VERSATILITY OF EOSINOPHILS IN IMMUNITY TO THE PARASITIC NEMATODE, *TRICHINELLA SPIRALIS*

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Lu Huang

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VERSATILITY OF EOSINOPHILS IN IMMUNITY TO THE PARASITIC NEMATODE, *TRICHINELLA SPIRALIS*

Lu Huang, Ph. D.

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Helminth infections continue to threaten human and animal health worldwide. Dissection of the interactions between helminths and helminth-induced Th2 immunity is necessary for the development of effective prophylactic and therapeutic approaches to disease control. *Trichinella spiralis* is natural pathogen of humans and rodents, providing a valuable context for study of the relationships between Th2 immunity and parasitic worms. Eosinophilia is a hallmark of Th2 immunity in helminth infections, yet the roles of eosinophils in worm infection have remained elusive. Our early studies have revealed unexpected functions of eosinophils in *T. spiralis* infection. Rather than promoting clearance of parasites, eosinophils supported muscle larvae survival and growth by regulating local immunity.

The results presented here define the eosinophil-dependent mechanisms that protect muscle larvae against nitric oxide-mediated killing in primary infection. Our results demonstrate that early recruitment of eosinophils to sites of infection is essential for driving local immunity that promotes larval survival. Eosinophil-derived IL-10 expands IL-10^{+} myeloid dendritic cells and $CD4^{+}$IL-10^{+} T cells that suppress iNOS expression and protect intracellular larvae. The results reveal a novel immunoregulatory function of eosinophils in helminth infection.

In secondary infection by *T. spiralis*, eosinophils are dispensable for intestinal immunity that clear adult worms while having a profound effect on the migration of newborn larvae. This interference limits further colonization of skeletal muscle. The protective effect is dependent on the presence of immune serum and reinforces the dogma that eosinophils adhere to larvae in the presences of antibodies.

Eosinophils regulate larval growth through a third distinct mechanism. Current results indicate that intrinsic STAT6 signaling of eosinophils and eosinophil-derived IL-4 are required for larval growth. Importantly, larval growth is supported by eosinophils independently of adaptive immunity. Gene expression array analysis of skeletal muscles of infected $Rag1^{-/-}$ mice revealed a signature compatible with muscle regeneration and a shift in the source of energy, indicating that eosinophils are likely promoting larval growth by regulating metabolism in local tissues, thereby creating a suitable microenvironment.

BIOGRAPHICAL SKETCH

Lu Huang was born and raised in Xi'an, Shaanxi Province, China. He spent his first eighteen years in Xi'an, one of the Four Great Ancient Capitals of China, with more than 3100 years of history. Lu developed strong interests in science during high school, especially in biology and chemistry. In 2002, he moved to Hangzhou, Zhejiang Province and started his college study at Zhejiang University, where he gained a strong background in general biology, including zoology, plant biology, microbiology, ecology, biochemistry, physiology, bioinformatics, cell biology and molecular biology. In 2006, he completed his undergraduate studies and received a B.S. with 1st class Honors, with a major in Bioinformatics. He spent one year in Dr. Jianzhong Shao's laboratory at Zhejiang University in order to study innate immunity in fish. In 2007, he came to Cornell and started his graduate study, and in 2008, he joined the Field of Comparative Biomedical Sciences and began his doctoral research with Dr. Susana Mendez to study immunity against Leishmania. In 2011, he moved to Dr. Judith Appleton's laboratory and joined the Field of Immunology and Infectious Disease. In the Appleton lab, he studied the role of eosinophils during infection by the parasitic nematode *Trichinella spiralis*. In 2014, he defended his Ph.D. thesis.

In memory of my grandfathers

Yangti Qin (秦养疐) and Huairen Huang (黄怀仁)

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CHAPTER 1

Functional versatility of eosinophils at the host-parasite interface[*]

[*] Lu Huang, Judith A. Appleton. Functional versatility of eosinophils at the host-parasite interface. Invited review for Clinical and Experimental Allergy. *In revision*.

Abstract

Eosinophilia is a central feature of Th2 immune responses induced by infections with parasitic worms. Although early work showed that eosinophils could adhere to and damage parasite larvae *in vitro*, a definitive role for eosinophils during worm infection remained elusive until recently. Newly created mouse strains in which the eosinophil lineage is ablated serve as powerful tools for the study of eosinophil function in disease, but also in health. Particularly exciting are recent findings that show an expanded functional repertoire for eosinophils in tissue homeostasis and metabolism, processes that are intricately related to the establishment and maintenance of parasitic worms in their hosts. In this review, we describe the value and potential of novel mouse models for studying eosinophils, emphasizing results from studies that have revealed functional properties of eosinophils in healthy tissues that also may be important in worm infection. Following a summary of findings in support of tissue-dependent eosinophil function in worm infection, we highlight experiments performed with *Trichinella spiralis*, in which eosinophils show considerable functional versatility by clearing parasites in one context and promoting parasite survival in another.

Introduction

Parasites depend upon their hosts to compensate for lost functions that otherwise enable a free-living lifestyle. The host provides a supportive physical environment for growth, reproduction, and maturation, and the metabolic needs of the parasite are intricately linked to the metabolism of the host. Parasitic worms are highly complex, macroscopic, invertebrate animals that incorporate nervous, excretory, gastrointestinal, muscular, and reproductive systems. Their size and motility is such that tissue trauma is a common feature of infection, further complicating their dependence upon the host for a physical home that also meets their nutritional needs. In spite of the complexities of this dependence and associated physical disruption, parasitic worms often colonize their hosts for long periods.

In parasitic infections of animals, a potent Th2-driven immune response is superimposed on the metabolic and physical interaction between host and worm. Effector mechanisms that clear worms from the body vary, likely depending upon the habitat and niche of the parasite. In some instances goblet cells are critical (1), in others mast cells (2) or antibodies (3-5) play important roles. These mediators may deprive the worm of energy or nutrients (1), or may disrupt the habitat (6). By a similarly varied array of mechanisms, worms evade or co-opt the immune response in order to complete their life cycles and be transmitted to the next host (7).

The eosinophil has been a prominent but enigmatic cell type in the immune response to parasitic worms. Representing less than 5% of leukocytes in the peripheral blood, Th2-derived Interleukin-5 (IL-5) induces activation and recruitment of eosinophils to tissues (8), and eosinophilia can be dramatic during worm infection (9).

Eosinophils contain numerous granules that store preformed cytotoxic proteins, including major basic protein-1 (MBP-1) (10, 11) and eosinophil peroxidase (EPX) (12, 13), and have been shown to degranulate on and kill larvae *in vitro* (14-16). Hence it has been widely accepted that eosinophils are effector cells that function as a downstream arm of Th2 immunity in defense against parasitic worms. The availability of eosinophil-ablated mouse models has enabled experimentation that addresses the functions of eosinophils *in vivo*, revealing that eosinophils are sometimes dispensable and other times beneficial to certain parasitic worms. In the latter context, it is relevant to note that eosinophils demonstrate immunoregulatory functions by virtue of their secretion of a wide variety of immunomodulators, including cytokines, chemokines, and growth factors (17, 18).

This review aims to summarize recent progress in our understanding of the role of eosinophils in worm infection. Many advances have been made possible by the availability of new mouse strains. In addition, we describe exciting findings relating to the role of eosinophils in processes that are relevant to parasitism, including tissue injury, metabolism, and antibody responses. Lastly, findings from mouse models of various helminth infections will be summarized, followed by a description of the versatility of eosinophils during *Trichinella spiralis* infection.

Mouse models for studying eosinophils

Early studies of eosinophil function in mouse models of worm infection relied upon antibody-mediated depletion of eosinophils or IL-5 (19, 20). Subsequently, mouse strains were engineered to be deficient in IL-5 (8), in eotaxins (21-23) or their

receptor CCR3 (21-24), or to overexpress IL-5 (IL-5Tg^{+}) (25). Although expansion and recruitment of eosinophils are profoundly altered in these strains, the lineage is not ablated, which compromises the interpretation of negative results. The development of two eosinophil-ablation models, PHIL and ΔdblGATA (26, 27), enabled definitive experiments that both confirmed some prior findings but also revealed previously unknown properties of eosinophils. The two strains were engineered using different approaches. In PHIL mice, diphtheria toxin (DT) A chain was inserted downstream of the eosinophil peroxidase promoter (26). In contrast, ΔdblGATA mice bear a deletion of the high-affinity double GATA site in the GATA1 promoter that blocks the development of eosinophil lineage (27). These strains, together with models developed earlier, have been reviewed recently (17, 28).

Useful tools for testing the effector function of eosinophils include mice engineered to be deficient in major proteins stored in the secondary granules, MBP-1 (29) or EPX (30). Recently, Doyle *et al*. showed that mice bearing both deficiencies manifest a specific and near complete loss of peripheral and tissue eosinophils (31). This strain provides an additional ablation model with some advantages. Unlike the PHIL mouse, it does not express DT A chain, eliminating risk of damage to neighboring cells or tissues. In addition, the targeting effect in MBP-1$^{-/-}$/EPX$^{-/-}$ mice appears to be highly specific for eosinophils, while ΔdblGATA mice have been reported to display mild anemia and impaired basophil function (32). Although the mechanisms underlying specific ablation of eosinophils in MBP-1$^{-/-}$/EPX$^{-/-}$ mice are unclear, this new strain provides an additional tool for investigating eosinophil function *in vivo*.

Adoptive transfer of eosinophils to eosinophil-ablated mice is an experimental design that considerably expands the utility of the model. Applied widely and creatively in recent years, transfer of eosinophils isolated from IL-5Tg^{+} mice into eosinophil-ablated or other genetically deficient recipients allows for testing of the significance and temporal requirements for eosinophil-derived factors *in vivo* (33). The method can be elaborated upon, for example, by crossing IL-5Tg^{+} mice with MHCII$^{-/-}$ mice to test the significance of antigen presentation by eosinophils in immunity (34). Obviously, this variation on the method requires time consuming and costly breeding of the desired strains. Alternatively, bone marrow-derived eosinophils can be propagated for adoptive transfer experiments (35). This system provides a more rapid way to generate specific gene deficient eosinophils, although the number of cells produced may be limiting.

A disadvantage of ablation models is that they do not enable contextual or temporal evaluation of eosinophil function in an otherwise eosinophil-replete animal. This challenge has been addressed by two groups, each using a knock-in strategy that inserted the gene for human DT receptor (DTR) into the EPX locus (36, 37). Depletion of eosinophils is induced by injection of DT. In the mouse model of asthma, inducible PHIL (iPHIL) mice were used to show that eosinophils were dispensable for sensitization to ovalbumin (OVA), although they were critical to the manifestation of disease following antigen challenge (36). These results document that the approach has value for time-limited depletion of eosinophils; however, complete depletion requires multiple DT treatments over a period of 14 days, a regimen that induces antibodies that prevent the toxin from reaching additional target cells. Thus, inducible

depletion is a useful tool for addressing the function of eosinophils during brief, but perhaps not prolonged intervals.

Eosinophils display a variety of receptors, and produce an array of chemokines and cytokines that are known to be key regulators in different physiological and pathological processes (17, 18), yet it has been difficult to determine whether the eosinophil is the source of influential mediators in the mechanism under investigation. A mouse model that addresses this need is one in which a gene knock-out is targeted specifically to eosinophils. The newly created eoCRE mouse, in which Cre recombinase is expressed under the control of the EPX promoter, supports this approach (38). By crossing with a strain bearing a floxed gene, mice are generated that bear eosinophils that are deficient in the corresponding protein. The availability of this strain will be invaluable to documenting the significance of eosinophil-derived immunoregulatory mediators, such as IL-4, IL-13, or TGF-β in different disease models. Moreover, as mentioned by Rosenberg (39), eoCRE mice will enable an indirect assay of human eosinophil function via expression of human genes in mouse eosinophil.

As with any experimental approach, caution must be applied in interpreting results from mouse studies of the sort described here, as different gene targeting strategies, depletion methods and transfer designs may create unique artifacts. The growing collection of mouse strains available for eosinophil manipulation provides an array of tools and approaches that will reduce this risk and significantly advance study of eosinophil function *in vivo*. The value of new mouse strains to research on

eosinophil function, and the generosity of spirit shown by their inventors in sharing such resources, must not go unrecognized.

Functions of eosinophils revealed in mouse models

Eosinophils and Th2 immunity

Early histologic studies documented that eosinophils enter tissues during inflammation induced by helminth infection (40) or hypersensitivity (41). Release of pre-formed mediators and granule-stored proteins in such tissues is compatible with eosinophils serving as effector cells in Th2-driven immunity. In the context of parasitic disease, this function received further support from *in vitro* experiments that documented larval killing by eosinophils (14-16). More recently, as the diversity of receptors and soluble mediators produced by eosinophils has been revealed, their potential to regulate, as well as effect Th2 responses has been tested.

Exposure to allergens and worm antigens induces eosinophils to upregulate surface MHCII and co-stimulatory molecules CD80, CD86 and CD40 (42, 43). Although murine eosinophils activate and present antigen to T cells in a variety of *in vitro* and *in vivo* experimental designs (34, 44, 45), antigen presentation has not yet been shown to impact the course of allergic disease in mice (33) or worm infection. In contrast, there is compelling evidence that eosinophils promote Th2 immunity by producing IL-4 following injection of schistosome eggs into mice (46) and by regulating recruitment and activation of dendritic cells (DC) and T cells (47-49). Accumulation of myeloid DCs in draining lymph nodes is eosinophil-dependent in allergic responses (33). Driven by eosinophil-derived IL-13, DCs and T cells work

cooperatively to restore allergic disease to eosinophil-ablated mice (48-50). These results are representative of broad, ongoing efforts to test the function of eosinophil-derived molecules in health and disease, discussed further below.

Eosinophils and tissue regeneration

Recently, eosinophils have been linked to IL-4 driven regenerative responses to tissue injury (51, 52). Following cardiotoxin injection into skeletal muscle, eosinophils are dominant among IL-4-secreting cells that infiltrate the site, and the regenerative response is severely compromised in ΔdblGATA mice. Fibro/adipogenic progenitor cells (FAPs) at the site of injection are induced to express IL-4Rα. IL-4 drives proliferation of FAPs, preventing their differentiation into adipocytes and supporting myogenic differentiation. Additional findings support the conclusion that FAPs promote muscle regeneration through their ability to phagocytose necrotic fibers (51). In the liver, infiltrating eosinophils are responsible for promoting IL-4-dependent regenerative responses to injury caused by CCl_4 (52). Liver regeneration in this model is dependent, in part, on IL-4Rα expression on hepatocytes.

These observations provide evidence that eosinophils contribute to tissue repair in two highly regenerative organs; however, direct evidence that the critical cytokine, IL-4 is derived from eosinophils is lacking, and other infiltrating cells also produce IL-4 in injured tissues (51, 52). This question can be addressed definitively using eoCre/IL-4 mice or experiments in which IL-4 deficient eosinophils are transferred to injured, eosinophil-ablated mice (38).

Comparison of results from injury models with those from experiments with parasitic worms provides evidence that the response to injury varies, depending on both the insult and the tissue. For example, liver injury is a prominent feature of the granulomatous response to *Schistosoma mansoni*, yet eosinophils do not influence the development of granulomas in the livers of infected mice (53). Similarly, eosinophils do not play a role in protecting the liver against migrating *Trichinella* larvae (54, 55). In contrast, eosinophils play key roles in the response to muscle injury and infection by *Trichinella* (56, 57).

Eosinophils and antibody responses

Plasma cells in the bone marrow are the main source of circulating antibodies. The mediators and mechanisms responsible for the prolonged survival of these cells are crucial to sustaining a state of immunity. A variety of cell types populate niches that provide contact-dependent and secreted survival signals to plasma cells within the bone marrow. These include mesenchymal stromal cells (58), megakaryocytes (59), DCs (60, 61), macrophages (62), basophils (63) and eosinophils (64). Bone marrow eosinophils have been implicated as the main cellular source of the plasma cell survival factors, APRIL and IL-6 (64). Using adoptive transfer protocols in eosinophil-ablated recipient mice, it was shown that plasma cell survival is further enhanced when eosinophils are first activated with adjuvant (65), consistent with a role for eosinophils in plasma cell survival and antibody production in response to infection. Eosinophils are dispensable for the parasite-specific IgM response during primary infection by *Strongyloides stercoralis* (66). Nevertheless, antibodies are

protective against re-infection with some parasitic worms (67-70). Thus, experiments designed to assess the role of eosinophils in plasma cell survival, antibody production, and protective immunity in worm infections are warranted and relevant to devising new vaccine strategies.

Eosinophils, obesity, and innate lymphoid type 2 cells

IL-5 is a critical cytokine in regulating activity of eosinophils. Long known to be produced by conventional Type 2 lymphocytes and myeloid cells, recent reports show that lineage negative, innate lymphoid type 2 cells (ILC2) produce large quantities of IL-5 and IL-13 in response to the epithelial derived-cytokines IL-25 and IL-33. Two studies have demonstrated that the accumulation of eosinophils and alternatively activated macrophages (arginase-1 positive; AAMs) in mouse visceral adipose tissue (VAT) requires IL-5 and IL-13, respectively (71, 72), and ILC2s in VAT are the major source of these cytokines (71).

IL-4 dependent AAMs in VAT are associated with metabolic syndrome, type 2 diabetes, and insulin resistance. Eosinophils are the predominant source of local IL-4 in VAT (72). The recruitment of IL-4-producing eosinophils to VAT by IL-5-producing ILC2s further promotes alternative activation of adipose resident macrophages, which are required for increasing systemic insulin sensitivity. Administration of exogenous IL-33, or infection with *Nippostrongylus brasiliensis*, activates ILC2s to produce IL-5 and IL-13, promoting eosinophil and AAM accumulation in VAT as well as glucose tolerance (71). IL-33 is an alarmin that is released by injured cells (73). Thus, worm-induced injury may stimulate ILC2s to

recruit eosinophils that deliver IL-4 to local macrophages that are needed for glucose homeostasis during infection.

