterinary Pathobiology, University of Missouri-Columbia). To study the primary response, mice were injected subcutaneously in the scruff of the neck with 100 or 500 L3 larvae in 100 or 200 µL HBSS respectively using a 21-26 gauge needle (BD). To study a rechallenge response, mice were first primed through an intraperitoneal injection of frozen larvae that were also homogenized using a Scilogex D160 Homogenizer (Lab Supply Network). Primed mice were challenged with 400 live L3 in 200 µL HBSS subcutaneously in the scruff of the neck two to four weeks later. Facial, superficial cervical, brachial, and axillary lymph nodes were analyzed at time points indicated in the Results.

Isolation of Single Cells for Flow Cytometry

For experiments not involving macrophages or stromal populations, lymph nodes were mechanically dissociated through a 70-µm nylon cell strainer (Falcon) in Dulbecco's modified Eagle medium (DMEM; Cellgro) supplemented with antibiotics (penicillin 100 IU/mL and 100 mg/mL streptomycin; Gibco, Carlsbad, Calif), L-glutamine (Gibco), 10 mmol/L HEPES (Gibco; supplemented DMEM), and 2-10% FBS (Gibco). For experiments involving macrophages and stromal populations, lymph nodes were first enzymatically digested instead of being mechanically dissociated through a cell strainer. Specifically, lymph nodes were placed in a 1.5mL Eppendorf tube and snipped in 1mL of digestion media containing Dispase II (0.8mg/mL) (Life Technologies Corporation, Cat#17105041), Collagenase D (0.2mg/mL) (Roche Applied Science, Cat#11088874103), and DNAse I (0.1mg/mL) (Sigma-Aldrich, Cat#10104159001). Tubes were then placed on a thermomixer at 37^{o}C and 800-1000rpm. After 10-20 minutes, tissue was further dissociated by pipetting, and supernatant transferred to separate container of 5mL iced FACS buffer (PBS with 2% FBS, and 1 mM EDTA (Gibco)). Undigested tissue was

added to a new solution of 1mL digestion media, placed back on the thermomixer, and dissociated through pipetting 10-20 minutes later. If the tissue was not fully dissociated, this cycle was repeated once more. Cell suspensions were then concentrated upon spinning at 350g, for 5 minutes at 4 ^{o}C. Final lymph node cell counts were accomplished through a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter).

Flow Cytometry Antibody Staining and Analysis

Three million cells per sample were stained in a 96 well plate. Basophils were detected either through YFP fluorescence in Basoph8 mice or detected as CD45^{int}, CD200R3^{hi}, and IgE^{hi}. Degranulation was detected by CD63 and IL-4 was determined through human CD2 expression in KN2 mice. Eosinophils were gated as CD11b^{hi}, Siglec-F^{hi}, Ly-6G^{int-lo}, neutrophils as CD11b^{hi}, Siglec-F^{int-lo}, Ly-6G^{hi}, inflammatory monocytes as CD11b^{hi}, Siglec-F^{lo},Ly-6G^{lo}, Ly-6C^{hi}, and fibroblastic reticular cells as CD45^{lo}GP38^{hi}, CD31^{lo}. YARG mice, which report Arginase-1 as YFP, were used to detect AAMs. When YARG was used to detect AAMs, monocyte-macrophages were considered one population and were distinguished by being CD11b^{hi}, I-Ab (MHC-II)^{hi}, CD64^{hi}. YARG YFP was gated in contrast to BV510 to separate true signal from monocyte-macrophage autofluorescence. Data were collected on an LSRFortessa (BD) with FACSDiva software (BD) and analyzed with FlowJo software (TreeStar). Surface staining was done at 4^{o}C for 20-60 minutes. For intracellular stains, cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD) for 20 minutes, then washed with Perm/Wash (BD) according to manufacturer's instructions. Intracellular proteins were then stained for 30 minutes at 4 ^{o}C.

Cytokine Detection of Restimulated T cells

Three million cells per lymph node were restimulated in 96 well plate in the presence of Ionomycin (Sigma-Aldrich, Cat#I0634-1MG), PMA (Sigma-Aldrich, Cat#P8139-1mg), and Brefeldin A (Life Technologies Corporation, Cat# 00-4506-51) for three hours at 37^{o}C and 5%

CO_2. After restimulation, cells were washed, and then stained for both surface and intracellular markers as described above.

Immunofluorescence Microscopy and Quantification

Lymph nodes from fluorescent reporter mice were prepared as described (Z. Yang et al., 2012). In brief, lymph nodes were dissected and fixed in 4% paraformaldehyde (diluted in PBS) (VWR International,Cat# 100504-858) for 2 hours. Fixed lymph nodes were washed with PBS 3x and then dehydrated in a 30% sucrose solution (diluted in PBS) (VWR International, Cat# EM-8510) overnight at 4^{o}C on a tube rotator. Subsequently, lymph nodes were embedded in Tissue-TEK OCT compound (Sakura) in cryo-molds and snap-frozen in a dry-ice ethanol bath and stored at -80^{o}C. When it was not necessary to detect intracellular components such as YFP, lymph nodes were immediately snap-frozen in OCT without prior fixation. Lymph node sections of 7-10 μm thickness were subsequently prepared on a Leica CM3050S cryostat. All sections were additionally fixed in cold acetone for 10 minutes. Sections were rehydrated in PBS and then stained with PBS containing 0.1% bovine serum albumin (Sigma-Aldrich, Cat# 03116956001), 1% normal mouse serum (Jackson ImmunoResearch, Cat#015-000-120), and diluted antibodies. Each stain step was done in a humidified chamber for 2-3 hours at 20-25^{o}C. Cover slips were mounted in Fluoromount-G (Fisher Scientific, Cat#OB10001) when using PBS as the primary buffering solution or MWL 4-88 glycerol/tris mounting solution (CITIFLUOR, Cat#MWL4-88-25) when using TBS as the primary buffering solution.