IL-5 is constitutively produced by ILC2s in healthy tissues (74). In contrast, IL-13 production is induced in lung ILC2s by worm infection, leading to local CCL11 production that promotes lung eosinophilia (74). Both lung and intestinal ILC2s express the functional receptors for a feeding-induced neuropeptide, vasoactive intestinal peptide (VIP), and increase the production of IL-5 in response to VIP stimulation. Thus, ILC2s respond to caloric intake by producing IL-5 and IL-13, thereby linking eosinophils to dietary cues. The response of eosinophils to food intake and their role in managing energy for the host, prompts speculation that they may influence the relationship of parasitic worms to their host's metabolism in ways that support or compromise infection.

Eosinophils in helminth infection

Laboratory experiments in which larvae are killed by eosinophils *in vitro*, in the presence of antibody and/or complement, have informed the dogma that eosinophils protect the host against infection with parasitic worms (14-16). The mechanism of killing was confirmed when purified EPX was shown to be toxic for *T. spiralis* larvae (15). Subsequent work identified IL-5 as a critical growth factor for generation and expansion of eosinophils (8, 75), an advance that eventually yielded new tools to study the role of eosinophils in helminth infection, including IL-5 neutralizing antibodies, IL-5 or IL-5 receptor knockout mice, and IL-5Tg^{+} mice (reviewed in Meeusen *et al.* (19) and Behm *et al.* (20)). Additional opportunities were

afforded by the identification and manipulation of the eotaxins and CCR3 (76). Studies of these molecules revealed important insights into their roles in directing eosinophil trafficking and expansion. Nevertheless, each of these regulatory mediators or receptors has multiple cellular targets (77, 78) and most importantly, their deficiency or depletion does not completely eliminate eosinophils from the body. Therefore, if a deficient mouse mounts a WT response, it is difficult to eliminate eosinophils as contributors to the mechanism under study.

Several helminth infections have been tested in mouse models of eosinophil ablation. The aggregated data reveal a marked disparity in eosinophil function among worms that colonize intestinal versus extra-intestinal sites. In PHIL and ΔdblGATA mice infected with *S. mansoni,* there are no obvious defects in immune responses, worm burdens, or egg deposition (53). Furthermore, egg-induced liver lesions, including granuloma formation and fibrosis, are not affected by eosinophil ablation (53). Larvae survival and development of primary and secondary immune responses are normal in PHIL mice infected with *S. stercoralis* (66). Similarly, eosinophils are dispensable for the development of intestinal Th2 responses to *Trichuris muris* and worm expulsion is normal in ΔdblGATA mice (79). Immune responses induced by primary infection with the murine hookworm, *N. brasiliensis,* are only marginally affected in ΔdblGATA mice (80). Infection of either PHIL or ΔdblGATA mice with *T. spiralis* showed that eosinophils are dispensable for intestinal immunity that clears adult worms (57). Mechanisms of immunity across these five infections vary considerably, and the negative results indicate that eosinophils are broadly ineffective in intestinal immunity mounted against primary worm infections.

Although intestinal *Trichinella* are not influenced by eosinophils, the outcome of muscle infection in either primary or secondary infections is profoundly altered in PHIL and ΔdblGATA mice. These findings are discussed in detail in the next section. Additional evidence that eosinophils are influential in extra-intestinal tissues was revealed by infection of eosinophil-ablated mice with the filarial nematode, *Litomosoides sigmodontis*. Growth of adult *L. sigmodontis* is impaired in the absence of eosinophils or IL-5, providing further support for the hypothesis that some helminths depend upon eosinophil-rich microenvironments (81).

Eosinophils and Trichinella infection

Among parasitic worms, *Trichinella* is unusual in that it completes its life cycle in a single animal host. Furthermore, growth and development of all life-stages occurs intracellularly. The infection occurs in two phases. First-stage larvae are ingested and develop into adult worms in the intestine, where they reproduce. Newborn larvae (NBL) migrate from the intestine to skeletal muscles, where they invade myotubes, grow rapidly, and establish chronic, intracellular infection **(Fig. 1.1)**. Eosinophilia is prominent during both phases of infection; however, eosinophils do not contribute to intestinal immunity that expels worms from the gut or limits their fecundity (57, 82), nor do they influence liver injury caused NBL as they migrate from the intestine (54, 55). In stark contrast, eosinophils have a profound effect on the growth and survival of intracellular larvae in skeletal muscle. We have reported that in either ΔdblGATA or PHIL mice, larval burdens in skeletal muscle are significantly reduced (56, 57). Poor larval survival correlates with reduced infiltration of infected

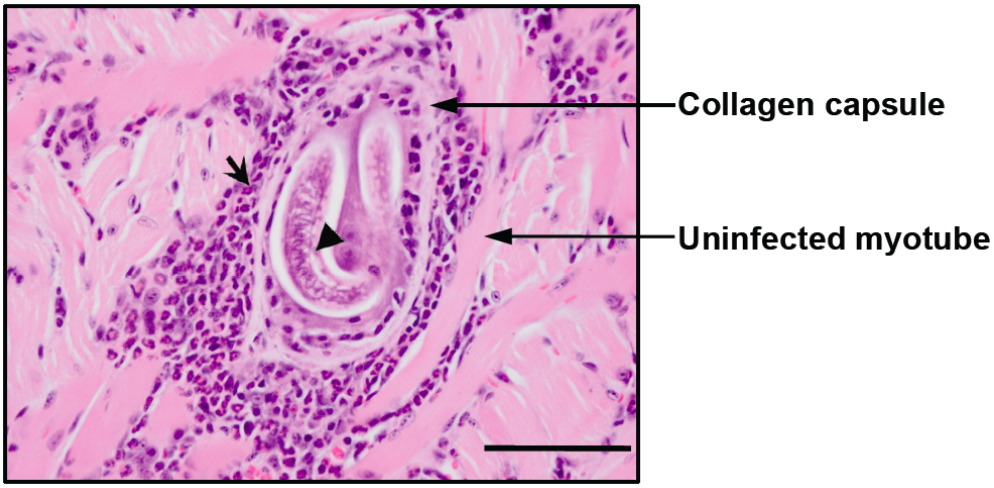

Figure 1.1. The *T. spiralis* nurse cell. Formalin-fixed, paraffin-embedded tongue from a C57BL/6 mice at 28 dpi was stained with hematoxylin and eosin. Features of the nurse cell are indicated. Arrow indicates eosinophil and arrowhead indicates muscle larva. Scale bar = 50 μm.

muscle by Th2 cells, in association with expanded local production of nitric oxide (NO) (56). NO is toxic for growing larvae (56). Among leukocytes that infiltrate infected muscle, IL-10 from $CD4^+CD25^-$ T cells suppresses IFN-γ production and induction of iNOS in macrophages and neutrophils (83). Moreover, larval burdens are reduced in IL-10 deficient mice (57, 83, 84) confirming a central role for IL-10 in promoting survival of intracellular larvae. In the absence of eosinophils, $IL\text{-}10^+CD4^+$ T cells are reduced, implicating eosinophils as drivers of the IL-10 response (56). Ongoing research in our lab has shown that, by producing IL-10 at the initiation of muscle infection, eosinophils expand IL-10-producing myeloid DCs and $CD4^+$ T cells which in turn inhibit the production of local NO and protect muscle larvae. Indeed, transfer of IL-10 competent DCs or $CD4^+$ T cells compensates for eosinophil deficiency (85). These findings are consistent with an earlier report that eosinophils were required for DC recruitment and accumulation in lung draining lymph nodes in the allergy model (33). Unsolved questions include the mechanism of DC recruitment by eosinophils and, most importantly, the trigger that induces eosinophils to make IL-10.

Following invasion of skeletal muscle cells, intracellular larvae undergo a period of rapid growth over the course of 20 days. Larvae grow poorly in muscles of eosinophil-ablated mice, an effect that is not dependent on local NO and is separable from immune-mediated killing (56). Results of pilot studies indicate that eosinophil-derived IL-4, but not IL-10, is critical for supporting larval growth (86). Although it is not known how eosinophil-derived IL-4 may support larval growth, it is intriguing to

consider that the parasite may be exploiting the IL-4 dependent regenerative response of muscle that would follow injury caused by invading larvae. Alternatively, the influence of eosinophil-derived IL-4 on glucose metabolism in adipose tissues may be relevant to infected muscle cells that require a source of energy for growing larvae. Currently, we are exploring these mechanisms.

During secondary worm infection, the functions of eosinophils appear to switch from immunoregulation to immune defense. Eosinophils have been demonstrated to be important for protective responses in secondary subcutaneous larval infection with *N. brasiliensis*, as revealed by the increased numbers of larvae migrating to the lungs in IL-$5^{-/-}$ and ΔdblGATA mice (80). Similarly, muscle burdens are increased in ΔdblGATA mice upon secondary infection with *T. spiralis*. Similar to *N. brasiliensis*, migration of *T. spiralis* NBL is increased in ablated versus WT mice (87). The results are compatible with earlier reports that eosinophils attach to *T. spiralis* NBL via specific antibodies and kill by releasing eosinophil peroxidase (EPX) (15). Confirmation of this mechanism requires experiments in which protection can be conferred upon naïve ΔdblGATA mice by transferring sera from infected mice together with eosinophils. Neither EPX nor MBP-1 is influential in primary infection (56); however re-infection of the knockout strains has not yet been performed. In summary, observations from mice infected with *N. brasiliensis* and *T. spiralis* are consistent with a mechanism in which antibodies compromise the rapid movement of larvae in tissue, enabling binding and killing by eosinophils. Further experimentation is required to confirm this hypothesis.

Collectively, the unexpected roles of eosinophils in *T. spiralis* infection provide new insights into the functions of this enigmatic cell. During primary infection, eosinophils manipulate host immune responses in ways that create a more favorable environment for the parasite. In secondary infection, eosinophils protect muscles against invading larvae, preventing accumulation of additional parasite burdens and thereby prolonging survival of both the host and the parasites already established in the muscle (**Fig. 1.2**).

Conclusion

Recent advances in eosinophil ablation and creation of new tools have advanced our understanding of what eosinophils do during helminth infection. It is surprising that eosinophils appear to be dispensable for intestinal immunity against worms. Data from studies of tissue-dwelling worms provide evidence that eosinophils may be influential in the delivery of essential host resources to the parasite. With further expansion of the toolset, the roles of the eosinophil at the intersections among immunity, injury, and parasitism will be revealed. New knowledge will enhance efforts to develop novel treatments and vaccination strategies for parasitic diseases.

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Conflict of interest

The authors have no conflicts of interest.

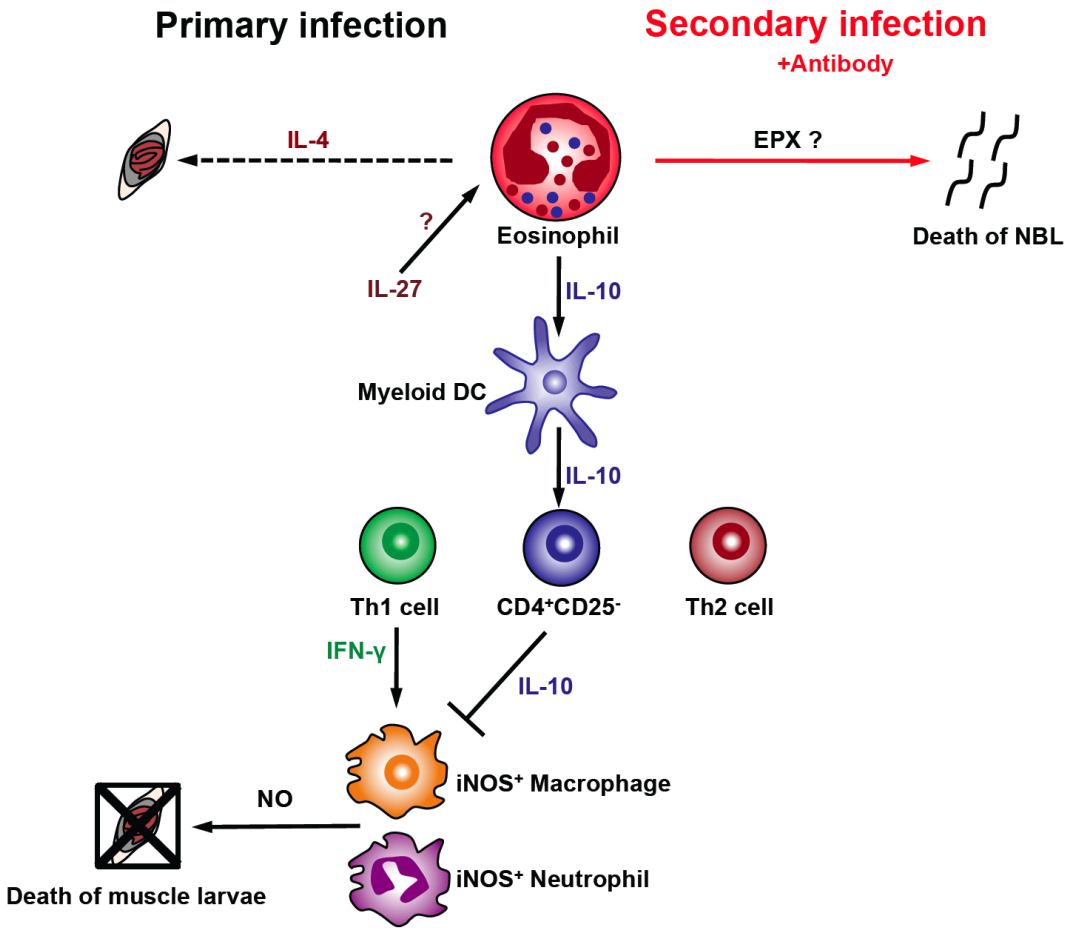

Figure 1.2. Functional versatility of eosinophils in *Trichinella* infection. In primary infection, *Trichinella* induces a mixed Th1 and Th2 immune response associated with eosinophilia in skeletal muscle. By secreting IL-10, eosinophils promote the expansion of IL-10 producing myeloid DCs, and enhance the production of IL-10 by $CD4^{+}CD25^{-}$ T cells. The production of NO by IFN-γ activated $iNOS^{+}$ macrophages and neutrophils is suppressed by IL-10, thereby promoting survival of muscle larvae. Through a different but poorly understood mechanism, eosinophils and IL-4 support larval growth in skeletal muscle. In secondary infection, *Trichinella*-specific antibodies promote adhesion and activate eosinophils to release granule proteins that destroy migrating NBL.

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CHAPTER 2

Eosinophil-derived IL-10 supports chronic nematode infection*

* Lu Huang, Nebiat Gebreselassie, Lucille Gagliardo, Maura Ruyechan, Nancy Lee, James J. Lee, Judith A. Appleton. Eosinophil-derived IL-10 supports chronic nematode infection. *Submitted*.

Abstract

Eosinophilia is a feature of the host immune response that distinguishes parasitic worms from other pathogens, yet a discrete function for eosinophils in worm infection has been elusive. The aim of this study was to clarify the mechanism(s) underlying the striking and unexpected observation that eosinophils protect intracellular, muscle-stage *Trichinella spiralis* larvae against NO-mediated killing. Our findings indicate that rapid, specific recruitment of eosinophils to sites of infection at the earliest stage of muscle infection, consistent with a local response to injury, is essential for larval survival. By producing IL-10 at the initiation of infection, eosinophils expand IL-10^{+} myeloid dendritic cells and $CD4^{+}$ IL-10^{+} T lymphocytes that inhibit *iNOS* expression and protect intracellular larvae. The results document a novel immunoregulatory function of eosinophils in helminth infection, in which eosinophil-derived IL-10 drives immune responses that eventually limit local NO production. In this way, the parasite co-opts an immune response in a way that enhances its own survival.

Introduction

Eosinophilia is a prominent consequence of Th2 immunity mounted in response to infections by parasitic helminths. Cytotoxic effects of eosinophils, mediated by cationic granule proteins, have been considered to be their central influence in worm infections (1, 2). Recent investigations of the roles of eosinophils in health and disease have provided new insights into the versatility of this cell population. In mouse models, eosinophils influence insulin resistance (3, 4), promote the regenerative response to toxic injury of skeletal muscle (5) and liver (6), and are required for recruitment of Th2 cells to the lung in allergy (7-9). Eosinophils constitutively express IL-4 (10) and production of IL-4 or IL-13 is key to the role of eosinophils in each of these contexts. Eosinophils also regulate adaptive immunity by producing cytokines (11), and this property has been tested in experiments that are relevant to the outcome of worm infection. In one example, eosinophils serve as an early source of IL-4, promoting Th2 cell polarization when *Schistosoma mansoni* eggs are injected into peritoneal cavities of mice (12). Moreover, eosinophils express MHCII and costimulatory molecules CD80 and CD86 on the cell surface and are capable of presenting allergens and helminth antigens to T cells (13-15). Perhaps equally likely is the potential for eosinophils to promote maturation of dendritic cells (DCs) in order to enhance antigen-specific Th2 immune responses (16, 17). While these studies provide evidence for immunoregulatory function of eosinophils in worm-induced Th2 immunity, the significance of antigen presentation or IL-4 secretion have yet to be confirmed in the course of an infection.

Despite their prominence in the response to infection by intestine-dwelling parasitic worms, including *Trichinella spiralis*, findings from experiments in eosinophil ablated strains of mice consistently indicate that eosinophils do not contribute in a discernable way to intestinal immunity (18-20). In contrast, eosinophils promote the growth and survival of *T. spiralis* larvae as they colonize skeletal muscle (20, 21). In two strains of mice in which the eosinophil lineage has been ablated (PHIL and ΔdblGATA) (22, 23), growing larvae in muscle are killed by a nitric oxide (NO)-dependent mechanism (21). Providing mice with eosinophils during the first 10 days of muscle infection prevents killing (21) suggesting that eosinophils may directly regulate inducible nitric oxide synthase *(iNOS)* expression by local macrophages and neutrophils. Other findings document that expression of *iNOS* in leukocytes that infiltrate sites of infection is regulated, in part, by $CD4^+$ T cells that produce IL-10 (24, 25). The relationship between eosinophils and $CD4^+$IL-10^+ T cells has not yet been elucidated.

In the studies reported here, we aimed to elucidate the specific activity of eosinophils that regulates local NO production. We found that eosinophils are rapidly and specifically recruited to sites of infection and that the presence of eosinophils at the earliest stage of muscle infection is necessary for larval survival four weeks later. Neither antigen presentation nor production of IL-4 by eosinophils is essential for preserving the parasite. In contrast, by producing IL-10, eosinophils expand IL-10^+ myeloid DCs and $CD4^+$IL-10^+ T cell that suppress local NO production and thus preserve larvae in muscle. Our results provide evidence that a parasitic worm co-opts the regulatory capacity of eosinophils in a way that supports its own survival.

Materials and Methods

Rats and mice

Adult Albino Oxford strain rats were produced and maintained in the Baker Institute vivarium. ΔdblGATA, PHIL, VertX, Rag1$^{-/-}$, IL-5-expressing transgenic (NJ.1638) (IL-5Tg^{+}), IL-5Tg^{+}/MHCII$^{-/-}$, IL-5Tg^{+}/IL-4$^{-/-}$ mice were bred at Cornell Transgenic Mouse Core Facility and offspring were transferred to the Baker Institute. IL-10$^{-/-}$ mice were purchased from The Jackson Laboratory. Rag2$^{-/-}$γc$^{-/-}$ mice were purchased from Taconic. Arg1$^{flox/flox}$;Tie2cre mice were a gift from Dr. Thomas Wynn (NIAID). PHIL mice were genotyped as described previously (22). All strains were on a C57BL/6 background. C57BL/6 ^{NHsd} mice were purchased from Taconic as wild type (WT) controls. Animal care was in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care and experiments were performed with the approval of the Institutional Animal Care and Use Committee of Cornell University.

Parasite and Antigens

Trichinella spiralis first-stage larvae (L_1) and newborn larvae (NBL) were recovered from rats as described previously (20). For oral infection, L_1 were suspended in 2% nutrient broth (Difco), 0.6% gelatin (Fisher Scientific) and doses of 300 L_1 were administered by gavage. For synchronous infection, 25,000 NBL were suspended in 0.25 ml serum-free DMDM (Mediatech, Inc.) and delivered by retro-orbital injection. Mice were euthanized by CO_2 inhalation at the times indicated in each experiment. Whole body muscle larvae burdens were assessed in whole carcasses

28 days postinfection (dpi) as described previously (20). In some experiments, larvae were recovered from diaphragms 17 dpi by digesting minced tissue for 15 min at 37 °C in 5 mg/ml collagenase I (Sigma), a method that allows recovery of larvae and leukocytes, enabling evaluation of the cellular response at a time that is relevant to larval survival. Crude somatic antigens from L_1 were prepared as previously described (20).

Eosinophil transfer experiments

Eosinophils were recovered from infected or uninfected IL-5Tg^{+}, IL-5Tg^{+}/MHCII$^{-/-}$, IL-5Tg^{+}/IL-4$^{-/-}$ or infected IL-10$^{-/-}$ mice 12-20 dpi. Cells were pooled from spleens and peritoneal lavage fluid and purified by magnetic bead selection as previously described (21). Briefly, eosinophils were labeled with PE-conjugated anti-Siglec-F antibody (BD) and anti-PE microbeads (Miltenyi Biotec). Average purity of eosinophils from this procedure was >93%. After washing twice with PBS, 5×10^6 eosinophils were resuspended in 200 µl sterile PBS and injected i.v. into ΔdblGATA mice every 48 h for 6 days.

Cell isolation from lymph node for DC phenotyping

Cells from cervical lymph nodes (dLNs) for DC phenotyping were isolated as described previously (35). Briefly, dLNs were digested at 37 °C on a shaker for 15 min with 1.75 Wunsch Units/ml Liberase CI (Roche) and 80 Units/ml DNase I (Sigma) in Hanks buffered saline (Cellgro) containing 1mM $MgCl_2$, 1.8mM $CaCl_2$, 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco). 100 µl 0.1M EDTA (pH 7.3) per

milliliter was then added to stop the reaction, and the tube was immediately diluted to 15 ml with DMEM containing 50 U/ml penicillin and 50 µg/ml streptomycin. Undigested tissue was manually dispersed on a stainless steel tea strainer, using 12 ml syringe pestle. Cell preparations were passed through 70 µm filters into a 50 ml tube to obtain a single-cell suspension. Cells were washed with PBS containing 2% FCS and analyzed by flow cytometry for DC phenotyping as described below.

$CD4^+$ T cell isolation

$CD4^+$ T cells were recovered from dLNs of infected WT and IL-$10^{-/-}$ donor mice on 17 dpi and enriched by negative selection on magnetic beads using CD4 T Cell Isolation Kit II (Miltenyi Biotec). Average purity was 94%. 3-5 × 10^6 $CD4^+$ T cells were suspended in PBS and injected i.v. into ΔdblGATA recipients that had been infected with 300 L_1 4 days previously.

Culture of bone marrow-derived dendritic cells (BMDCs)

BMDCs were generated as described previously (69). Briefly, bone marrow cells were isolated from naïve WT and IL-$10^{-/-}$ donor mice. Cells were plated (5 × 10^5/ml) in complete RPMI medium in the presence of recombinant murine GM-CSF (20 ng/ml; Peprotech) for 3 days. Fresh medium containing GM-CSF (20 ng/ml) was added to cultures on 3 and 6 days. On day 9, non-adherent cells were harvested and stained for CD11c, MHCII and CD11b to evaluate the purity (>90%) by flow cytometry. BMDCs were then primed with 50 µg/ml L_1 crude somatic antigen for 18 h. Primed cells were washed in PBS, suspended in PBS, and 5 × 10^6 BMDCs were

injected i.v. into ΔdblGATA mice that had been infected with 300 L_1 4 days previously.

Flow cytometry

Cells from individual diaphragms were recovered following perfusion of blood from tissues, as described previously (21), and cultured ex vivo for 6 h with 250 ng/ml ionomycin (Sigma-Aldrich), 50 ng/ml PMA (Sigma-Aldrich), and 1 μg/ml brefeldin A (BD Pharmingen). After a 15 min incubation with Fc block (eBioscience) and 10% normal mouse serum, cells were incubated for 15 min with PE-Cy5-conjugated anti-CD4 (eBioscience). Samples were then treated with fixation/permeabilization buffer (eBioscience), and permeabilized cells were incubated for 1 h with PE-conjugated anti-IL-4 (eBioscience) or anti-IL-10 (eBioscience).

For dendritic cell phenotyping, cells from dLNs and diaphragms were incubated FITC-conjugated anti-CD11b, PE-conjugated anti-CD11c and Pacific blue-conjugated anti-MHCII. Data were acquired using a Gallios flow cytometer (Beckman Coulter) and analyzed with FlowJo software (Tree Star)

Parasite measurement

Body area was measured as described previously (21). Briefly, developing L_1 larvae were recovered (17 dpi) by digesting minced diaphragms for 15 min at 37 °C in PBS containing 2% FCS and 5 mg/ml collagenase I (Sigma). Larvae were treated with 70% ethanol (warm up at 56 °C) overnight. Larvae were then centrifuged and resuspended in 5% glycerol/70% ethanol for one day and then transferred to slides

using a Cytospin. Slides were fixed with methanol and stained with HEMA-3 (Fisher Healthcare), and measurements were performed using a BX51 microscope. The area of each larva was calculated by Microsuite Basic Olympus software. At least 25 larvae were measured per mouse, and values are expressed in μm^2.

Multiplexed cytokine assay

Cytokines were measured in serum using the Bio-Plex 200 multiplex system (Bio-Rad) and the Bio-Plex Pro TM mouse cytokine 23-plex Assay kit (M60-009RDPD), following manufacturer's instructions.

Quantitative RT-PCR

Total RNA was isolated from masseter tissue using TRIzol reagent (Invitrogen). Reverse transcription of the RNA (1 μg) was performed using SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative RT-PCR (qRT-PCR) was performed using the Applied Biosystems PRISM 7500 Sequence Detection System and its analysis software, SDS 2.3 and RQ Manager. The reactions were performed using the Taqman Universal PCR Master Mix. All primers were purchased from Applied Biosystems.

Statistical analysis

All experiments were performed twice with similar results. Means ± SD were calculated from data collected from individual mice unless otherwise indicated. Significant differences were determined using Student's *t* test or one-way ANOVA

with Tukey's post hoc test for multiple means. Statistical analysis was performed with GraphPad Prism 5 software.

Results

Arginase 1 is required for the suppression of NO-mediated larval clearance but does not support larval growth

Infective first-stage larvae of *T. spiralis* colonize the intestine, develop into adult worms, and release migratory newborn larvae (NBL) that eventually enter skeletal muscle, invade myotubes, grow rapidly, and establish chronic, intracellular infection. Diaphragm and masseter are preferred muscles for infection in mice and the life cycle is complete in 28 days. At this time, first-stage larvae in muscle are fully infectious. In the absence of eosinophils, larval growth is impaired and larval survival is reduced (20, 21).

We have reported previously that *Arginase 1* (*Arg1*) expression is decreased in skeletal muscles of eosinophil-ablated mice at 17 dpi (21) and that infiltrating neutrophils and macrophages were $iNOS^{+}$ in WT mice. With the knowledge that Arg1 competes with iNOS in controlling the production of NO, and that NO is toxic for growing *T. spiralis* larvae (21), we aimed to test larval clearance in mice deficient in Arg1. Compared with WT mice, larval burdens were reduced in $Arg1^{flox/flox}$;Tie2cre mice **(Fig. 2.1. A)**, although larvae grew normally **(Fig. 2.1. B)**. The reduction in muscle burden was associated with enhanced NO production in antigen stimulated draining lymph node (dLN) cell cultures **(Fig. 2.1. C)**. These results, together with those obtained previously (20, 21) support a role for NO producing myeloid cells in

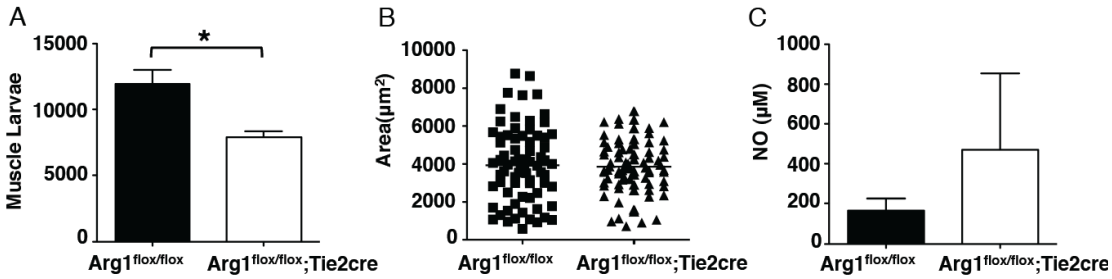

Figure 2.1. Arg1 is required for larval survival but not growth. (A) Larval burdens in muscle of $Arg1^{flox/flox}$;Tie2cre and $Arg1^{flox/flox}$ mice, 26 dpi. **(B)** Body size (area) of larvae recovered from infected $Arg1^{flox/flox}$;Tie2cre and $Arg1^{flox/flox}$ mice, 17 dpi. Bars represent means from at least 75 larvae pooled from masseters of 3 mice. **(C)** NO production in antigen-stimulated dLN cell cultures. Each data set was collected from two experiments with similar results. Values represent mean ± SD; n = 3 - 4 mice. Significant differences were determined by Student's *t* test or ANOVA and Tukey's test. *$p < 0.05$

larval clearance. Importantly, this toxicity is separable from the compromise of larval growth that also occurs in eosinophil-ablated mice.

IL-10 derived from $CD4^+$ T cells compensates for eosinophil deficiency

We have shown previously that NO production in infected muscles of IL-10-deficient mice was reduced by adoptive transfer of IL-10-competent $CD4^+$ T cells (24). By testing the impact of eosinophil-ablation on $CD4^+$ T cells at sites of infection, we found that reduced larval survival correlated with impaired recruitment of $CD4^+IL\text{-}10^+$, as well as $CD4^+IL\text{-}4^+$ T cells, to sites of infection (compare WT to PBS treated eosinophil-ablated mice in **Fig. 2.2. A - C**). When $CD4^+$ T cells from dLN of WT infected mice were transferred to ΔdblGATA mice, they infiltrated skeletal muscle and promoted leukocyte infiltration (data not shown), documenting that the local environment in ablated mice was not deficient in chemoattractants or endothelial cell surface molecules necessary for T cell and leukocyte recruitment. Indeed, local gene expression of chemokines that recruit Th2 cells was unaltered in ablated mice **(Fig. 2.2. D)**. Furthermore, expression of markers of T cell activation was similar in cells recovered from diaphragms of *T. spiralis*-infected WT, PHIL or ΔdblGATA mice **(Fig. 2.2. E and F)**. Thus, the local environment was compatible with $CD4^+$ T cell recruitment and $CD4^+$ T cells were being activated, but they were compromised in their abilities to produce IL-4 and IL-10. Transfer of $CD4^+$ T cells from infected WT donors to eosinophil-ablated mice (4 dpi) improved larval burdens and promoted infiltration of $CD4^+IL\text{-}10^+$ cells **(Fig. 2.2. A and B)**. In contrast, transfer of $CD4^+$ T

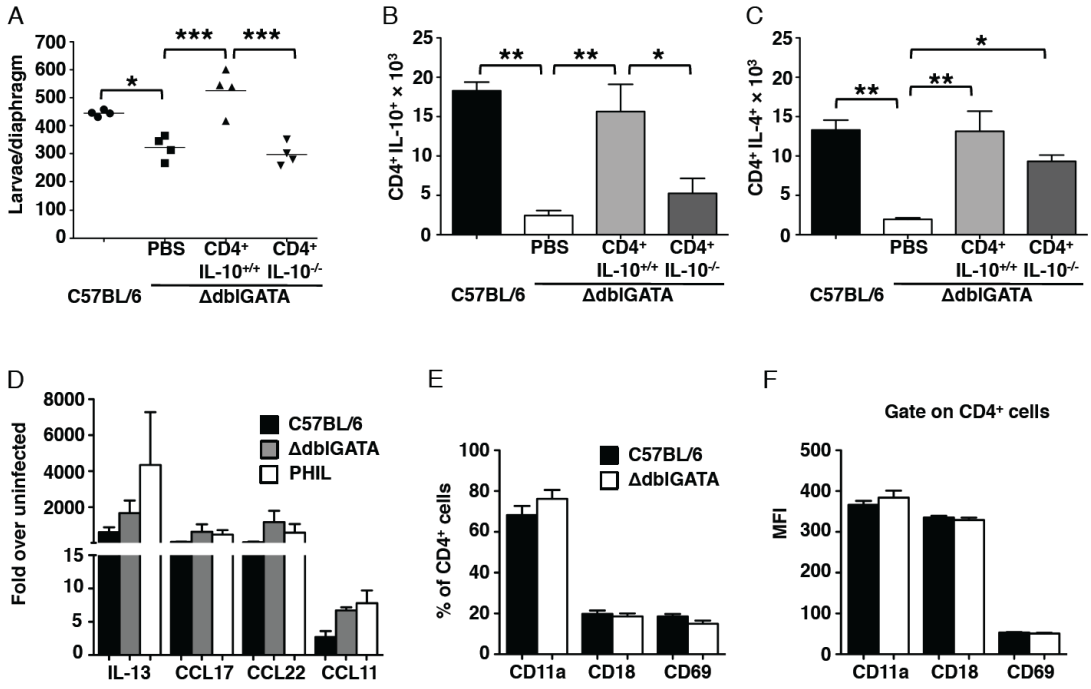

Figure 2.2. IL-10 derived from CD4$^+$ T cells protects larvae in muscle. (A) – (C), 5 × 10^6 CD4$^+$ T cells from infected WT or IL-10$^{-/-}$ mice were transferred to ΔdblGATA recipient mice on 4 dpi. **(A)** Larval burdens in diaphragms, 17 dpi. **(B)** Number of CD4$^+$IL-10$^+$ cells and **(C)** CD4$^+$IL-4$^+$ cells in diaphragms of mice, 17 dpi. **(D)** qPCR analysis of IL-13, CCL11, CCL17 and CCL22 in masseter muscles of WT, PHIL, ΔdblGATA mice, 14 dpi. **(E)** Percentage and **(F)** MFI of CD11a$^+$, CD18$^+$ and CD69$^+$ on gated CD4$^+$ T cells recovered from dLNs of WT and ΔdblGATA mice, 14 dpi. A – F, Each data set was collected from two experiments with similar results. Values represent mean ± SD; n = 3 - 4 mice. Significant differences were determined by Student's *t* test or ANOVA and Tukey's test. *p < 0.05, **p < 0.001, ***p < 0.0001.

cells from infected IL-10$^{-/-}$ donors had neither effect **(Fig. 2.2 A and B)**. CD4^{+}IL-4^{+} cells were recruited independently of the IL-10 status of donor cells **(Fig. 2.2. C)**. Thus, CD4^{+}IL-10^{+} T cells were capable of compensating for the absence of eosinophils in controlling local NO production, supporting the conclusion that the direct action of eosinophils on NO-producing leukocytes was not required to protect larvae from killing.