An Axioscan Z1 slide scanning microscope (Zeiss) was used to collect tiled images of all lymph nodes on slides after immunofluorescence staining. Analysis of inflammatory cluster number, area, and mean fluorescence intensities was performed using Zen Blue software (Zeiss). Regions were drawn around inflammatory foci consisting of dense clusters of eosinophils and/or alternatively activated macrophages. For most analysis, clusters with an area less than 5,000

μm^2 were considered too insignificant for inclusion. The first three sections per lymph node, spaced approximately 100 μm apart, were analyzed and values averaged. For Figure 3.5, foci with an area less than 10,000 μm^2 were not included and six sections were taken instead of three. For multinucleated giant cell analysis, only one section per lymph node was analyzed. Multinucleated cells were determined using DAPI, to detect nuclei number and a combination of CD11c, and YARG YFP signal to distinguish individual cells. Regions were drawn around each individual cell that possessed more than one nucleus to determine area per cell. The number of nuclei per cell was counted manually.

To quantify basophil localization in histological sections, we detected YFP^+ basophils using the Basoph8 reporter amplified with Rabbit-anti-GFP antibody (Life Technologies) in lymph nodes that had been sliced from the cortical to the medullary region. Imaris (Bitplane) software was used to detect YFP^+ basophils, which were converted into "spots." The subcapsular sinus, HEVs, lymphatic endothelium, $CD169^+$ cells, B cell follicles, and interfollicular regions were converted into "surfaces." Distances of basophils to various regions or cells was calculated using the distance transformation function. To determine contacts of basophils with $CD169^+$ cells, the "distance transformation" function was used to find basophils that were in very close proximity to CD169+ surfaces, then true basophil-CD169 contacts were confirmed manually.

Two-photon Imaging and Data Analysis

Explanted lymph nodes were prepared as detailed in the methods of Sullivan et al 2011 (Sullivan et al., 2011). Samples were imaged on a LSM 7 MP Indimo two-photon microscope (Carl Zeiss MicroImaging) customized with three laser lines derived from two Ultra II Chameleon lasers (Coherent) and a Compact OPO (Coherent), as well as 4 sensitive GaAsP and 1 far red sensitive non-descanned detectors. For live imaging, GFP and YFP fluorophores were excited between 930-1020 nm and recorded simultaneously. Alexa-647 (conjugated to IgY) was excited

at 1200 nm using the Compact OPO. Collagen was visualized using second harmonic generation. For live imaging, *z*-stacks were typically collected every 30 s for 30 mins. For imaging *B. pahangi*, z-stacks were collected every 4 seconds for several minutes. Band pass filters used to record fluorescence signals from each fluorophore are listed in Table 1. Each *xy* plane was collected at a resolution of 512 x 512 pixels with variable zoom. The spacing between planes in the *z* dimension was set to three to five times the *xy* pixel size for a given zoom setting as described in Sullivan et al 2011. Zen Black (Zeiss) was used to acquire images from the LSM 7 MP. Volocity (PerkinElmer) or Imaris software were used for further analysis of acquired images.

Table 3.1: Filters for LSM 7 MP

Fluorescence	Band Pass Filter	Vendor
Second Harmonic	472/30	Semrock
Broad GFP/YFP	525/50	Chroma
Narrow GFP	510/20	Semrock
Narrow YFP	539/30	Semrock
Alexa 647	670/50	Chroma

Table 3.2: Filters for Axioscan Z1

Fluorescence	Excitation	Dichroic	Emission	Vendor
DAPI, eFluor 450	370/60	425	460/50	Chroma
Alexa 488	470/40	495	525/50	Zeiss
PE	546/12	560	607/80	Zeiss
Alexa 647, APC, eFluor 660	620/60	660	700/75	Chroma

Table 3.3: List of Antibodies Used

Antigen	Fluorophores	Vendors	Clone	Assay
CD200R3	APC, PE	Biolegend	Ba13	FC, IF
CD131	PE	BD Biosciences	JORO 50	FC
IgE	FITC	Biolegend	RME-1	FC
IgE	Biotin	BD Biosciences	R35-118	FC
Ly-6C	Pcpcy5.5, BV711, PE, Biotin	Biolegend	HK1.4	FC, IF
CD19	PECy7	BD Biosciences	1D3	FC
CD19	PEDazzle594	Biolegend	6D5	FC
CD45	A700	Biolegend	30-F11	FC
--	eFluor780 (Viability Dye)	Life Technologies	--	FC
Siglec F	BV421, PE	BD Biosciences	E50-2440	FC, IF
Ly-6G	BV510, Biotin	Biolegend	1A8	FC, IF

Antigen	Fluorophores	Vendors	Clone	Assay
CD11b	BV785, A647, Biotin	Biolegend	M1/70	FC, IF
CD11c	PE, A647, BV650, A647	Biolegend	N418	FC, IF
Biotin	Streptavidin-A647,	Life Technologies	--	IF, FC
Biotin	Streptavidin-Qdot605	Life Technologies	--	IF, FC
Rabbit IgG	A488	Jackson ImmunoResearch	--	IF
IgD	eFluor450	Fisher Scientific	11-26c	IF
CD169	eFluor660	eBioscience	Ser-4	IF
Rat IgM μ chain	Cy3	Jackson ImmunoResearch	Polyclonal	IF
Rat IgG (H+L chains)	Cy3	Jackson ImmunoResearch	Polyclonal	IF
LYVE-1	PE	Life Technologies	ALY7	IF
Arm. Hamster IgG	AMCA	Jackson ImmunoResearch	Polyclonal	IF
GP38	Biotin, APC	eBioscience	8.1	IF, FC
VCAM-1	PE	Life Technologies	429	FC
CD4	PECy7	Life Technologies	GK1.5	FC
--	NP-APC	--	--	FC
CD64	PECy7	Biolegend	X54-5/7.1	FC
I-Ab	A647	Biolegend	KH74	FC
CD24	BV421	Biolegend	M1/69	FC
CD63	PECy7, APC	Biolegend eBioscience	NVG-2	FC
CD49b	Biotin, APC	Biolegend	DX5	FC
--	IgY-A647	Jackson ImmunoResearch	--	FC, 2P
Thy1.2	PE	SRW	30-H12	FC
CD31	Biotin	Biolegend	390	FC, IF
hCD2	Biotin	Biolegend	RPA-2.10	FC
--	DAPI	Sigma-Aldrich	--	FC, IF
VCAM-1	Purified	eBioscience	429	IF
GFP	Purified	Invitrogen	Polyclonal	IF
CD3e	Purified	eBioscience	eBio500A2	IF
PNAd	Purified	Biolegend	MECA-79	IF
CD16/32	Purified	Biolegend	93	FC

*IF – Immunofluorescence, FC – flow cytometry, 2P – 2 Photon
** All antigen are mouse unless otherwise specified

Chapter 4: Conclusion

Through this work we have gained greater insight into the in vivo importance of basophil function, particularly through IgE/FcεRI-mediated signaling. Our work highlights the importance of IgE-allergen affinity in basophil activation, as revealed through in vitro and in vivo studies using low and high affinity antigen. This research also reveals how IgE/FcεRI-activated basophils promote allergic inflammation and the formation of organized inflammatory cell clusters on a spatial-temporal level. Lastly, we have provided evidence that a primary function for the recruitment of basophils into the lymph node is to augment a local protective response to a perceived threat (e.g. parasite) that has entered into the organ from the afferent lymphatics. This work therefore further establishing the lymph node as a site of barrier defense, in particular, against parasites that migrate through lymphatic vessels.

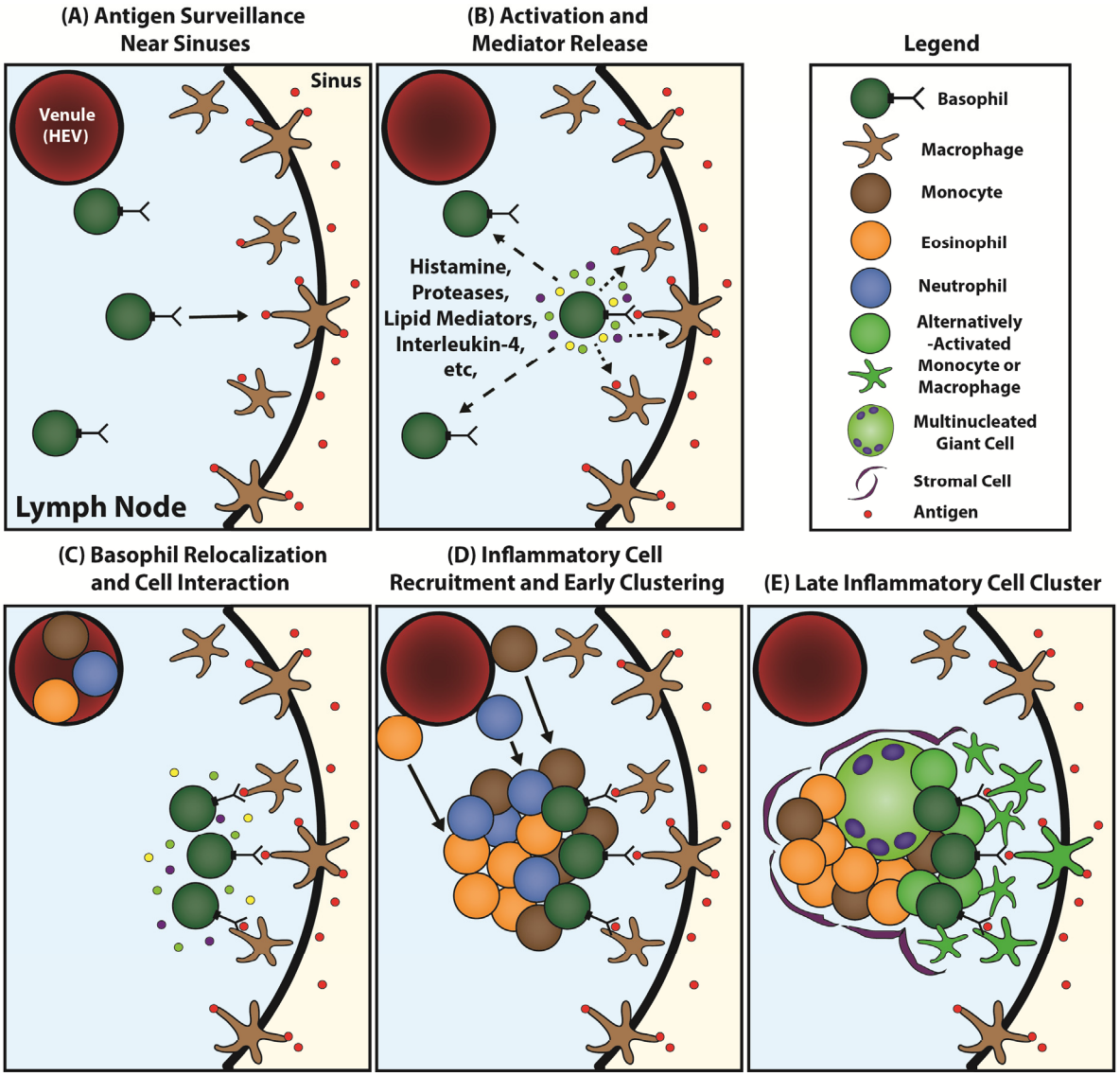

Figure 4.1: Diagram of Basophil-Mediated Inflammatory Response in the Lymph Node. (A) Upon entry, basophils localize to regions with high potential for foreign antigen exposure. (B) Basophil activation by antigen through IgE/FcεRI leads to the release of various inflammatory mediators. (C). Following activation, basophils relocalize closer to the subcapsular sinus, increase interactions with nearby macrophages, and continue to secrete inflammatory mediators, such as IL-4. Basophils directly or indirectly (e.g. macrophages) promote the recruitment of eosinophils, neutrophils, and monocytes. (D) Inflammatory cells are recruited and cluster at the site of first antigen exposure. (E) In later stages, monocytes/macrophages differentiate to become alternatively activated or multinucleated giant cells, neutrophils undergo apoptosis, and stromal cells reorganize around the emerging inflammatory cell cluster.