Eosinophils promote accumulation of IL-10^{+} myeloid DCs and IL-10-producing DCs compensate for eosinophil deficiency

The finding that eosinophils impact production of CD4^{+}IL-10^{+} T cells, together with the knowledge that eosinophils influence DCs in allergy (16), prompted us to test the impact of eosinophil-ablation on DCs in *T. spiralis* infection. Quantification of CD11c^{+} cells in dLNs of infected WT, PHIL and ΔdblGATA mice revealed no differences on 7, 9 and 11 days of infection (data not shown); however, fewer CD11c^{+}CD11b^{+}MHCII^{high} cells were recovered from dLN and diaphragm on day 11 in eosinophil-ablated mice **(Fig. 2.3. A - C)**. The results are consistent with earlier findings that eosinophils were required for DC trafficking and accumulation in lung dLNs following aeroallergen provocation (26). Indeed, accumulation of CD11c^{+}CD11b^{+}MHCII^{high} cells (myeloid DCs, mDCs) in *T. spiralis*-infected diaphragm significantly improved when eosinophils were restored to ΔdblGATA mice **(Fig. 2.3. C)**, confirming that recruitment or expansion of mDCs was dependent upon eosinophils. Moreover, the percentage of diaphragm mDCs that were IL-10^{+} was

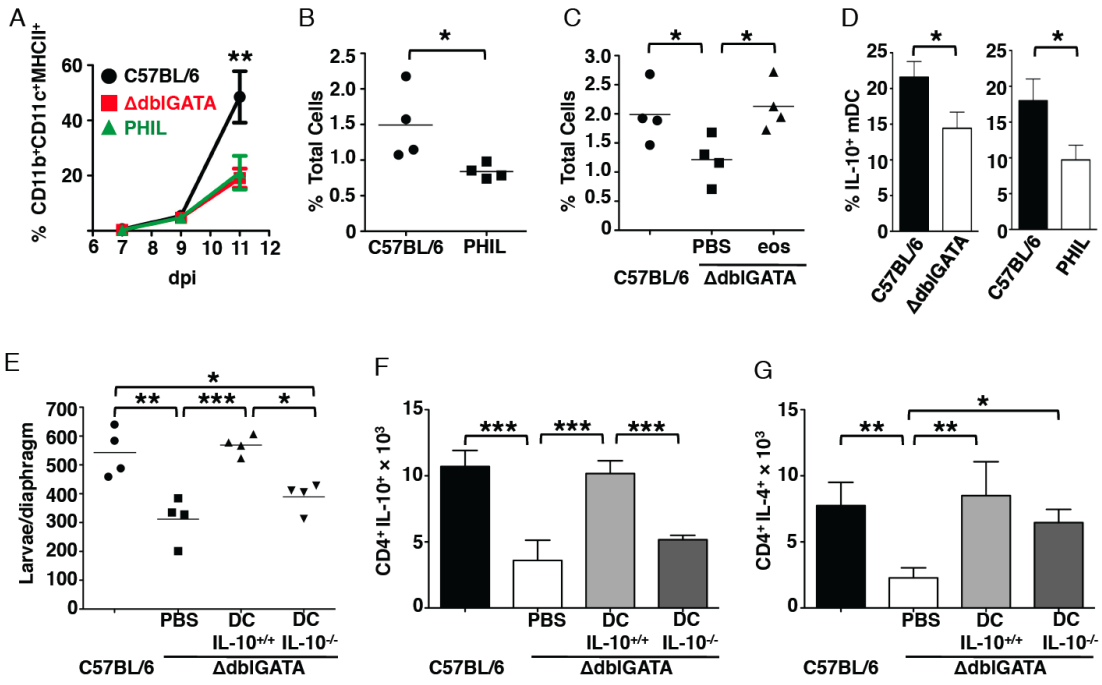

Figure 2.3. Eosinophils promote accumulation of IL-10$^+$ mDC that protect larvae. **(A)** Percentage of CD11b$^+$CD11c$^+$MHCII$^+$ mDCs in total CD11c$^+$ cells in dLN 7, 9 and 11 dpi. **(B)** Percentage of mDC of total diaphragm cells, 11 dpi. **(C)** Numbers of mDC cells recovered from diaphragms of ΔdblGATA mice (11 dpi) that received 5 × 10^6 eosinophils or PBS on alternate days from 5 – 9 dpi. **(D)** Percentage of total diaphragm cells that were IL-10$^+$ mDCs, 11 dpi. **(E) – (G)**, ΔdblGATA mice received 5 × 10^6 antigen-primed BMDCs from WT or IL-10$^{-/-}$ mice, 4 dpi. **(E)** Larval burdens in diaphragms, 17 dpi. **(F)** Number of CD4$^+$IL-10$^+$ cells and **(G)** CD4$^+$IL-4$^+$ cells in diaphragms, 17 dpi. Each data set was collected from two experiments with similar results. Values represent mean ± SD; n = 3 - 4 mice. Significant differences were determined by Student's *t* test or ANOVA and Tukey's test. *p < 0.05, **p < 0.001, ***p < 0.0001.

reduced in eosinophil-ablated mice **(Fig. 2.3. D)**. Bone marrow-derived DCs (BMDC) that were incubated with larval antigens prior to transfer to ΔdblGATA mice on 4 dpi increased survival of larvae **(Fig. 2.3. E)** and improved recruitment of $CD4^+IL\text{-}4^+$ and $CD4^+IL\text{-}10^+$ T cells to diaphragms **(Fig. 2.3. F and G)**. In contrast, transfer of larval antigen primed BMDC from $IL\text{-}10^{-/-}$ donors only improved the number of $CD4^+IL\text{-}4^+$ T cells in diaphragm without affecting either $CD4^+IL\text{-}10^+$ T cells or larval survival **(Fig. 2.3. E - G)**. Thus, eosinophils both recruit mDCs and support their production of IL-10. Such cells likely drive expansion of $CD4^+IL\text{-}10^+$ cells that limit *iNOS* expression (24) and NO-mediated larval killing.

Eosinophils arrive and exert their influence immediately following muscle infection

To further investigate the function of eosinophils, we sought to determine the time at which eosinophils normally extravascate at sites of skeletal muscle infection. In order to synchronize infection of the muscle, we injected NBL intravenously into WT mice. Eosinophil numbers increased dramatically in the diaphragm between 6 and 12 h following injection **(Fig. 2.4. A).** This was preceded by increases in serum IL-5, RANTES and KC **(Fig. 2.4. B)**, three important drivers of eosinophil chemotaxis. IL-6 in serum also increased upon injection **(Fig. 2.4. B).** Results for other mediators in the panel were not significant. Neutrophils did not extravascate in diaphragm until 12-24 h following injection **(Fig. 2.4. A)**, documenting a strong

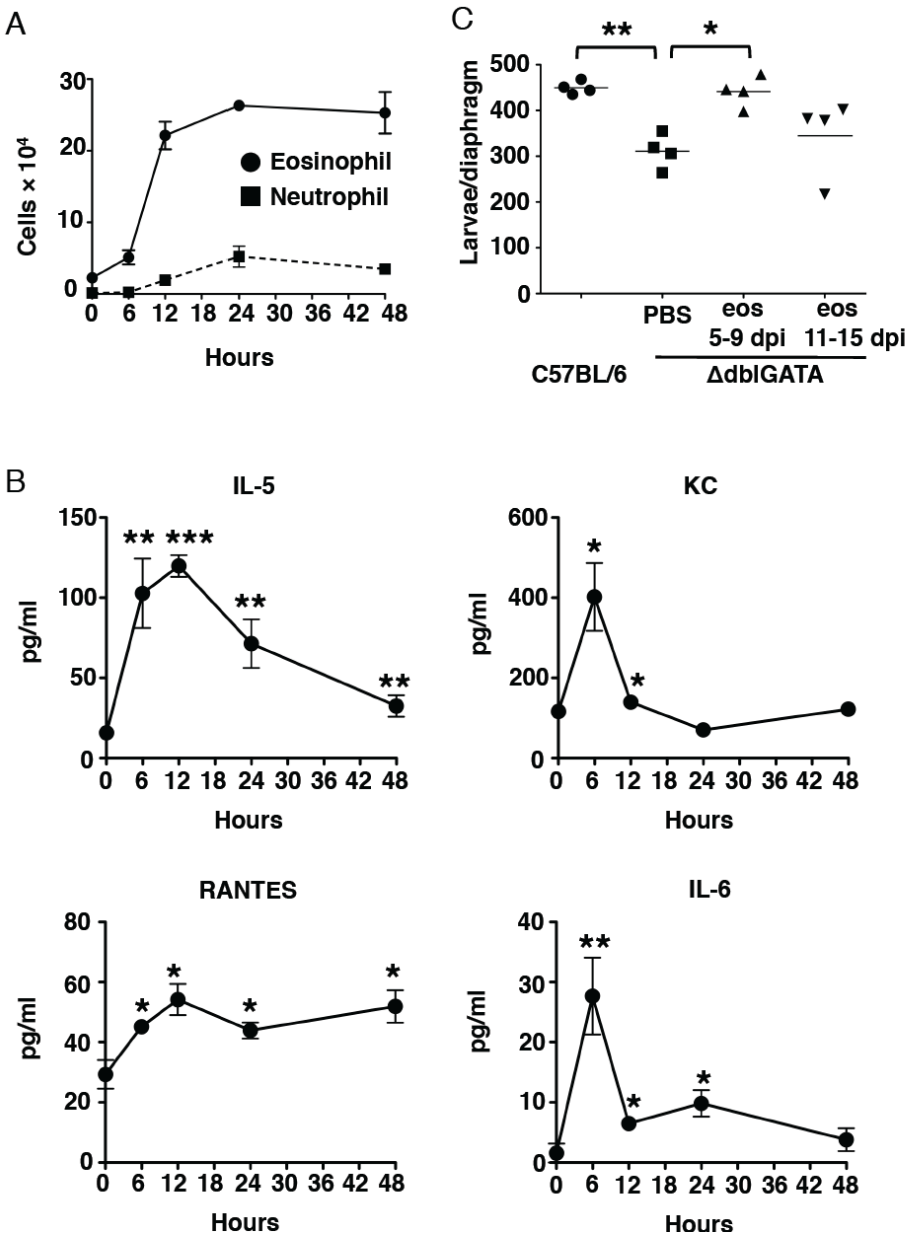

Figure 2.4. Eosinophils are rapidly and specifically recruited to muscle to promote larval survival during infection. (A) Numbers of eosinophils (Siglec-F^+ cells) and neutrophils (Ly-$6G^+$ (clone 1A8) cells) in diaphragms of naïve or WT mice, 6, 12, 24 and 48 h post injection with 25,000 NBL. **(B)** Concentrations of IL-5, KC, RANTES and IL-6 in sera from mice in (A). **(C)** Larval burdens in diaphragms, 17 dpi, of ΔdblGATA mice that received $5x10^6$ eosinophils or PBS every 48 h between 5 – 9 dpi or 11 – 15 dpi (oral infection). Each data set was collected from two experiments with similar results. Values represent mean ± SD; n = 3 - 4 mice. Significant differences were determined by Student's *t* test or ANOVA and Tukey's test. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

selection for eosinophils in the early recruitment of leukocytes to infected tissue (the ratio of neutrophils to eosinophils in the blood is approximately 25:1). Transfer of eosinophils collected from IL-5Tg^{+} mice to ΔdblGATA mice during two different intervals confirmed that larval survival was dependent upon the presence of eosinophils between day 5 and 9 following oral infection, a period during which NBL first arrive in muscle **(Fig. 2.4. C)**. Transfer of similar numbers of eosinophils between days 11 and 15 had a modest but not statistically significant effect on larval survival. Experimental outcomes were similar when eosinophil donors were either uninfected or infected (data not shown). Taken together, the results support the conclusion that rapid recruitment of eosinophils to sites of infection positions them to influence the initiation of the immune response.

Type 2 innate lymphoid cells are not required to promote muscle eosinophilia and larval survival

The newly discovered type 2 innate lymphoid cells (ILC2) have been demonstrated to recruit eosinophils by releasing preformed IL-5 (3, 27). In order to determine whether ILC2 influence recruitment of eosinophils to sites of infection, as well as larval survival, we injected NBL into ILC-ablated Rag2$^{-/-}$γc$^{-/-}$ mice. Intravenous infection controls the dose of NBL that are delivered to the muscle by circumventing the prolonged survival of fecund intestinal worms that occurs in lymphocyte-deficient mice. Although there were fewer eosinophils in the spleens in Rag2$^{-/-}$γc$^{-/-}$ compared to Rag1$^{-/-}$ mice **(Fig. 2.5. A)**, the recruitment of eosinophils to

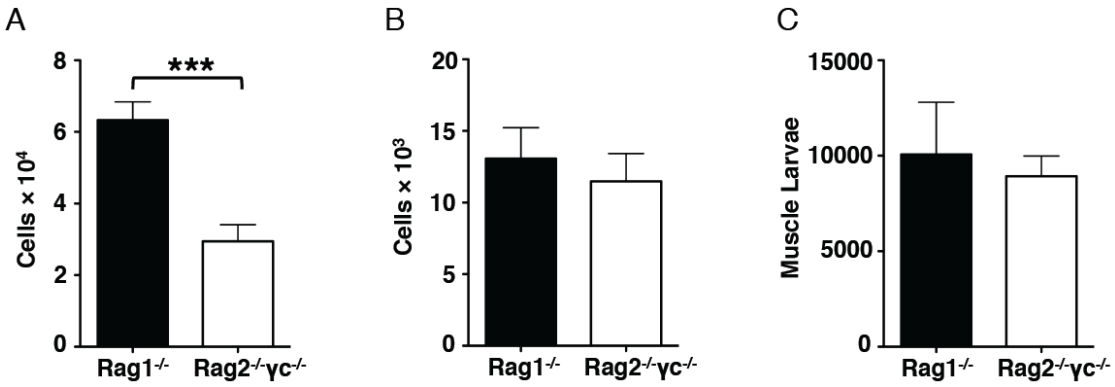

Figure 2.5. ILC2 are dispensable for muscle eosinophilia and larval survival. (A) – (C), Rag1$^{-/-}$ and Rag2$^{-/-}$γc$^{-/-}$ mice were injected 25,000 NBL intravenously. Numbers of eosinophils in **(A)** spleens and **(B)** diaphragms, 13 dpi. **(C)** Total body larval burdens in muscle, 24 dpi. Each data set was collected from two experiments with similar results. Values represent mean ± SD; n = 4 mice. Significant differences were determined by Student's *t* test. ***p < 0.0001.

sites of infection was normal in the absence of ILC2 **(Fig. 2.5. B)**. More importantly, larval burdens were equal in both strains of mice **(Fig. 2.5. C)**. Taken together, the results indicate that muscle eosinophilia and larval survival are not dependent on ILC2.

Antigen presentation by eosinophils is not required to preserve larvae

The requirement for eosinophils during the earliest phase of the IL-10-dependent immune response suggested that, in addition to promoting IL-10 production by mDCs **(Fig. 2.3. D)**, eosinophils may themselves present antigen. To better understand whether larval survival is a consequence of MHCII-dependent antigen presentation by eosinophils, we transferred $MHCII^{-/-}$ eosinophils isolated from infected $IL\text{-}5Tg^{+}/MHCII^{-/-}$ mice to infected ΔdblGATA mice (5 - 9 dpi). Both $MHCII^{+/+}$ AND $MHCII^{-/-}$ eosinophils supported larval burdens, larval growth **(Fig. 2.6 A and B)**, and accumulation of $CD4^{+}IL\text{-}4^{+}$ cells in diaphragm **(Fig. 2.6. C)**. Thus, antigen presentation by eosinophils is not required to promote larval survival or to drive Th2 responses.

Eosinophil-derived IL-10, but not IL-4 is required for larval survival

We next examined whether eosinophil-derived IL-4 promotes larval survival in skeletal muscle. Transfer of $IL\text{-}4^{-/-}$ eosinophils isolated from $IL\text{-}5Tg^{+}/IL\text{-}4^{-/-}$ mice to infected ΔdblGATA mice improved larval burdens and this correlated with an increase in the number of $CD4^{+}IL\text{-}10^{+}$ cells in diaphragms **(Fig. 2.7. A and B)** but not $CD4^{+}IL$-

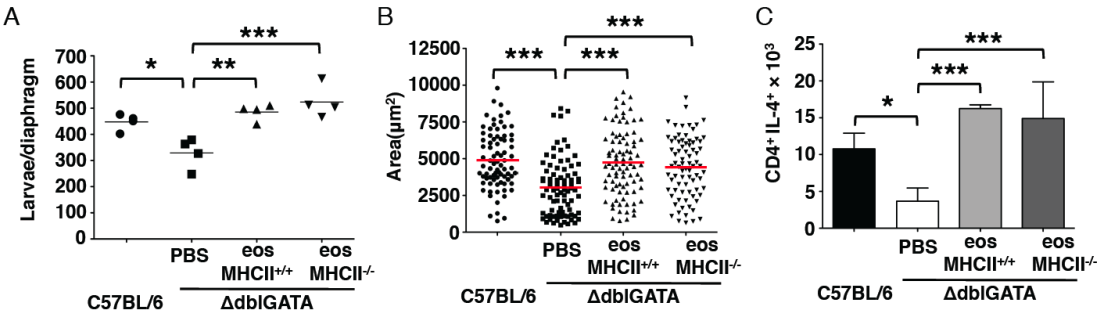

Figure 2.6. Antigen presentation by eosinophils is not required to preserve larvae. **(A) – (C)**, ΔdblGATA mice received PBS or 5×10^6 eosinophils from infected IL-$5Tg^+$ or IL-$5Tg^+$/$MHCII^{-/-}$ mice every 48 h from 5 – 9 dpi. **(A)** Larval burdens in diaphragms, 17 dpi. **(B)** Body size (area) of larvae, 17 dpi. **(C)** Numbers of $CD4^+IL\text{-}4^+$ cells in diaphragms, 17 dpi. Each data set was collected from two experiments with similar results. Values represent mean ± SD; n = 4 mice. Significant differences were determined by ANOVA and Tukey's test. *p < 0.05, **p < 0.001, ***p < 0.0001.

4^+ T cells **(Fig. 2.7. C)**. In other experiments, IL-$13^{-/-}$ and WT mice responded similarly to infection with *T. spiralis* (data not shown). The results support the conclusions that both IL-4 and IL-13 are dispensable in eosinophil-mediated regulation of immunity that preserves *T. spiralis* larvae by preventing local production of NO. We detected eosinophils producing IL-10 in infected reporter mice (VertX strain (28)) confirming that eosinophils in dLN and diaphragms expressed the gene **(Fig. 2.7. D)**. Furthermore, transfer of eosinophils from IL-$10^{-/-}$ mice to ΔdblGATA recipients failed to improve burdens **(Fig. 2.7. E)**, while IL-10 competent eosinophils rescued larvae and enhanced recruitment of $CD4^+$IL-10^+, but not $CD4^+$IL-4^+ T cells to sites of infection **(Fig. 2.7. E - G)**. Thus, the mechanism of action of eosinophils in preserving larvae in muscles depended upon intrinsic production of IL-10 that directed the adaptive immune response via IL-10^+ mDCs and $CD4^+$IL-10^+ T cells, ultimately inhibiting expression of *iNOS* and preventing NO-mediated killing of the parasite.

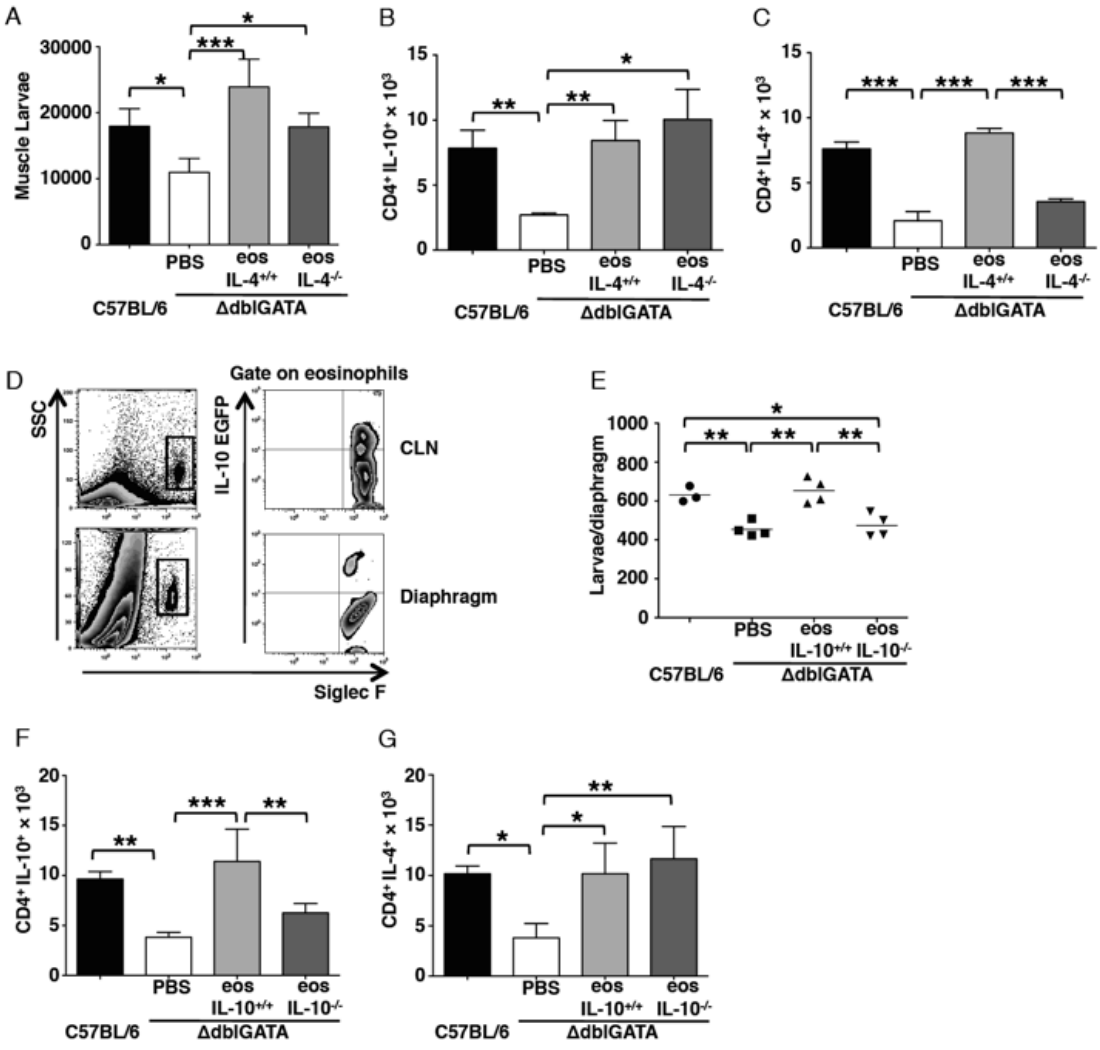

Figure 2.7. Eosinophil derived IL-10 preserves larvae. (A) – (C), ΔdblGATA mice received PBS or 5×10^6 eosinophils from infected IL-5Tg$^+$ or IL-5Tg$^+$/IL-4$^{-/-}$ mice every 48 h from 5 – 9 dpi. **(A)** Total body larval burdens in muscle, 28 dpi. **(B)** Number of CD4$^+$IL-10$^+$ cells and **(C)** CD4$^+$IL-4$^+$ cells in diaphragms of mice, 17 dpi. **(D)** IL-10 detected in eosinophils recovered from dLNs and diaphragms of infected Vertx mice, 13 dpi. **(E) – (G)**, ΔdblGATA mice received 5×10^6 eosinophils from infected WT or IL-10$^{-/-}$ mice, or PBS every 48 h from 5 – 9 dpi. **(E)** Larval burdens in diaphragms, 17 dpi. **(F)** Numbers of CD4$^+$IL-10$^+$ cells and **(G)** CD4$^+$IL-4$^+$ cells in diaphragms of mice, 17 dpi. Each data set was collected from two experiments with similar results. Values represent mean ± SD; n = 3 - 4 mice. Significant differences were determined by Student's *t* test or ANOVA and Tukey's test. *p < 0.05, **p < 0.001, ***p < 0.0001.

Discussion

The aim of this study was to bring clarity to the striking and unexpected observation that eosinophils protect intracellular, muscle-stage *T. spiralis* larvae against NO-mediated killing. Testing two strains of eosinophil-ablated mice, engineered by two very different approaches, reduced the likelihood that the results are artifactual, and our experiments consistently replicate results in PHIL and ΔdblGATA mice. Adoptive transfer methods are powerful tools for examining the contribution of effector cell populations in such mice. We set out to address three questions: what are the crucial players in killing by NO, do eosinophils prevent killing via $CD4^{+}IL\text{-}10^{+}$ T cells, and when and how do eosinophils exert their influence? We discovered a dominant role for IL-10 throughout the immune response that culminates in the protection of larvae in muscle.

Our previous findings showed that growing larvae were at greatest risk for NO mediated killing and suggested that the combination of impaired growth and local NO that was evident in eosinophil-ablated mice was responsible for the high rate of larval clearance. Infection of Arg1-deficient mice showed that larval killing could occur in the absence of impaired growth. Similarly, growth is normal in $IL\text{-}10^{-/-}$ mice while local *iNOS* expression is high, and larval killing is evident (21, 29). Reduction in larval burdens at 28 days following oral infection is similar in these two strains (44% in $Arg1^{flox/flox}$;Tie2cre, 50% in $IL\text{-}10^{-/-}$) and in ΔdblGATA (48%). PHIL mice consistently show a higher rate of clearance (67-77%), a finding that is unexplained. We speculate that IL-10 and Arg1 deficiencies yield much higher local NO that overcomes the contribution of impaired growth to net larval clearance that may occur

in eosinophil-ablated mice. The relationship between impaired growth and larval clearance requires further investigation. While $CD4^+CD25^-$ T cells are critical sources of IL-10 that limits NO production by cells that infiltrate *Trichinella*-infected muscle (21, 24), the functions of eosinophils in supporting larval growth appear to operate independently of adaptive immunity (30), and are being investigated separately. In this report, we describe the processes associated with eosinophil-driven adaptive immunity that give rise to control of local NO production by T cells.

Taken together with earlier findings that inhibition of iNOS by drug treatment correlated with improved larval survival (20), that macrophages account for 80% of leukocytes at sites of infection, and that $iNOS^+$ cells at sites of infection are largely macrophages and neutrophils (21), the results from $Arg1^{flox/flox}$;Tie2cre mice support the conclusion that myeloid cells are important targets of eosinophil-dependent immune regulation during *T. spiralis* infection. Moreover, adoptive transfer of $IL\text{-}10^+$ mDCs or $CD4^+$ T cells to eosinophil-ablated mice compensated for eosinophils in protecting larvae from killing. This result indicates that eosinophils do not need to interact directly with macrophages or neutrophils in order to limit NO production, and further supports the conclusion that their influence is effected by promoting an adaptive immune response that is rich in IL-10.

In addition to limiting NO production, Arg1 is the key enzyme in collagen deposition by macrophages (31) . A collagen capsule is a defining feature of the *Trichinella* nurse cell and large numbers of macrophages are apposed to the capsule (32, 33). Nevertheless, the nurse cell has been reported to supply collagen for the capsule (32). Although the expression of Arg1 is decreased in diaphragms of

eosinophil-ablated mice, capsule formation is normal in surviving nurse cells (20), indicating myeloid cells may be dispensable for collagen deposition. Normal larval growth in $Arg1^{flox/flox}$;Tie2cre mice provides further, indirect evidence that local myeloid cells are not required for collagen capsule formation.

Eosinophils induce T cell responses by distinct mechanisms in different systems(13-16, 34). Restoring eosinophils to ablated, infected mice promoted the expansion of mDCs and $CD4^+IL\text{-}10^+$ T cells. We found no evidence that eosinophils promoted $CD4^+$ T cell activation via antigen presentation, rather our data are consistent with an eosinophil influence on DC recruitment and activation that subsequently drives T cell differentiation. Importantly, $CD11b^+$ mDCs, a DC subset, can initiate the production of IL-10 from T cells in helminth infections by secreting IL-10 (35-39). In allergic asthma, eosinophils have been shown to be required for accumulation of mDCs in the lung and draining lymph node (26). In *T. spiralis* infected, eosinophil-ablated mice, the reduction in $CD11b^+$ mDCs at sites of infection and CLN was not likely the result of a deficient chemokine response (as is observed in asthma (26)) as gene expression of several chemokines known to be essential for recruitment of monocytes was normal in eosinophil-deficient infected tissue (data not shown). Although the deficiency in total cells is not readily explained by our data, clearly the cytokine profile of mDCs was influenced by eosinophils. IL-10 induces maturation of DCs, and triggers the secretion of IL-10 from DCs (39, 40). Thus, we speculate that in addition to promoting the accumulation of mDCs, eosinophils provide IL-10 that stimulates DCs to become IL-10 producers. This, in turn, creates a

suitable microenvironment for the development of $CD4^{+}IL\text{-}10^{+}$ T cells that limit *iNOS* expression and thereby protect larvae.

We speculate that mice mount innate and adaptive IL-10 responses in order to limit tissue injury and suppress immune-mediated inflammation caused by NBL, and that such a response coincidentally supports *T. spiralis* in establishment of intracellular infection. Eosinophils were selectively recruited to skeletal muscle within hours of intravenous injection of NBL. This response is distinct from the well-documented, T cell-dependent eosinophilia induced by *T. spiralis* that occurs several days after oral infection (41). The innate response included increases in serum concentrations of three key mediators of eosinophil chemotaxis: IL-5, KC and RANTES (42). The nature of the assay performed did not reveal the source(s) of the chemokines and it is possible that they were derived from muscle, lung or other tissues that larvae enter during migration. The timing of detection in blood is consistent with the mediators being preformed rather than induced. We speculate that the release of these mediators is triggered by injury caused by large NBL (150 μm) that deploy a stylet to aid their exit from the blood and penetration of tissue. Endothelial cells express KC and RANTES, and rapidly secret them in response to injury (43, 44). Moreover, NBL activate the complement system (45), which may trigger tissue resident mast cells to release IL-5, KC and RANTES (46-49).

The cellular sources and significance of IL-10 varies among helminth infections (50-55). By infecting IL-10 reporter mice, we observed that eosinophils in dLN and muscle produce IL-10 during *T. spiralis* infection. IL-10 was not detected in eosinophils in uninfected mice, indicating that it was induced by infection. The

immediate release of chemokines and recruitment of eosinophils to sites of infection are consistent with an innate response to injury caused by NBL. IL-10 is a feature of the response to injury, inhibiting inflammation and minimizing tissue damage (56). By regulating macrophage phenotype, IL-10 promotes muscle regeneration and growth (57).

Injured tissues can release mediators known for initiating the IL-10 response, including IL-6 and IL-27 (58, 59). In *T. spiralis* infection, serum IL-6 was dramatically increased at 6 h after NBL injection. Exercise-associated muscle injury induces increase of muscle-derived IL-6 in plasma (58), and the transient increase of IL-6 may enhance production of IL-10 (60). Both IL-6 and IL-27 influence T cell populations to produce IL-10 (61-64). We detected increased expression of IL-27 as well as increased numbers of IL-27 receptor expressing eosinophils at sites of infection (Huang and Appleton, unpublished data), suggesting a potential role for IL-27 in triggering eosinophils to secret IL-10; however, pilot experiments showed only a modest trend towards decreased larval burdens in IL-27 receptor deficient mice, with a partial improvement in larval survival following adoptive transfer of normal eosinophils to receptor deficient mice. Thus, although IL-27 may contribute, it does not fully account for IL-10 induction in eosinophils at sites of infection. The influence of IL-6 on eosinophils merits further investigation.

IL-25 and IL-33 are found in endothelial cells and function as alarmins during inflammation. Both are known to stimulate ILC2 (65) to release IL-5 and IL-13, which in turn activate T helper cells and recruit eosinophils (66). The timing of eosinophil recruitment was not consistent with a role for ILC2 in nucleating this innate eosinophil

response, and indeed, infection of Rag2$^{-/-}$γc$^{-/-}$ mice showed normal muscle eosinophilia and larval survival. Although it has been shown that ILC2 is essential for maintaining the integrity of mucosal sites, such as clearance of intestinal helminths and promoting tissue repair after helminth infections (67-69), our data support a dispensable role for ILC2 in muscle infection.

Study of *T. spiralis* infection in mice is particularly valuable because there are very few natural animal models of either tissue-dwelling helminth infection or skeletal muscle responses to injury caused by infection. By combining eosinophil ablation and restoration with this natural infection, our findings document a novel immunoregulatory function of eosinophils. By entering sites of infection immediately following tissue invasion by *T. spiralis* larvae, eosinophils deploy IL-10 that expands IL-10-secreting DCs and CD4^{+} T cells, thereby controlling the activation of proinflammatory macrophages and neutrophils that otherwise kill parasite larvae by releasing NO. We speculate that *Trichinella* has adapted to its host by exploiting a mechanism that is in place to limit tissue injury. The remarkable functional versatility of eosinophils that has become increasingly evident recently is an important consideration in devising strategies for new prophylactic and therapeutic approaches to reducing the burden of parasitic worm infections.

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CHAPTER 3

Eosinophils mediate protective immunity against secondary nematode infection[*]

[*] Lu Huang, Nebiat Gebreselassie, Lucille Gagliardo, Maura Ruyechan, Kierstin Luber, Nancy Lee, James J. Lee, Judith A. Appleton. Eosinophils mediate protective immunity against secondary nematode infection. *Manuscript in preparation*.

Abstract

Eosinophils are versatile cells that regulate innate and adaptive immunity, influence metabolism and tissue repair, and contribute to allergic lung disease. Within the context of immunity to parasitic worm infections, eosinophils are prominent yet highly varied in function. We have shown previously that when mice undergo primary infection with the parasitic nematode, *Trichinella spiralis*, eosinophils play an important, immune regulatory role that promotes larval growth and survival in skeletal muscle. In this study, we aimed to address the function of eosinophils in secondary infection with *T. spiralis*. By infecting eosinophil-ablated mice, we found that eosinophils are dispensable for immunity that clears adult worms or controls fecundity in secondary infection. In contrast, eosinophil ablation had a pronounced effect on secondary infection of skeletal muscle by migratory newborn larvae. Restoring eosinophils to previously infected, ablated mice caused them to limit muscle larvae burdens. Passive immunization of naïve, ablated mice with sera from infected donors, together with transfer of eosinophils, was effective in limiting the number of newborn larvae that migrated in tissue and colonized skeletal muscle. Our results are compatible with earlier reports from *in vitro* experiments that have informed the dogma that eosinophils bind to larvae in the presences of antibodies. These findings document a third function for eosinophils in *T. spiralis* infection.

Introduction

Parasitic worms are estimated to infect two billion people worldwide and nearly 1 billion children live in areas of high transmission (1). Although drug therapy is often effective in clearing infections, reinfection rates are high (2), consistent with poorly sustained immunity. A relatively small number of anthelmintic drugs are available and the efficacy of anthelmintics is known to be limited by the emergence of drug resistant parasites (3-5). Advancing vaccination as a more sustainable alternative to chemotherapy requires understanding of immune mechanisms that are effective in preventing or clearing infection.

Clearance of worm infections occurs by mechanisms that vary among parasites, the hosts they infect, and the tissues they colonize (6). As is the case with other pathogens, mechanisms of immunity in primary worm infection often differ from those that are effective in preventing or clearing secondary infection. There is extensive evidence supporting key roles for Th2 cells in clearing primary, intestinal infections, although the effector mechanisms vary (7-9). Additional evidence documents important contributions of B cells and antibodies in controlling secondary infections and also in vaccine induced protection (10-13). In the case of the parasitic nematode, *Trichinella spiralis*, T cell-mediated immunity that drives intestinal mastocytosis in primary infection is central to the mechanism of worm clearance in rats and mice (14-16); however, mast cell activation is neither necessary nor sufficient for antibody-mediated protection that clears larval stages from rats during a secondary intestinal infection (17-19). Mice do not demonstrate this same, antibody-mediated

immunity to secondary infection but instead manifest worm expulsion that is accelerated but similar to that observed in primary infection (20).

Intestinal mastocytosis during primary *T. spiralis* infection occurs simultaneously with a pronounced tissue eosinophilia (21). *In vitro* studies have shown that eosinophils are capable of adhering to and killing several species of parasitic worms. In some species, including *T. spiralis*, killing occurs only in the presence of specific antibodies (22, 23). These findings served to set a paradigm for eosinophils as cytotoxic effector cells in worm infection. Early investigations of eosinophil effector function *in vivo* were performed by antibody-mediated depletion of eosinophils or manipulating IL-5 in mice. Findings from studies conducted with various worm infections supported a role for IL-5 in some cases (24, 25) and not in others (26). Subsequently, experiments with eosinophil-ablated mice have shown that eosinophils are dispensable in immunity to primary infections with intestinal worms (27-31). Secondary infections are less well studied, but challenge of eosinophil-ablated mice revealed that eosinophils contribute to protective immunity against *Nippostrongylus brasiliensis* by interfering with larval migration (30).