The Role of Affinity on Basophil Activation through IgE/FcεRI

Basophil activation through IgE/FcεRI is crucially dependent upon the affinity of IgE for its cognate antigen. While some studies have revealed the importance of affinity on basophil activation, the extent to which IgE affinity intrinsically enhances FcεRI downstream responses had not been elucidated prior to our work. It was unclear whether higher affinity antigen results in enhanced basophil activation merely because of an increase in IgE/FcεRI receptor occupancy at a given concentration of antigen or because higher affinity IgE-antigen interaction inherently promotes greater FcεRI-mediated signaling. We found that, even when IgE/FcεRI receptor occupancy by antigen was comparable, higher IgE affinity enhanced basophil degranulation and IL-4 production compared to lower IgE affinity. The affinity of IgE also had a similar effect on mast cell degranulation in vitro, basophil degranulation in vivo, and anaphylaxis. Ultimately, our results reveal that IgE-antigen affinity is inherently critical for IgE/FcεRI-mediated signaling and downstream pathological responses.

Our work suggests that IgE affinity is a primary predictor for the severity of downstream allergic responses. We found that the subcutaneous injection of high affinity antigen in passively sensitized mice resulted in systemic anaphylaxis; however, the injection of low affinity antigen did not result in anaphylaxis, even when relative antigen concentrations were adjusted to achieve similar receptor occupancy on mast cells and basophils. The physiological significance of this experiment may be found in events during which allergen is injected such as stings or bites. In these situations, some injected allergen inevitably escapes from the skin into the bloodstream where, in previously sensitized individuals, mast cells and basophils can respond through allergen-specific IgE/FcεRI. Nonetheless, in some incidences of skin exposure such as a sting, the amount of allergen that enters into the bloodstream may actually be very limited. Because of this low antigen concentration, we would predict the affinity of IgE to be especially critical for whether an individual experiences no detectable response, a mild response, or a

severe, systemic anaphylactic response. Other allergic reactions due to different routes of exposure would also be worsened by higher affinity IgE, though increased intake of allergen (such as in the case of ingestion) may compensate for lower affinity IgE, still leading to poor outcome in some circumstances.

The kinetic proofreading model used to explain why IgE affinity is critically important for FcεRI-mediated activation had been studied in the literature but less clearly demonstrated in primary basophils. Previous work in both RBL cells and primary mast cells revealed that higher affinity antigen led to specific increases in Syk phosphorylation, degranulation, and the synthesis of numerous cytokines (Goldstein et al., 2008; Z. J. Liu et al., 2001b; R. Suzuki et al., 2014; C. Torigoe et al., 1998). The superiority of higher affinity antigen to lead to these effects remained true even when the levels of FcεRI phosphorylation induced by high and low affinity antigen were comparable. This phenomenon supported the notion of a kinetic proofreading mechanism in FcεRI-bearing cells (explained in the Introduction) (Lyons et al., 1996; McKeithan, 1995; Rabinowitz et al., 1996). In our study, basophil degranulation and IL-4 production were both significantly induced by high affinity antigen-IgE/FcεRI interaction compared to low affinity interaction even when antigen-receptor occupancy was comparable between the two affinity conditions. This result supports the notion that that FcεRI-mediated signaling leading to degranulation and IL-4 production in primary basophils is also constrained by a kinetic proofreading mechanism.

Future Directions

Given our data, it is tempting to conclude that the activation of IgE/FcεRI with higher affinity antigen, relative to lower affinity antigen, merely results in a uniform increase of all associated signaling pathways; however, that may not be true. As mentioned previously (in the

Introduction), the production of some molecules, particularly certain chemokines, have been found to escape kinetic proofreading of FcεRI signaling (Gonzalez-Espinosa et al., 2003b; Hlavacek et al., 2001; Z. J. Liu et al., 2001b; R. Suzuki et al., 2014; C. Torigoe et al., 1998). A more recent report had demonstrated that mast cells can productively signal in response to lower affinity antigen stimulation of IgE/FcεRI through different pathways than higher affinity antigen stimulation. This was attributed to higher affinity antigen stimulation more effectively activating the Syk and LAT1 pathway, whereas lower affinity antigen stimulation signaled through a Fgr/LAT2-associated pathway. Through this divergent signaling cascade, higher affinity antigen led to enhanced degranulation, leukotriene synthesis, and inflammatory cytokine synthesis (TNF-alpha, IL-6, IL-13), and in vivo neutrophil recruitment while lower affinity antigen provoked the production of more chemokines (CCL2,3,4) and in vivo monocyte recruitment (R. Suzuki et al., 2014). Worth mentioning, separate studies have demonstrated that the signaling requirements for the induction of IL-4 transcription and secretion are different than degranulation, appearing more similar to some chemokines such as CCL2 (MCP-1) (Gonzalez-Espinosa et al., 2003b; S. L. Schwartz et al., 2017). Nonetheless, in our study, both basophil degranulation and IL-4 production were similarly affected by IgE affinity. It is unclear whether the production of other basophil-derived compounds would be constrained by IgE affinity. It would be worth investigating how affinity affects both the differential phosphorylation of basophil-associated signaling proteins as well the production of other mediators, especially chemokines.

In addition to testing how IgE affinity differentially affects basophils to produce various compounds, this study also provides a basis in which to test the importance of IgE affinity on downstream inflammatory processes *in vivo*. In our studies as well as other studies, basophils have been shown to recruit neutrophils, monocytes, eosinophils, and to polarize macrophages to become alternatively activated. Basophils have also been demonstrated to organize these

inflammatory cells at relevant sites of antigen (or potentially parasitic) exposure. Basophils may even act directly on certain epithelial cells (or any nearby stromal cells) and induce changes such as proliferation, leading to compartmental thickening and exclusion (Hayes et al., 2020; Tabakawa et al., 2018). Given that effective basophil-dependent models of inflammation in the skin (chronic allergic inflammation) and lymph node (allergic lymphadenitis) now exist, a next step would be to test how affinity affects the downstream function of basophils in these contexts. Since our work demonstrated that basophil degranulation was highly dependent upon IgE affinity, it would not be surprising for inflammatory events related to histamine or protease release to be impacted in a likewise manner. Similarly, since IL-4 production was also highly dependent on IgE affinity, it would be worth investigating downstream responses to IL-4 such as macrophage polarization or eosinophil recruitment. Physiologically, IgE contributes to protection from reinfection with numerous parasites such as *N. brasiliensis*, *H. polygyrus*, and ticks. It would be useful to confirm just how important IgE affinity is in these contexts. Given that some parasite infections can lead to massive IgE production, some of which may not be specific to the parasite itself, antigen affinity for a minor parasite-specific subset of IgE molecules (out of total bound IgE on basophils) may have even greater importance. Ultimately, the findings of our study have provided a foundation on which to better explore the importance of IgE affinity on basophil function in vivo. It will be of great interest to know whether affinity is preferentially more important for certain functions over others.

Basophil Effector Function in the Lymph Node

Basophils in the lymph node were found to promote a local allergic inflammatory response after re-exposure of the lymph node to allergen. Though numerous reports had suggested that basophils accumulate into lymph nodes to enhance an adaptive immune response, in our study, the most striking contribution of basophils was in promoting an inflammatory response within the lymph node. Initially we had been optimistic about the potential for basophils to enhance an IgE

response since basophils produce IL-4, a key cytokine in IgE B cell differentiation, but we could not detect any consistent reduction of IgE plasma or germinal center cells in basophil-depleted mice using a variety of immunization conditions (data not shown). This suggested to us that the role of basophils in the lymph node may not primarily be to enhance the adaptive response, although that has been the predominant hypothesis. Instead, we found that basophils could respond to a re-exposure of allergen in the lymph node by promoting the recruitment of neutrophils, eosinophils, and monocytes; organizing these cells, along with local stroma and macrophages, into clustered foci; and enabling the differentiation of monocytes or macrophages to become alternatively activated or fused multinucleated giant cells. FcεRI signaling was found to be important for the organization of these clusters. Moreover, IgE-induced activation of basophils was found to be sufficient to induce an allergic inflammatory response and cell cluster formation after antigen challenge, independent of T cell restimulation by cognate antigen. We had also studied the spatial and behavioral changes associated with basophil restimulation by antigen in the lymph node. Basophils primarily localize in regions close to lymph node sinuses, where antigen exposure is more likely to occur. However, upon antigen re-exposure, basophils relocalize more tightly to the sinuses and increase contact with CD169+ cells, most of which are likely macrophages. Live imaging revealed that basophils commonly interact with CX3CR1+ macrophages for long durations. Antigen re-exposure may also increase the propensity for basophil-macrophage interactions (data not shown). At the same time, activation of basophils also resulted in degranulation and enhanced production of IL-4, both of which likely promoted the allergic inflammation. To examine whether basophils were involved in a more physiologically relevant condition of allergic lymphadenitis compared to our IgY and calcipotriol model, we infected mice with the lymphatic-dwelling helminth, *Brugia pahangi*. After infection, larvae from this parasite were clearly detected in the lymph nodes of mice, leading to a massive inflammatory response of similar cellular composition to what we had observed in our IgY and calcipotriol model. Importantly, basophils were found to be associated with inflammatory cell

clusters that had formed in response to the parasitic invasion of the lymph node. Altogether, these data suggest that the recruitment of basophils into the lymph node is primarily a defensive measure by which basophils enhance a local allergic inflammatory response.

Our data has revealed useful insights into the spatial-temporal nature of inflammatory cluster formation as it relates to basophils. Based on our study, we propose a revised model for basophil-induced inflammation (Figure 1). Though the context of this model comes from the lymph node, features of this model likely apply to other organs such as the skin or lungs. First, upon entry, basophils migrate to regions in the tissue which possess high exposure to potential antigen. In the lymph node, these locations are those in proximity to where antigens from afferent lymphatic vessels drain: regions above the follicle closer to the sinuses, interfollicular regions, and regions near medullary and cortical-medullary sinuses. Various chemokines released in response to immunogenic stimulation may fine tune exactly where basophils ultimately concentrate. Once at these sites, basophils possess a scanning behavior which may increase the chance of coming into contact with danger-associated stimuli. This behavior may even lead to the cells leaving the parenchyma of the tissue to gain entrance to the more superficial regions (with even higher exposure to antigen) such as lymph node sinuses (Y. Zhang et al., 2016) or in the airways of the lung. Indeed, using two-photon imaging we had observed some basophils within the subcapsular sinuses (data not shown). Second, in response to antigen re-exposure (or possibly stimulation via innate receptors), basophils become activated, degranulate, and begin to generate additional inflammatory mediators. Some of these released mediators include histamine, proteases (MCPT-8/11), leukotrienes, and IL-4. Third, quickly after activation, basophils collectively reposition closer to the site of antigen exposure. In our lymph node model, this site was the subcapsular sinus. Basophils also increase their interactions with nearby macrophages. These interactions may enhance the activity of basophils and/or stimulate macrophages to produce inflammatory mediators. Fourth,