In contrast, results from infections with worms that colonize extraintestinal sites, including *T. spiralis*, show that eosinophils are beneficial to the parasite during primary infection (29, 32, 33). *Trichinella* completes its life cycle in a single host. Infective first-stage larvae are ingested and mature into adult worms in the intestine, where they reproduce and release newborn larvae (NBL). NBL enter the circulatory system and transit the lung (34) prior to arriving in skeletal muscles. Larvae invade myotubes and establish chronic, intracellular infection. By infecting eosinophil-

ablated mice, we have shown that in primary infections, eosinophils are required for efficient growth as well as survival of muscle larvae (29, 32). Survival is promoted via control of local NO-production by an eosinophil-driven IL-10 response (29, 32, 35, 36). The effect of eosinophil ablation on secondary infection has not been tested. Previous studies in IL-5-deficient or -depleted mice yielded contradictory results (26, 37). The unexpected properties of eosinophils in primary *T. spiralis* infection and the contradictory results between *in vitro* and *in vivo* studies of eosinophil function prompted us to test the contribution of eosinophils to secondary immunity to *T. spiralis* using eosinophil lineage-ablated mice.

We report here that eosinophils are required for effective immunity to secondary infection by *T. spiralis*. Although eosinophil ablation had no effect on intestinal immunity, it was associated with enhanced colonization of skeletal muscle. Transfer experiments showed that eosinophils interfered with migration of NBL, an effect that was dependent on the presence of immune serum. The results provide evidence in support of an antibody-dependent mechanism in which eosinophils limit secondary infection by *T. spiralis*.

Materials and Methods

Rats and mice

Adult Albino Oxford strain rats were produced and maintained in the Baker Institute vivarium. PHIL, ΔdblGATA, $MBP^{-/-}$, $EPX^{-/-}$ and IL-5-expressing transgenic (NJ.1638) ($IL\text{-}5Tg^{+}$) mice were bred at Cornell Transgenic Mouse Core Facility and offspring were transferred to the Baker Institute. All strains were on a C57BL/6 background. C57BL/6 ^{NHsd} mice were purchased from Taconic as wild type (WT) controls. Animal care was in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care and experiments were performed with the approval of the Institutional Animal Care and Use Committee of Cornell University.

Parasite and Antigens

Trichinella spiralis first-stage larvae (L_1) and newborn larvae (NBL) were recovered from rats as described previously (29). For oral infection, L_1 were suspended in 2% nutrient broth (Difco), 0.6% gelatin (Fisher Scientific) and doses of 300 L_1 were administered by gavage. For synchronous infection, 25,000 NBL were suspended in 0.25 ml serum-free DMEM (Mediatech, Inc.) and delivered by retro-orbital injection. Mice were euthanized by CO_2 inhalation at the times indicated in each experiment. Whole body muscle larvae burdens were assessed in whole carcasses 28 days post-infection (dpi) as described previously (29). Intestinal worm burdens were estimated as described previously. To determine the numbers of NBL in the lung, the whole lung was surgically isolated and disrupted on a tea strainer. Tissue suspensions were

centrifuged and resuspended in 10% formalin. NBL were counted using a phase contrast microscope. Crude somatic antigens from L_1 were prepared as previously described (29).

Challenge infection, sera collection and passive immunization

Mice were infected with 300 L_1 and challenged with the same dose after 90 days. Immune sera were collected from WT or ΔdblGATA mice 28 days following re-infection. Normal sera were collected from naïve WT mice. For passive immunization, mice were injected intraperitoneally with 0.2 ml serum from naïve or infected mice.

Enzyme-linked immunosorbent assay (ELISA)

Cells from cervical lymph nodes (CLN) or mesenteric lymph nodes (MLN) were cultured, stimulated with antigen, and supernatants were assayed for IL-4, IL-5, IL-10, IL-13 and IFN-γ, as described previously (32). Serum antibodies specific for *T. spiralis* antigens were measured as described previously (17). Mouse sera were diluted 1:2000 for IgG1, or 1:100 for IgG2c. IgG1 was detected with rat anti-mouse IgG1 (BD PharMingen) and biotinylated mouse anti-rat IgG (BD PharMingen) followed by HRP-conjugated streptavidin (BD Biosciences). IgG2c was detected with goat anti-mouse IgG2c (Immunology Consultants Laboratory) and HRP-conjugated rabbit anti-goat IgG (Immunology Consultants Laboratory).

Eosinophil transfer experiments

Eosinophils were recovered from infected IL-5Tg^{+} mice 12-20 dpi. Cells were pooled from spleens and peritoneal lavage fluid and purified by positive selection on magnetic beads, as previously described (32). Briefly, eosinophils were labeled with PE-conjugated anti-Siglec-F antibody (BD) and anti-PE microbeads (Miltenyi Biotec). Average purity of eosinophils from this procedure was >93%. After washing twice with PBS, 5×10^6 eosinophils were resuspended in 200 µl sterile PBS and injected i.v. into ΔdblGATA mice. Cells were transferred 4 times, on alternate days, for 8 days.

Flow cytometry

Cells from individual diaphragms were recovered as described previously (32). After a 15 min incubation with Fc block (eBioscience) and 10% normal mouse serum, cells were incubated for 15 min with PE conjugated anti-SiglecF (BD Pharmingen). Data were acquired using a Gallios flow cytometer (Beckman Coulter) and analyzed with FlowJo software (Tree Star)

Worm fecundity

Worm fecundity was determined as described (38). Briefly, female worms were recovered from the small intestines of C57BL/6 and ΔdblGATA mice, 6 days following primary or secondary infection with 300 L_1. Individual female worms were placed in 100 µl of RPMI1640 containing 10% FCS in a single well of a 96-well plate and incubated at 37 °C for 24 h in 5% CO_2. The number of shed NBL was counted under a microscope. At least ten female worms were collected from each mouse, and the mean NBL value per mouse was used to calculate means for each treatment group.

ATP content measurement

100 adult worms were collected from individual mice as described (39). Worms were washed three times and boiled for 15 min in 100 µl PBS. Lysates were centrifuged 10,000 × g for 10 min at 4 °C. ATP was measured in supernatants with an ENLITEN ATP Assay System Bioluminescence Detection Kit (FF2000, Promega, Madison, WI).

Statistical analysis

All experiments were performed twice with similar results. Means ± SD were calculated from data collected from individual mice unless otherwise indicated. Significant differences were determined using Student's t test or one-way ANOVA with Tukey's post hoc test for multiple means. Statistical analysis was performed with GraphPad Prism 5 software.

Results

Eosinophils are dispensable for intestinal immunity against secondary T. spiralis infection.

To investigate the role of eosinophils in secondary infection of *T. spiralis*, we first examined parameters of intestinal infection in eosinophil-ablated mice. We infected WT and ΔdblGATA mice with 300 larvae and then challenged them with the same dose 90 days later (1°+2°). A group of naïve mice received larvae at the 90 day time point in order to document the primary immune response (challenge control) **(Fig. 3.1. A)**. Cytokine production by antigen-stimulated MLN cultures was similar in WT and ΔdblGATA mice **(Fig. 3.1. B)**. The rate of intestinal worm expulsion was similar in the two strains in primary infection, and was similarly accelerated in secondary infection **(Fig. 3.1. C)**. Worm fecundity and ATP content of male and female worms were not significantly different between strains in either infection **(Fig. 3.1. D and E)**. Taken together, the results indicate that eosinophil ablation did not influence the outcome of intestinal infection by *T. spiralis*. Therefore, any differences in either NBL migration or muscle larvae burden in eosinophil-ablated vs. WT mice that may be observed following intestinal infection would be attributed to extraintestinal effects of eosinophils and not to differences in 'doses' of NBL produced by intestinal worms.

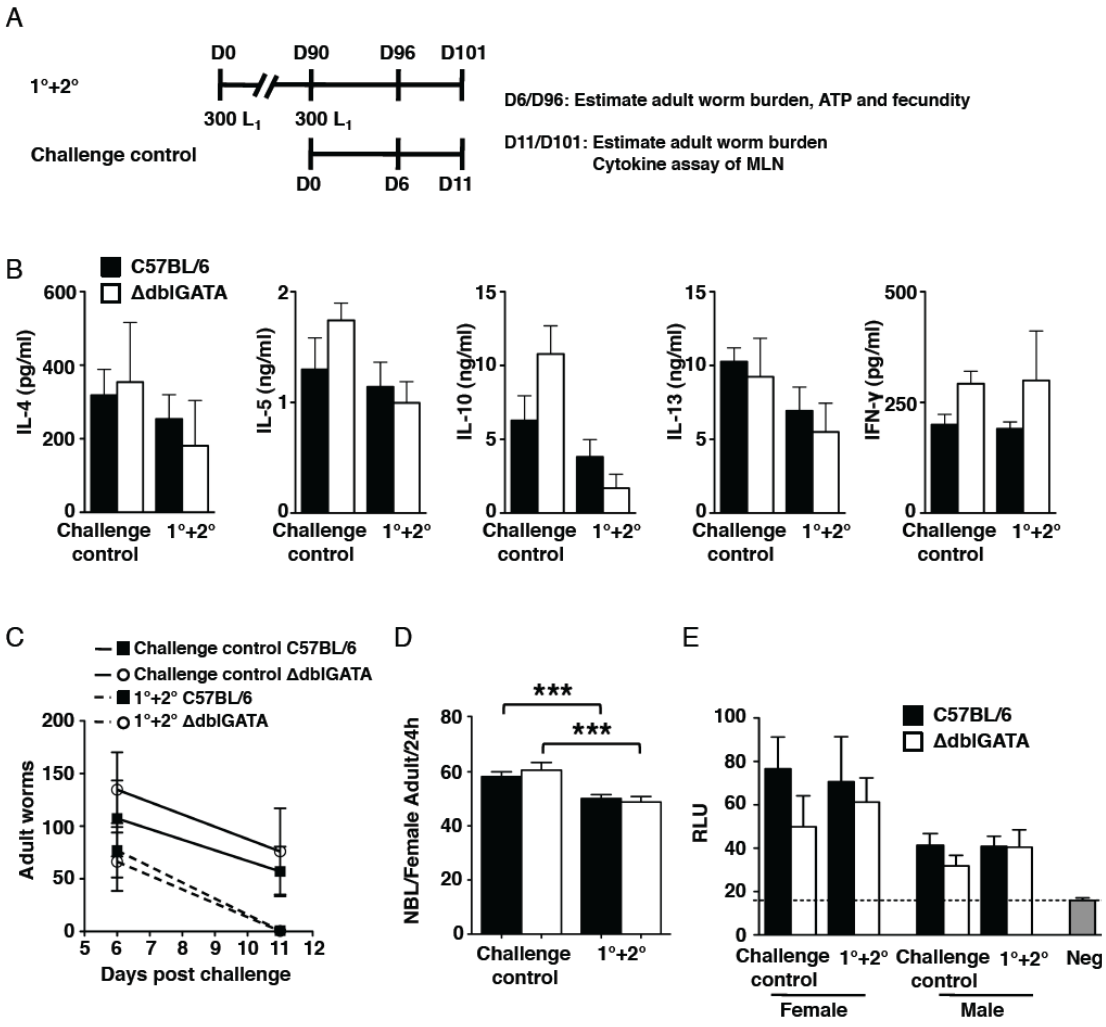

Figure 3.1. Eosinophils are dispensable for intestinal immunity against *T.spiralis* infection. (A) Design of experiments indicating timing of sample collections. D, day. **(B)** Cytokine responses in MLN of WT and ΔdblGATA mice. **(C)** Adult worms in intestines of WT and ΔdblGATA mice during primary or secondary infection. **(D)** Fecundity of female adult worms and **(E)** ATP content of adult males and females recovered from the intestines of WT and ΔdblGATA mice during primary or secondary infection. Each data set was collected from two experiments with similar results. Values represent mean ± SD; n = 3 - 4 mice. Significant differences were determined by ANOVA and Tukey's test. ***$p < 0.0001$.

Eosinophils prevent the accumulation of muscle larvae in secondary infection

The influence of eosinophils on extraintestinal parasites, specifically migrating NBL and larvae that colonize skeletal muscle, was tested in experiments similar in design to those described above, but with an additional control group that received only the first infection (infection control) **(Fig. 3.2. A)**. Cytokines were assayed in CLN cultures, as these nodes drain the tongue and masseter, preferred sites of colonization by NBL. Antigen-stimulated CLN cultures prepared from ΔdblGATA mice 17 days after secondary infection produced less IL-4 and than WT cells. This finding is similar to that observed in primary infection and reported previously (29, 32). IL-10 was reduced in both primary and secondary infection. No differences were observed in IL-5, IL-13 and IFN-γ **(Fig. 3.2. B)** in secondary infection, although IFN-γ was increased in cultures from ablated mice undergoing primary infection, as reported previously (29, 32).

WT mice prevented the accumulation of additional muscle stage larvae while ΔdblGATA mice did not show this resistance, i.e. their muscle burdens equaled the combined burdens of the two infection control groups **(Fig. 3.2. C)**. While few if any migrating NBL were recovered from lungs of WT mice ten days post-secondary infection, the number recovered from lungs of ΔdblGATA mice was significantly higher and was not different from that of the challenge control mice **(Fig. 3.2. D)**. Thus, eosinophils appeared to limit larval establishment in muscle by interfering with migration of NBL.

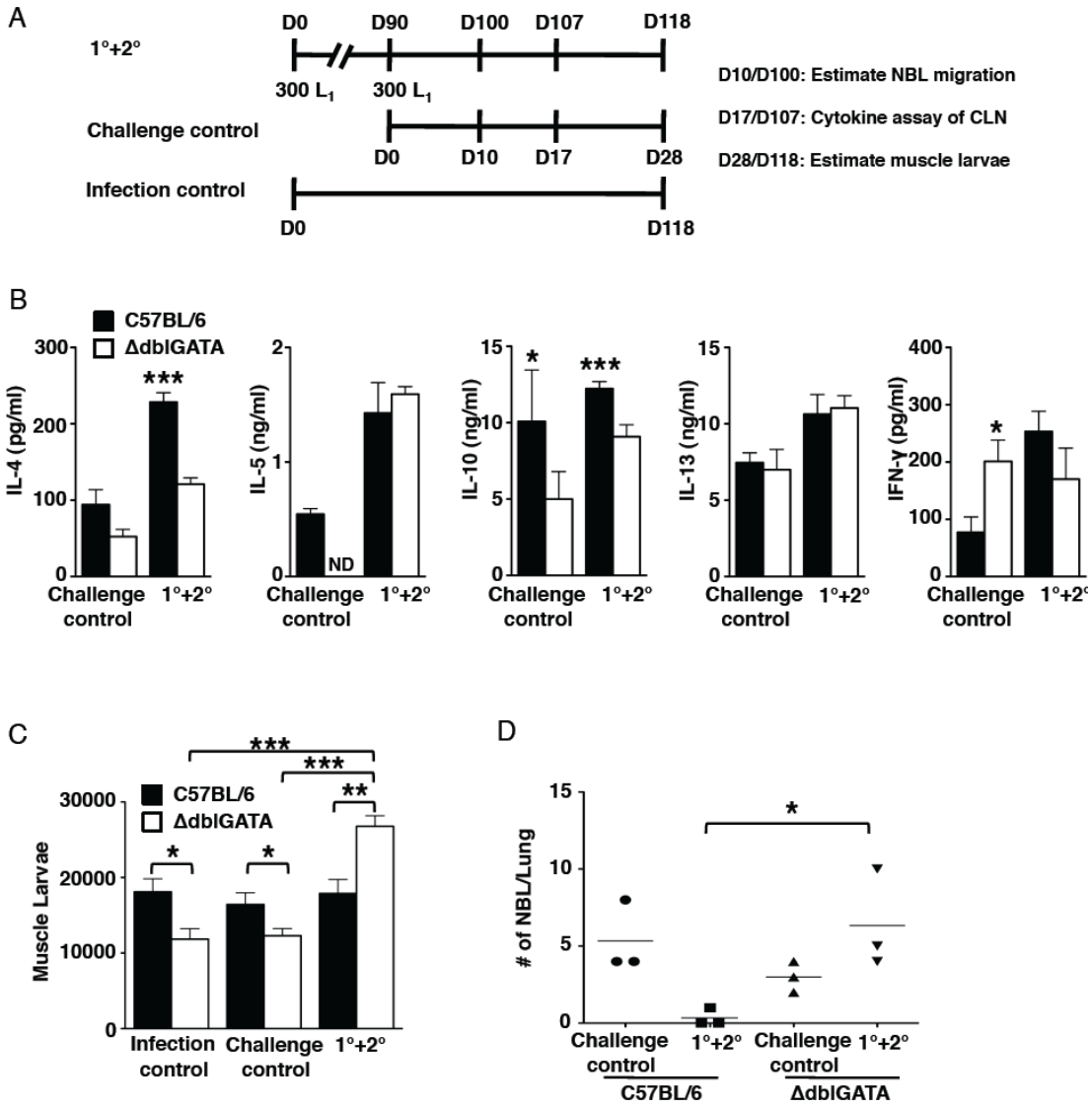

Figure 3.2. Eosinophils influence secondary immunity to muscle infection and limit the migration of NBL. (A) Design of experiments indicating timing of sample collections. D, day. **(B)** Cytokine responses in CLN cultures of WT and ΔdblGATA mice. **(C)** Whole body muscle larval burdens in WT and ΔdblGATA mice. **(D)** Numbers of NBL in lungs recovered from WT and ΔdblGATA mice. Each data set was collected from two experiments with similar results. Values represent mean ± SD; n = 3 - 5 mice. Significant differences were determined by Student's *t* test or ANOVA and Tukey's test. *p < 0.05, **p < 0.001, ***p < 0.0001.