within six hours, effector molecules produced by basophils, resident macrophages, and/or stromal cells promote the recruitment and clustering of recruited leukocytes including neutrophils, monocytes, and eosinophils. Eosinophils and neutrophils, in turn, secrete additional chemoattractants such as leukotrienes, which may provide a positive feedback loop in recruiting more inflammatory cells, including basophils, to the region, ultimately leading to the formation of "inflammatory clusters" (M. Chen et al., 2006; Patnode, Bando, Krummel, Locksley, & Rosen, 2014). Resident macrophages also reorganize to form what resembles a meshwork within each individual cluster. Fifth, by 24 hours (possibly sooner), stromal cells which highly express GP38, VCAM-1, and ICAM-1 (ICAM-1 data not shown) as well as macrophages (many also highly expressing VCAM-1) appear to encapsulate the clusters. These encircling cell populations through their expression of adhesion molecules (particularly, VCAM-1 and ICAM-1) may facilitate adhesion of inflammatory cells to the region (Shi et al., 2010). Alternatively, just as VCAM-1 and ICAM-1 on endothelium regulate cell entry into tissue, adhesion molecules on these encircling macrophages and stromal cells may also regulate cell entry into or exit out of the clusters (Kong, Kim, Kim, Jang, & Lee, 2018; Long, 2011; Shi et al., 2010). By 24 hours basophil-derived IL-4 (among other cytokines) also acts on resident macrophages and recruited monocytes, leading to their differentiation to become AAMs or multinucleated giant cells. AAMs may contribute to protection against parasites (Obata-Ninomiya et al., 2013; Turner et al., 2018), though it is also likely that during the later stages of the inflammatory response, they facilitate the resolution of inflammation (Egawa et al., 2013). Additionally, multinucleated giant cells may enhance the clearance of debris, especially larger particles such as dying cells (e.g. neutrophils) or partially degraded foreign material (e.g. killed parasites) (Milde et al., 2015). Overall, our study has provided significant insight and clues on a spatial-temporal level as to how basophils promote the organization of type-2 inflammatory clusters after antigen- and IgE/FcεRI-induced activation.

An important contribution of our work was in demonstrating the involvement of basophils in allergic inflammation directed against the lymphatic traversing parasite, *B. pahangi,* at the lymph node. As previously mentioned (in the Introduction), many parasites have been shown to transit through lymphatic vessels and lymph nodes, including parasites that later take residence in non-lymphatic tissues. Worth noting, even *B. pahangi* has been shown to migrate from the external lymphatic system to the deeper internal lymphatics and coelomic cavities (Bain et al., 1994). In light of all this, lymph nodes appear to be optimally positioned so as to have great potential in facilitating the detection and response to lymphatic-traversing parasites. Indeed, infection of lymph nodes with *B. pahangi* resulted in pronounced allergic inflammation. The association of basophils in the inflammatory response against the parasite strongly suggested that basophils could contribute to protection. A specific mechanism for how basophils might contribute has not yet been resolved, but our study using the IgY and calcipotriol model along with other reported studies on basophils or mouse models of Brugia infection (*B. pahangi* and *B. malayi* often used interchangeably) have provided great insights to consider.

Basophils most likely to contribute the greatest to protection against lymphatic traversing parasites, such as *B. pahangi*, early after a secondary infection. One recent study had revealed that IL-4, macrophages, and eosinophils were protective against infection by *B. malayi*, a filarial worm more commonly known to infect humans. According to this study, IL-4 acts on macrophages to promote their polarization toward an alternatively activated state. These macrophages in turn secrete mediators such as eotaxin-1 to enhance the recruitment of eosinophils, which protect against the infective larvae (Turner et al., 2018). Though these experiments were performed within the peritoneum, a less-physiological site of infection, the mechanism is likely relevant in more common infection sites such as lymphatic channels and lymph nodes. Since basophils can rapidly produce IL-4 in an IgE-dependent manner, it is likely that these cells are the primary producers of IL-4 early after re-infection. In this way, basophils

may be recruited to the site of infection and secrete IL-4 to induce macrophage recruitment of eosinophils. A previous report had shown that basophils were important for early trapping and killing of *N. brasiliensis* in the skin upon re-infection, thereby preventing further migration to the lung (Obata-Ninomiya et al., 2013). In a similar manner, basophils may be valuable in reducing the spread of lymphatic-migrating parasites by rapidly accumulating into lymph nodes and performing IL-4-related effector function before the arrival of many memory Th2 cells. It is also known that basophils can be induced to accumulate into lymph nodes in response to parasites, even if the parasites do not actually enter the organ itself (Giacomin et al., 2012; S. Kim et al., 2010; C. Schwartz, Turqueti-Neves, et al., 2014). Whether this lymph node response is a direct reaction to secreted proteases or host-derived signaling molecules that have drained into the organ is unclear; nonetheless, in the context of a true lymphatic-migrating parasite, this mechanism may provide protective benefit by allowing the draining lymph node to increase its defenses prior to the arrival of the organism. Once the parasite reaches the lymph node, basophil-associated inflammation, comprised of alternatively-activated macrophages and eosinophils, may facilitate killing of the larvae. Indeed, four days after re-infection, significant amounts of alternatively-activated macrophages and eosinophils had infiltrated the lymph node and were surrounding parasitic bodies. Taken together, our data suggest that basophils may enhance allergic inflammation in lymph nodes in response to invasive filarial larvae, although the specific contributions of basophils in these models have not yet been directly tested.

Though the IgY and calcipotriol model that we used to induce allergic lymphadenitis may at first appear contrived, it may nonetheless reflect a disease that is known to occur in humans, independent of parasite infection. In our study, we have referred to the inflammatory response we observed in lymph nodes as “allergic lymphadenitis” due to the recruitment of cells typically associated with allergic responses. Clinical terms, however, may vary. Similar diseases are