Specific antibodies are required for eosinophil-mediated protection against secondary muscle infection

To confirm that eosinophils are required for the resistance to re-infection, we performed eosinophil transfer experiments. Restoring eosinophils to previously infected ΔdblGATA mice between days 4 and 10 following oral challenge infection resulted in fewer larvae maturing in skeletal muscle **(Fig. 3.3. A)**. [This result is the opposite of that observed when a nearly identical transfer protocol is applied to primary infection. In that case, larval burdens are increased in ΔdblGATA eosinophil recipients (32) (**Fig. 3.3. D**, see naïve sera plus eosinophils).] With this result, we hypothesized the eosinophils clear migrating larvae from tissues by working in concert with immune effectors that were induced by prior infection. Although it has not been documented *in vivo*, binding of antibodies to the body surfaces of newborn larvae has been shown to promote eosinophil adhesion and degranulation *in vitro* (23). In order to test the protective effects of serum antibodies, we passively immunized naïve ΔdblGATA mice with sera from naïve or immune WT mice. The next day, eosinophils were restored to recipients and mice were infected intravenously with NBL **(Fig. 3.3. B)**. [Note that we used intravenous infection rather than oral infection in order to avoid any unknown effects of immune sera on intestinal infection that might have altered the release of NBL by adult worms.] Compared to ΔdblGATA mice that received naïve serum and eosinophils, there were fewer NBL in lungs of ΔdblGATA mice that received both immune serum and eosinophils **(Fig. 3.3. C)**. Consistent with previous findings, eosinophils improved muscle survival in ΔdblGATA mice that received

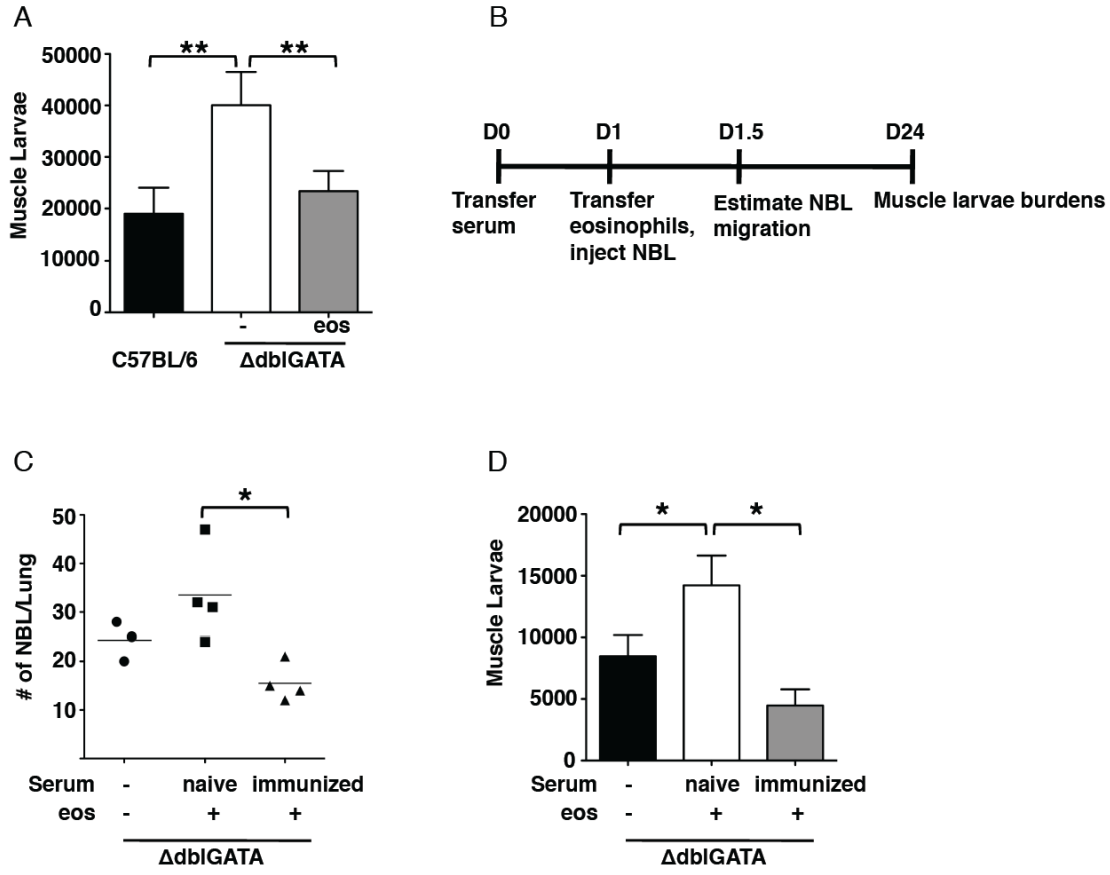

Figure 3.3. Eosinophils cooperate with immune sera. (A) Whole body muscle larval burdens in WT and ΔdblGATA mice, 28 days post-challenge. ΔdblGATA mice received 5×10^6 eosinophils or PBS every 48 h between 4 and 10 days post-challenge. **(B)** Design of experiments indicating timing of sample collections. D, day. **(C)** Numbers of NBL in lungs recovered from ΔdblGATA mice, 12 h post infection. **(D)** Whole body muscle larval burdens in ΔdblGATA mice, 24 dpi. Each data set was collected from two experiments with similar results. Values represent mean ± SD; n = 4 mice. Significant differences were determined by ANOVA and Tukey's test. *$p < 0.05$, **$p < 0.001$.

naïve serum; however, transfer of eosinophils to ΔdblGATA mice that received immune serum did not improve burdens **(Fig. 3.3. D)**. Taken together, the results support a mechanism in which specific antibodies enable eosinophils to defend against muscle infection by limiting the migration of NBL. In addition, eosinophils and immune sera were sufficient to limit larval colonization of skeletal muscle in otherwise naïve mice, indicating that other immune effectors are not required.

Effect of eosinophils on plasma cells and generation of specific antibodies

Eosinophils have been demonstrated to be required for long-term maintenance of plasma cells in the bone marrow (40, 41). We tested whether eosinophils influence the number of plasma cells or the quantity and qualities of antibodies produced during *T. spiralis* infection. Consistent with published findings, there were fewer $CD138^+$ plasma cells in the bone marrow in naïve, eosinophil-ablated mice **(Fig. 3.4. A)**; however, the difference was not evident after 90 days of infection **(Fig. 3.4. A)**. The number of plasma cells in spleen was not influenced by eosinophil ablation **(Fig. 3.4. B)**. We next compared the IgG1 and IgG2c responses mounted against parasite antigens by WT and ΔdblGATA mice. Serum IgG1 was similar between the two strains **(Fig. 3.4. C)**; however, serum IgG2c was significantly reduced in eosinophil-ablated mice that were infected and challenged **(Fig. 3.4. D)**. To test the significance of the difference in isotype composition, we passively immunized naïve ΔdblGATA mice with sera from naïve WT, immune WT, or immune ΔdblGATA mice, then restored eosinophils and infected mice with NBL **(Fig. 3.3. B)**. Immune sera from WT or ΔdblGATA mice were equally effective in limiting NBL migration to the lung **(Fig.**

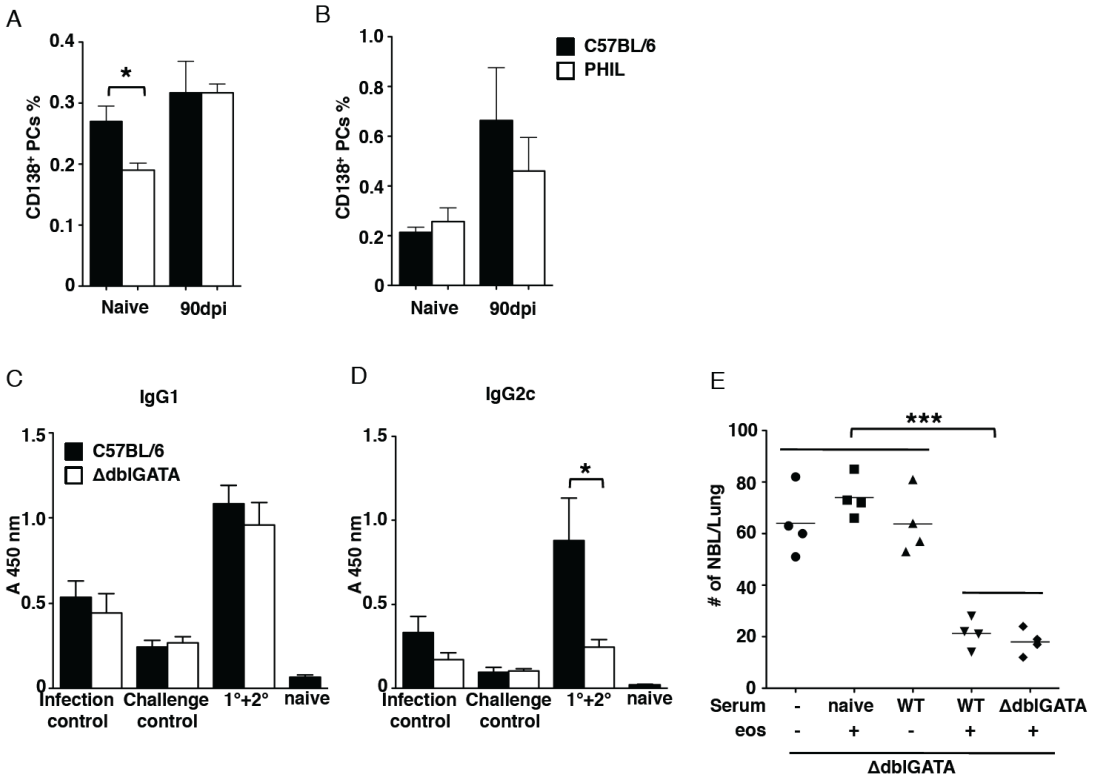

Figure 3.4. Isotype response is influenced by eosinophils. Percentage of $CD138^+$ plasma cells in **(A)** bone marrow and **(B)** spleen of naive or 90 dpi WT and PHIL mice. Level of *T.spiralis* crude antigen specific **(C)** IgG1 and **(D)** IgG2c in serum collected from WT and ΔdblGATA mice. 1° + 2°, mice were infected on day 0 (1°), reinfected on day 90 (2°), and serum collected 28 days after 2° infection. Infection ctrl, serum collected 118 dpi. Challenge ctrl, serum collected 28 dpi. **(E)** Numbers of NBL in lungs recovered from ΔdblGATA mice, 12 h post infection. ΔdblGATA mice were passively immunized with sera from naïve or immune WT or ΔdblGATA mice, intravenously infected with 25000 NBL following eosinophils transfer. Each data set was collected from two experiments with similar results. Values represent mean ± SD; n = 3-4 mice. Significant differences were determined by Student *t* test or ANOVA and Tukey's test. *$p < 0.05$, ***$p < 0.0001$.

3.4. E). Thus, the difference in antibody isotype did not influence the protection afforded by immune sera and immunes sera and eosinophils were sufficient to confer protection.

Eosinophils are recruited to sites of infection in secondary infection

Our previous studies have demonstrated that eosinophils promote muscle larvae survival in primary infection by limiting local production of toxic NO (29, 32). When eosinophils are absent, NO is produced and growing larvae are killed. We tested the hypothesis that during secondary infection, eosinophils are prevented from reaching skeletal muscle, speculating that they may become encumbered in tissues by immune complexes formed by antibodies and NBL antigens, and that this would prevent eosinophil traffic to muscle. By recovering cells from skeletal muscle following our protocol of passive immunization with eosinophil transfer, we found that recruitment of eosinophils to muscle after NBL injection was normal in ΔdblGATA mice **(Fig. 3.5. A)**. In a different experiment, eosinophils infiltrated muscle normally in WT mice following oral challenge **(Fig. 3.5. B)**. Both experiments documented that eosinophils access skeletal muscle efficiently in secondary infection and support the conclusion that the effector mechanism that limits colonization of muscle involves antibody-dependent, eosinophil-mediated interference with NBL migration.

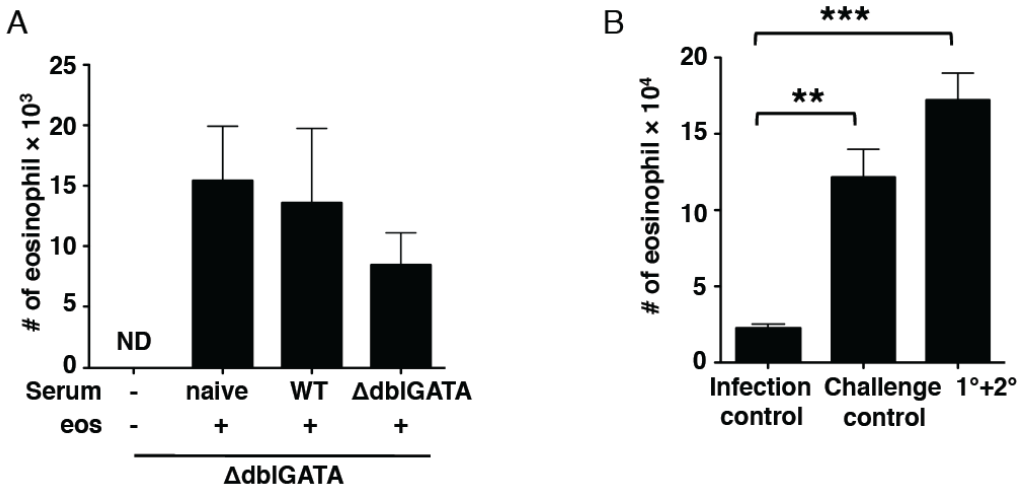

Figure 3.5. Eosinophils are recruited to sites of infection in secondary infection. **(A)** Numbers of eosinophils in diaphragms recovered from ΔdblGATA mice, 12 h post infection with NBL. Experimental design is diagramed in Fig. 3.3. B. **(B)** Numbers of eosinophils in diaphragms recovered from WT mice. Experimental design is diagramed in Fig. 3.2. A. Each data set was collected from two experiments with similar results. Values represent mean ± SD; n = 4 mice. Significant differences were determined by Student *t* test or ANOVA and Tukey's test. **p < 0.001, ***p < 0.0001.

Discussion

Parasitic worms deploy a variety of strategies to evade or co-opt the immune response. *Trichinella spiralis* is a natural parasite of rodents, providing an accessible and readily manipulated life-cycle for the study of these mechanisms. When combined with passive transfer and adoptive transfer methods, eosinophil-ablated mice have proven to be powerful tools for investigating the role of eosinophils at the host/parasite interface. We observed that when re-infected with *T.* spiralis, ablated mice failed to manifest immunologic memory that normally limits colonization of skeletal muscle by larvae. This observation supported a key role for eosinophils in protection against super-infection.

Three life stages of *T. spiralis* encounter eosinophils during the course of infection: adult worms in the intestine, migrating NBL that enter a variety of tissues, and muscle larvae that mature intracellularly in skeletal muscle. Despite the abundance and the prominence of eosinophils in the intestinal immune response, they are not required for adult worm clearance in primary infection (29). Similarly, eosinophils fail to influence both worm expulsion and Th2 immunity in primary infection by the closely related nematode, *Trichuris muris* (28). In secondary infection by *T. spiralis*, intestinal worm expulsion was accelerated; however, the absence of eosinophils did not influence the clearance of worms, the fecundity of female adult worms, or ATP content of male and female adults. Although the mechanism of secondary intestinal immunity against *T. spiralis* adult worms has not been thoroughly investigated, memory T cells are important for expulsion of intestinal worms (42, 43) and IL-4R signaling is crucial to worm expulsion in primary infection (7). Cytokine

production in MLN of ablated and WT mice supported the conclusion that antigen-induced IL-4 production was not altered by eosinophil ablation. Our results indicate that eosinophils are not required for the expression of intestinal immunity during secondary infection by *T. spiralis.*

The finding that eosinophil-ablated mice failed to prevent the accumulation of muscle larvae during secondary infection suggested that immunologic memory may be compromised. Th2 memory cells express IL-25 receptor and eosinophils are capable of influencing Th2 memory cell function by secreting IL-25 (44). During secondary infection by *T. spiralis*, IL-4 and IL-10 were significantly reduced in CLN of ΔdblGATA mice, consistent with memory Th2 responses being impacted in muscle. Despite this deficiency, transfer of eosinophils to previously infected ΔdblGATA mice conferred protection against the secondary infection. Furthermore, transfer of eosinophils and immune serum to naïve mice was protective. Moreover, serum from ΔdblGATA mice was as effective as serum from WT mice in conferring immunity. Therefore, although T cell responses and development of memory T cells was influenced by eosinophils, activation of memory T cells was not required for eosinophils to limit migration of NBL. Similary, although antibody production was influenced by eosinophils, the influence did not compromise the protection afforded by immune serum.

In accordance with early *in vitro* studies (23, 45-47), the influence of eosinophils on NBL *in vivo* was dependent on the presence of immune serum, most probably specific antibodies. Mouse eosinophils express Fcα and Fcγ receptors (48, 49), but do not express Fcε receptors (50). Complement has been shown to be

important for immunity to parasitic infections (51-53), and *T. spiralis* NBL are potent activators of complement (54). Furthermore, mouse eosinophils express functional complement receptors (55). The effect of immune serum may be mediated via complement receptors, Fc receptors, or some combination and requires further investigation.

Eosinophil granule proteins have been implicated as key to effector function. The two most abundant granule proteins in mouse eosinophils, major basic protein (MBP) and eosinophil peroxidase (EPX) (56), are required for protective immunity against *Strongyloides stercoralis* and *Litomosoides sigmodontis* in mice (31, 57). Both MBP and EPX are capable of killing *T. spiralis* NBL *in vitro* (47, 58); however, deletion of the genes encoding either MBP or EPX does not affect the capacity of mice to control primary *T. spiralis* infection (32). In secondary infection, results of pilot studies indicate that infected $MBP^{-/-}$ and $EPX^{-/-}$ mice were similar to WT mice in resisting secondary infection (Huang and Appleton, not shown). It is possible that MBP and EPX are functionally redundant, so that deleting both genes would be required to document their action against NBL *in vivo*; however, testing this hypothesis directly is not possible, because MBP and EPX double knockout mice lack eosinophils (59). Other granule proteins, such as eosinophil cationic protein (ECP), appear to be potent toxins and may be important (46). Alternatively, eosinophils may act through a granule protein-independent mechanism, for example, by adhering to and entrapping NBL as they move through tissue. This may be a two-step process, in which antibodies impede the movement of highly motile NBL such that the much less motile eosinophil can approach and adhere. Recent reports suggest that antibodies are

capable of cooperating with basophils or alternatively activated macrophages to impede the mobilization of larvae (60, 61).