more often termed eosinophilic lymphadenitis/lymphadenopathy because of the obvious histological presence of numerous eosinophils, yet I refrain from that term in this dissertation because eosinophils are certainly not the only cellular component of the disease. Many disorders not involving parasites have been associated with the presence of allergic lymphadenitis such as hyper-IgE syndromes, dermatitis, Churg-Straus syndrome (which involves blood vessel inflammation), drug-induced hypersensitivity, and Kimura's disease (allergic inflammatory disease involving the head and neck areas) (H. Chen et al., 2004; Churg et al., 1995; Garcia Carretero et al., 2016; Gowani et al., 2018; Kaplinsky et al., 1988; Makis et al., 2010; Nonaka et al., 2017; Swanson et al., 2017; Winter et al., 2007). The actual cause of many of these disorders is unclear but may involve inflammation of the skin and the corresponding skin-draining lymph nodes. This is interesting since one of the reagents we use for inducing pronounced lymphadenitis, calcipotriol, is a dermal irritant that promotes allergic inflammation of the skin through excessive TSLP production (M. Li et al., 2006). Therefore, though the experimental procedure we used was primarily a proof-of-concept for basophil involvement in allergic lymphadenitis, this experimentally induced response may, in fact, have useful implications toward better understanding non-parasitic forms of allergic lymphadenitis in general. Whether basophils are important contributors to these inflammatory responses in humans is unknown. Nonetheless, at least for the case of Kimura's disease, basophils have been found to associate with the inflammatory response, including being found in lymphoid tissue (Nonaka et al., 2017). Future work attempting to better understand allergic lymphadenitis may therefore benefit from using the model we have established.

Future Directions

In the context of our model of allergic lymphadenitis, basophils were observed to interact with many different cells in lymph nodes, especially macrophages near subcapsular sinuses;

however, it remains undetermined what functional purpose this interaction serves. Our two-photon time-lapse imaging revealed that macrophages can interact with basophils for relatively prolonged periods. Basophil-macrophage interactions were common and often extended beyond the usual duration of imaging (30 minutes). While most basophils displayed a motile, scanning behavior in the vicinity of macrophages near sinuses, after antigen rechallenge, the number of extended basophil-macrophage interactions appeared to increase (though a detailed quantitative analysis has not yet been performed). Subcapsular sinus macrophages are known to capture and display antigen that has drained into the sinus of the lymph node (Carrasco & Batista, 2007; Junt et al., 2007; Phan, Grigorova, Okada, & Cyster, 2007). Therefore, the scanning behavior of basophils around macrophages may increase their likelihood to detect foreign antigen. Upon antigen recognition through FcεRI, basophils may then upregulate (or activate) adhesive receptors to promote strong interactions with nearby macrophages. Antigen-activated B cells have also been demonstrated to reduce motility and accumulate near the subcapsular sinus in a manner that may be facilitated through the high expression of ICAM-1 and VCAM-1 on the surface of SCS macrophages (Junt et al., 2007).

It would be worth investigating whether these or other adhesive molecules are also involved in basophil interaction with lymph node macrophages. Through extended interactions, basophils and macrophages might act synergistically, providing complementary stimulatory signals in a direct and highly concentrated manner. Activated basophils may display stimulatory ligands or secrete IL-4 or M-CSF onto macrophages. These signals may then induce the secretion of various inflammatory mediators, alternative activation, fusion to become multinucleated giant cells, or proliferation. Additionally, macrophages may also enhance the activity of basophils through cytokine and surface bound ligands. One recent study had demonstrated that Notch signaling is important for enhancing the activity of basophils in vivo (Webb et al., 2019). Whether macrophages commonly express Notch ligands or other surface bound ligands that help to enhance basophil activity has not been determined. After basophil activation,

relocalization, and interaction with macrophages, we observed early inflammatory clusters began to form. Given the high potential for macrophage involvement in basophil-mediated inflammation of the lymph node, one important future experiment would involve the depletion of resident macrophages during our IgY and calcipotriol model. It would be valuable to know whether macrophages promote various stages of the basophil-mediated response such as basophil relocalization, the recruitment of inflammatory cells, and/or the clustering of recruited cells.

IgE and FcεRI are most likely just one of many stimuli that provoke basophil mediator release and effector function in the lymph node. Certainly, other innate stimuli derived from pathogens or damage-associated signals like IL-33 could induce or enhance basophil function. We did not, however, investigate the role of non-basophil derived signals in enhancing the inflammatory response other than IL-3 and IL-4, which were necessary for basophil recruitment. As it pertains to activation of basophils through antigen and antibodies, IgE was clearly involved in enhancing inflammation through basophils, but whether other isotypes were involved was less clear. Of note, the knockout of FcεRIα revealed clear effects on inflammatory cluster formation, which we showed to be basophil-dependent, but unexpectedly, eosinophil and neutrophil cell counts appeared less affected compared to the knockout of FcRγ. Since FcRγ is shared by both FcεRI and Fcγ receptors, this suggests that basophils have the potential to be activated by and perform some, but not all, effector functions (such as eosinophil recruitment) through IgG. At the same time, it is not clear whether IgG is a normal part of the physiological process of basophil activation since removal of FcεRIα, which competes for the FcRγ chain, may lead to a compensatory increase in FcγRIII on the surface of basophils. Some reports have suggested that basophils can be activated through IgG to induce anaphylaxis, but other reports have since contradicted this finding (C. Ohnmacht et al., 2010; Tsujimura et al., 2008). Overall, a variety of

activating receptors and corresponding stimuli may be involved in enhancing basophil effector function at the lymph node, which future studies may help to clarify.

It remains to be determined what molecules basophils produce to provoke effector function in nearby cells at the lymph node. IL-4 is the primary candidate for many of the functions that we had observed, including eosinophil recruitment, macrophage polarization, and macrophage differentiation. Nonetheless, we have not yet determined the importance of basophil-derived IL-4 in our model. Other candidates of interest include M-CSF, TNF-alpha, histamine, or leukotrienes. Basoph8 mice, which express Cre recombinase in a basophil specific manner, will be important for determining the contribution of these various effector molecules in combination with floxed alleles. While some strong candidates for basophil effector function exist (as mentioned), further RNA-sequencing may be useful to discover additional mediators. Surprisingly, to my knowledge, RNA-seq has not been carefully performed comparing steady state basophils and IgE-activated basophils in vivo (especially in inflamed tissue) to determine what mediators are upregulated upon IgE-induced activation. This may be particularly helpful since many in vitro experiments have used questionably relevant conditions such as cell lines or IL-3 expanded bone marrow-derived “basophil” populations to probe mediator release, which may not necessarily reflect what primary basophils in vivo actually produce. As an additional benefit, single cell RNA-seq may also yield useful information regarding other cluster-associated cells, including macrophages and stromal cells. Based upon expression profiles of basophils and other cluster-associated cells, an interactome of various mediators, receptors, and ligands could also be constructed which may provide a broader understanding into the role of basophils in allergic inflammation (Cohen et al., 2018).

Further studies are needed to determine whether basophils have an impact on the adaptive immune system. Most studies using genetic-depletion models have thus far shown little impact

on the T or B cell responses that were assessed. Nonetheless, it still appears plausible that basophils could enhance the differentiation of T cells or B cells, especially since basophils produce relevant cytokines (i.e., IL-4) and localize in sites consistent with early T and B cell activation, in particular, interfollicular regions. Physiologically though, in most conditions, basophils are actually recruited in very low numbers compared to the total cell population, suggesting basophils are unlikely to have a major impact on the adaptive response in the lymph node as a whole. One possibility is that basophils may actually have a greater impact on the adaptive response at peripheral sites rather than the lymph node itself. This possibility is suggested by the observation that basophils have been found to interact with T cells in non-lymphoid, inflamed peripheral tissue (Sullivan et al., 2011). B cells, which may accumulate in inflamed tissue, especially during chronic inflammatory settings, might have also opportunity to interact with basophils. Overall, more research is still needed to better clarify whether basophils can directly impact B cells or T cells and thereby modulate adaptive immune responses, whether at a secondary lymphoid organ or in other inflamed tissues.

Though our experimental model of IgY and calcipotriol immunization led to a profound accumulation of basophils within the draining lymph node, the mechanism for basophil accumulation remains unclear. Many experimental stimuli have previously been demonstrated to induce basophil recruitment into the lymph node. These stimuli likely engage danger or damage-associated pathways within the local lymph node environment that emulate pathogenic components. For example, papain, a protease, may mimic parasitic proteases that are used to disrupt tissue barriers and migrate through the host. Previous reports have suggested that many of these stimuli involved in basophil recruitment converge and rely upon the production of IL-3 (Kim et al., 2013; S. Kim et al., 2010; Leyva-Castillo, Hener, Michea, et al., 2013). Other reports had also revealed TSLP to be important in mediating many of the responses of calcipotriol, including basophil recruitment (Leyva-Castillo, Hener, Jiang, & Li, 2013; Leyva-Castillo, Hener,

Michea, et al., 2013; M. Li et al., 2006). Indeed, we demonstrated in our study that both IL-3 and TSLP were necessary for the accumulation of basophils into the lymph node. Moreover, we also found IL-4 to be required as well, though how IL-4 contributed to recruitment was unclear. Unique to this model was the extent to which basophil accumulation occurred. While testing many combinations of adjuvants and proteins, only when IgY and calcipotriol were combined did a basophils accumulate in massive quantities in the lymph node, often 10 to 100 fold higher than in other conditions (e.g. calcipotriol or papain alone). Correlating with basophil recruitment, the number of IL-3 capable T cells also increased significantly when calcipotriol was combined with IgY compared to goat IgG. This data revealed IgY to play an instrumental role in the recruitment process, yet there is no clear mechanistic basis for why IgY should have such a profound impact on the basophil response or IL-3 production. In fact, IgY has often been touted within therapeutic contexts for its inert nature because it does not react with any known mouse or human Fc receptors. Nonetheless, a few reports have correlated IgY or FGC (a serum fraction containing a similar protein to IgY in fowl) to enhanced asthma or an antigen-specific Th2 cell bias, respectively (Mosmann, Cherwinski, Bond, Giedlin, & Coffman, 1986; Yao et al., 2015). Precisely why IgY might be particularly involved in Th2 and basophil responses remains to be determined. Future research should be done to determine what property of IgY is important for basophil recruitment. Such a discovery would not only reveal a potentially important mechanism for basophil recruitment but also may reveal a novel receptor or pathway involved in stimulating allergic inflammation and defense against helminths.

Summary

Basophils have the capacity to orchestrate allergic inflammation in the lymph node through IgE. The affinity of IgE is of particular importance in promoting basophils to degranulate and produce IL-4 in response to specific antigen. Our research supports the existence of a proofreading property within FcεRI signaling which prevents basophil effector responses to specific antigen

when IgE affinity is low. Even when IgE/FcεRI receptor occupancy of antigen is comparable to that of high affinity antigen, low affinity antigen cannot induce basophil responses to a similar extent. Physiologically, this mechanism most likely restrains basophils from orchestrating an inflammatory response to antigen that is unlikely to be a threat. More specifically, low affinity antigen may merely represent cross reactivity of previously generated IgE to an unrelated inert foreign or self-antigen. Conversely, high affinity antigen led to potent activation of basophils through IgE, resulting in degranulation and IL-4 production. In the context of allergy, high affinity antigen will activate basophils and exacerbate disease. However, in response to a secondary infection by a parasite, the production of IgE with high affinity to parasite-derived molecules may enable basophils to respond in a rapid and potent manner. Upon IgE-induced activation, tissue-recruited basophils would likely relocalize closer to the source of antigen and exert effector functions through their release of various mediators, such as IL-4. Through increased interactions, basophils may then promote macrophages to enhance the recruitment of circulating leukocytes as well as differentiate to become alternatively activated. Alternatively activated macrophages, eosinophils, and other cell populations would then provide active protection, ultimately surrounding the parasite to form an inflammatory foci of cells, possibly maturing to become a granuloma. When parasites transit through the lymphatics, lymph nodes can become a site of detection and defense. In that case, the lymph node recruits type 2 inflammatory cells such as eosinophils and basophils to participate in a local protective response to kill and prevent the parasite from migrating further into the host. In this way, basophils recruited into the lymph node may respond to parasitic antigen through IgE and enhance an allergic inflammatory response which neutralizes the invading organism.

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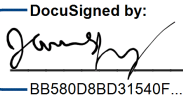
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