Our results show that the longstanding paradigm of defensive function for eosinophils against helminth infection is valid and highly significant in *T. spiralis* infection. Previously, we have reported that eosinophils support rather than limit colonization of skeletal muscle during primary infection by *T. spiralis* (29, 32). In this context, the mechanism by which eosinophils support larval growth in muscle is distinct from the mechanism of immune regulation that limits local NO synthesis (36, 62). We have speculated that these two, distinct eosinophil-mediated effects cooperate to promote survival of larvae in muscle. The host-protective function of eosinophils in the response to challenge represents a third role for eosinophils in *T. spiralis* infection. Understanding all of the contributions of eosinophils to immunity mounted in response to parasitic worms will be crucial to the development of effective vaccines and therapeutic interventions.

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CHAPTER 4

Eosinophil-derived IL-4 promotes nematode growth in an innate context[*]

[*] Lu Huang, Daniel Beiting, Nebiat Gebreselassie, Lucille Gagliardo, Nancy Lee, James J. Lee, Judith A. Appleton. Eosinophil-derived IL-4 promotes nematode growth in an innate context. *Manuscript in preparation*.

Abstract

It has become increasingly clear in recent years that the function for eosinophils in health and disease is highly varied. Recent studies have revealed novel aspects of eosinophil function in metabolism and tissue regeneration. Such mechanisms provide new clues in the investigation of the roles of eosinophils in parasitic worm infections. The aim of this study was to investigate the mechanism underlying the observation that eosinophils promote growth of *Trichinella spiralis* larvae in skeletal muscle. Our results indicate that normal larval growth requires IL-4 and STAT6 signaling that are intrinsic to eosinophils. The effect occurs independently of adaptive immunity. Gene expression array data were compatible with a muscle regenerative response to newborn larvae and a shift in the source of energy in infected cells. The results suggest that eosinophil-derived IL-4 promotes larval growth by enhancing glucose uptake in nurse cells. Our findings document a novel interaction between worms and host, in which worms have evolved a strategy to co-opt a host cell function to facilitate their own growth.

Introduction

Genomic analysis of diverse members of the Nematoda has revealed that free-living nematodes evolved to become parasitic not once, but several times (1). The process of host adaptation is associated with loss of functions that enable a free-living lifestyle. Understanding these dependencies is invaluable to devising therapeutic and prophylactic approaches to controlling parasitic disease. Animal hosts often mount potent Th2 immune responses that can be highly effective in limiting worm infections, although effecter mechanisms vary by host, tissue and parasite. As counterpoint, helminths deploy mechanisms to evade or co-opt the host immune response in ways that ensure completion of life cycle and transmission to the next host. In one example, schistosomes are capable of manipulating innate immune signals and Th2 immunity to facilitate their development (2, 3).

Eosinophilia is a hallmark of the response to parasitic worms. Long considered to be cytotoxic effector cells that kill parasitic larvae, recent studies in eosinophil-ablated mice have shown that the contributions of eosinophils are dispensable or beneficial to certain parasitic worms (4-7). For instance, the filarial worm *Litomosoides sigmodontis* develops faster and alters reproduction in response to IL-5 induced eosinophilia (8). Moreover, recent studies have revealed novel contexts in which eosinophils regulate metabolism and tissue regeneration (9-12). Such mechanisms have potential to be highly significant in the intimate, dependent relationship between parasite and host.

After being released from female adult worms in the intestine, *Trichinella spiralis* newborn larvae (NBL) enter the circulatory system, migrate in a variety of

tissues and eventually establish intracellular infection in skeletal muscle. Following invasion of skeletal muscle cells, NBL remain inactive for 4 days prior to initiating growth from 130 μm to 1 mm in length over the course of 20 days (13). Our previous studies demonstrated that eosinophils are recruited to muscle upon NBL arrival and positively regulate both survival and growth of larvae (4, 6, 14). Support of larval survival is dependent on IL-10 production that is intrinsic to eosinophils, which in turn drives production of $CD4^{+}IL\text{-}10^{+}$ cells that suppress local nitric oxide (NO) production. NO is toxic for NBL and growing larvae (4, 14).

Support of growth occurs independently of IL-10 by a distinct mechanism (4). In the current study, we aimed to reveal the mechanism underlying eosinophil-dependent growth of muscle larvae. Our results reveal a requirement for IL-4/STAT6 signaling in eosinophils, and eosinophils supported larval growth independently of adaptive immunity. Microarray analysis indicated that infection by NBL induces dynamic changes on genes transcription, consistent with a muscle regenerative response and a shift in the source of energy in infected tissue. Redistribution of glucose transporter 4 (GLUT4) and phosphorylation of Akt were observed in nurse cells, compatible with enhancement of glucose uptake. The results implicate eosinophil-derived IL-4 in promoting larval growth by enhancing glucose uptake in nurse cells. These findings provide evidence for a parasitic adaptation in which the recruitment of eosinophils provides a nutrient-rich environment that facilitates larval growth.

Materials and Methods

Rats and mice

Adult Albino Oxford strain rats were produced and maintained in the Baker Institute vivarium. PHIL, ΔdblGATA, Rag1$^{-/-}$, PHIL/Rag1$^{-/-}$ and IL-5-expressing transgenic (NJ.1638) (IL-5Tg^{+}), IL-5Tg^{+}/IL-4$^{-/-}$ and IL-5Tg^{+}/STAT6$^{-/-}$ mice were bred at Cornell Transgenic Mouse Core Facility (TMCF) and offspring were transferred to the Baker Institute. IL-4$^{-/-}$ and STAT6$^{-/-}$ were purchase from The Jackson Laboratory and the bred at TMCF. PHIL/Rag1$^{-/-}$, IL-5Tg^{+}/IL-4$^{-/-}$ and IL-5Tg^{+}/STAT6$^{-/-}$ mice were generated by crossing and backcrossing on the deficient strains and genotype was confirmed by PCR. Rag2$^{-/-}\gamma c^{-/-}$ and B6.SJL mice were purchased from Taconic. IL-13$^{-/-}$ mice were a gift from Dr. Avery August (Cornell) and Dr. Thomas Wynn (NIAID). PHIL mice were genotyped as described previously (15). All strains were on a C57BL/6 background. C57BL/6 ^{NHsd} mice were purchased from Taconic as wild type (WT) control. Animal care was in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care and experiments were performed with the approval of the Institutional Animal Care and Use Committee of Cornell University.

Parasite and Antigens

Trichinella spiralis first-stage larvae (L_1) and newborn larvae (NBL) were recovered from rats as described previously (6). For oral infection, L_1 were suspended in 2% nutrient broth (Difco), 0.6% gelatin (Fisher Scientific) and doses of 300 L_1 were administered by gavage. For synchronous infection, 25,000 NBL were suspended in

0.25 ml serum-free DMEM (Mediatech, Inc.) and delivered by retro-orbital injection. Mice were euthanized by CO_2 inhalation at the times indicated in each experiment. Whole body muscle larvae burdens were assessed in carcasses 28 days post- oral infection (dpi) or 24 days post-NBL injection as described previously (6). Crude somatic antigens from L_1 were prepared as previously described (6).

Parasite measurement

Body area was measured as described previously (4). Briefly, developing L_1 larvae were recovered (17 dpi) by digesting minced diaphragms for 15 min at 37°C in PBS containing 2% FCS and 5 mg/ml collagenase I (Sigma). Larvae were treated with 70% ethanol (warm up at 56°C) overnight. Larvae were then centrifuged and resuspended in 5% glycerol/70% ethanol for one day and then transferred to slides using a Cytospin. Slides were fixed with methanol and stained with HEMA-3 (Fisher Healthcare), and measurements were performed using a BX51 microscope. The area of each larva was calculated using Microsuite Basic Olympus software. At least 25 larvae were measured per mouse, and values are expressed in μm^2.

Bone marrow chimeras and flow cytometry

6- to 8-week old B6.SJL (CD45.1) or $STAT6^{-/-}$ (CD45.2) were provided with acidified water (pH 2-3) containing 1 mg/ml gentamicin sulfate solution 1 week prior to lethal gamma irradiation (950 cGy), followed by retro-orbital injection with 10^7 bone marrow cells isolated from femurs of $STAT6^{-/-}$ or B6.SJL mice, respectively. Reconstitution was confirmed by detection of leukocytes bearing congenic markers by

flow cytometry eight weeks after transplantation. Spleen cells from recipients were incubated with Fc block in 2% FBS containing PBS, followed by staining with FITC-conjugated anti-mouse CD45.1 (Biolegend) and Alexa Fluor 700-conjugated anti-mouse CD45.2 (eBioscience). Data were acquired using a Gallios flow cytometer (Beckman Coulter) and analyzed with FlowJo software (Tree Star).

Eosinophil transfer experiments

Eosinophils were recovered from infected IL-5Tg^{+}, IL-5Tg^{+}/IL-4$^{-/-}$ or IL-5Tg^{+}/STAT6$^{-/-}$ mice 12-20 dpi. Cells were pooled from spleens and peritoneal lavage fluid and purified on magnetic beads as previously described (16). Briefly, eosinophils were labeled with PE-conjugated anti-Siglec-F antibody (BD) and anti-PE microbeads (Miltenyi Biotec). Average purity of eosinophils from this procedure was >93%. After washing twice with PBS, 5×10^6 eosinophils were resuspended in 200 µl sterile PBS and injected i.v. into ΔdblGATA mice every 48 h for 6 days, beginning 5 days post oral infection.

Microarray-based expression profiling and analysis of Gene Ontology enrichment

Diaphragms from Rag1$^{-/-}$ mice were harvested at 0, 2 and 7 days post-injection of NBL and preserved in RNAlater (Qiagen). Individual, whole diaphragms were homogenized using a rotor-stator and RNA was isolated using the RNeasy Plus kit (Qiagen). Biotin-labeled complementary RNA (cRNA) was generated using the Illumina TotalPrep RNA amplification kit (Ambion). Illumina MouseWG-6 version 2

expression beadchips were hybridized with cRNA from three diaphragms collected on 2 and 7 days post-injection. Two diaphragms were collected from $Rag1^{-/-}$ uninfected mice. Scanned images were converted to raw expression using GenomeStudio v1.8 software (Illumina). Data analysis was carried out using the statistical computing environment, R (v3.0.2). Raw data was background subtracted, variance stabilized and normalized by robust spline normalization using the Lumi package (17). Differentially expressed genes (≥2-fold, $P<0.05$) were identified by linear modeling and Bayesian statistics using the Limma package (18). Clusters of co-regulated genes were identified by pearson correlation using the hclust function of the stats package in R. Gene Ontology enrichment analysis was conducted in the Database for Visualization and Integrative Discovery (DAVID) (19).

Immunohistochemistry

Immunohistochemistry was conducted as described previously (20). Rabbit polyclonal anti-GLUT4 antibody (Novus Biological), rabbit monoclonal anti-phospho-Akt (Ser473, Cell Signaling) and anti-total Akt (Cell Signaling) antibodies were used. Sections were examined and photographed on a BX51 microscope fitted with DP-12 digital camera system (Olympus, Melville, N.Y.).

Statistical analysis

All experiments were performed twice with similar results. Means ± SD were calculated from data collected from individual mice unless otherwise indicated. Significant differences were determined using Student's *t* test or one-way ANOVA

with Tukey's post hoc test for multiple means. Statistical analysis was performed with GraphPad Prism 5 software.

Results:

Larval growth is regulated by IL-4/STAT6 signaling pathway

Our previous studies showed that the reduced numbers of Th2 cells in *T. spiralis* infected muscle in eosinophil-ablated mice were associated with compromised larval growth. To further dissect the interaction between Th2 immunity and larval development, we first synchronously infected STAT6$^{-/-}$ mice with newborn larvae. Larval growth was impaired in the absence of STAT6 **(Fig. 4.1. A)**; however, larval survival was not affected **(Fig. 4.1. D)**, suggesting STAT6 signaling is essential for parasite growth but is not required for the IL-10 dependent protection of larvae from NO. IL-4 and IL-13 signal via the STAT6 pathway. Larval burdens were increased in orally infected Il-13 mice due to the prolonged period of intestinal colonization by adult worms (data not shown). Intravenously infected IL-13$^{-/-}$ mice supported larval growth normally **(Fig. 4.1. C and F)**. In contrast, growth of larvae in IL-4$^{-/-}$ mice was impaired while larval survival was normal **(Fig. 4.1. B and E)**. The results provide further evidence that larval growth and survival are controlled by distinct mechanisms, and show that IL-4 and STAT6 are essential for larval growth.

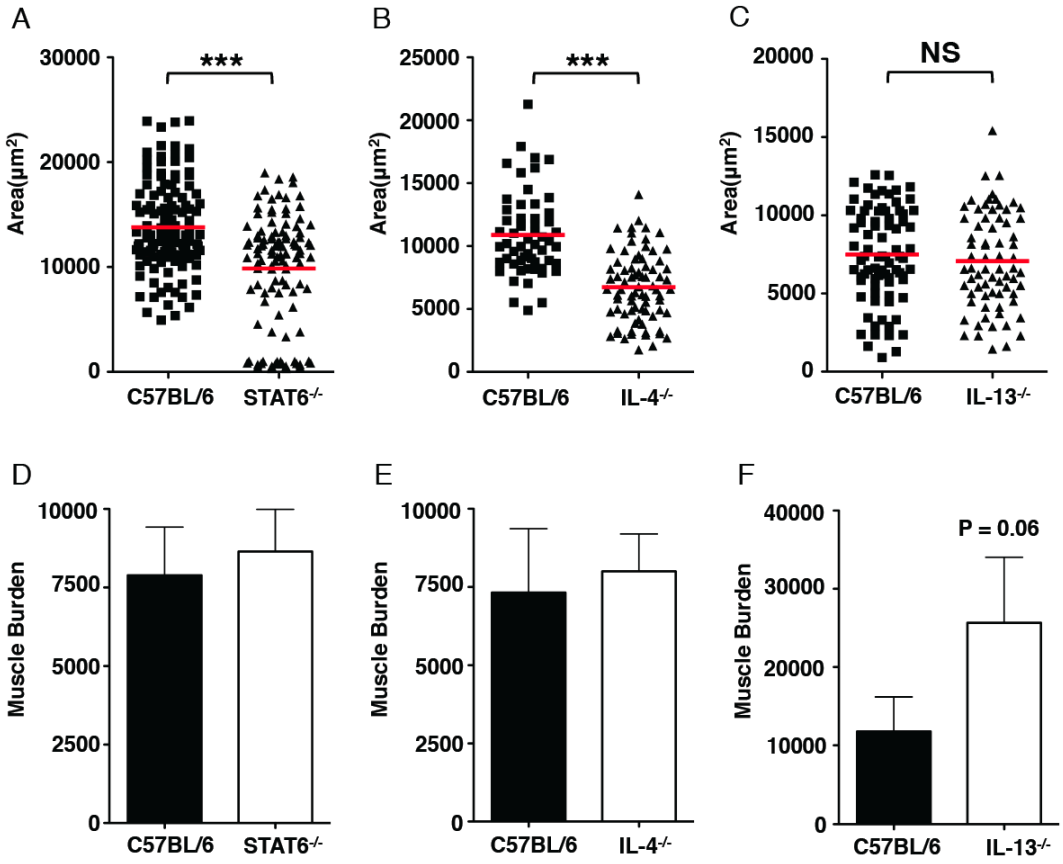


Figure 4.1. IL-4/STAT6 signaling pathway is essential for larval growth. Area of larvae recovered from **(A) – (C)**, C57BL/6, STAT6$^{-/-}$, IL-4$^{-/-}$ and IL-13$^{-/-}$ mice injected 25,000 NBL intravenously. Total body larval burdens in muscles of **(D)** WT and STAT6$^{-/-}$ and **(E)** WT and IL-4$^{-/-}$, 24 days post injection, and **(F)** WT and IL-13$^{-/-}$ mice infected orally with 300 L_1, 28 days post infection. Each data set was collected from two experiments with similar results. Values represent mean ± SD; n = 4 mice. Significant differences were determined by Student's *t* test. ***p < 0.0001.

STAT6 signaling in bone marrow-derived cells regulates larval growth

To further examine STAT6-dependent larval growth, we infected bone marrow chimeric mice prepared from B6.SJL and STAT6$^{-/-}$ mice. The results showed that only mice with STAT6 competent bone marrow were able to support larval growth **(Fig. 4.2)**. Thus, larval growth is dependent on STAT6 signaling in bone marrow-derived cells and is unlikely to involve STAT6 signaling in infected skeletal muscle cells.

IL-4/STAT6 signaling in eosinophils promotes larval growth

Transfer of eosinophils isolated from either infected IL-5Tg^{+} or IL-5Tg^{+} /STAT6$^{-/-}$ mice to infected ΔdblGATA mice (5-9 dpi) improved larval burdens **(Fig. 4.3. A)**; however, only recipients of STAT6$^{+/+}$ eosinophils supported larval growth **(Fig. 4.3. B)**. Similarly, transfer of IL-4$^{-/-}$ eosinophils isolated from infected IL-5Tg^{+}/ IL-4$^{-/-}$ mice did not improve larval growth in ΔdblGATA mice **(Fig. 4.3. C)**. Thus, STAT6 signaling in eosinophils and eosinophil-derived IL-4 are required for parasite growth.

Eosinophils support larval growth independently of adaptive immunity

Larvae grew normally in Rag1$^{-/-}$ mice, while larval growth was impaired in PHIL/Rag1$^{-/-}$ mice **(Fig. 4.4. A)**, indicating that eosinophils promote larval growth in an innate context. Type 2 innate lymphoid cells (ILC2) are IL-5 producers that can promote recruitment and activation of eosinophils. We questioned whether ILC2 contributed to larval growth by recruiting and activating eosinophils. We found that

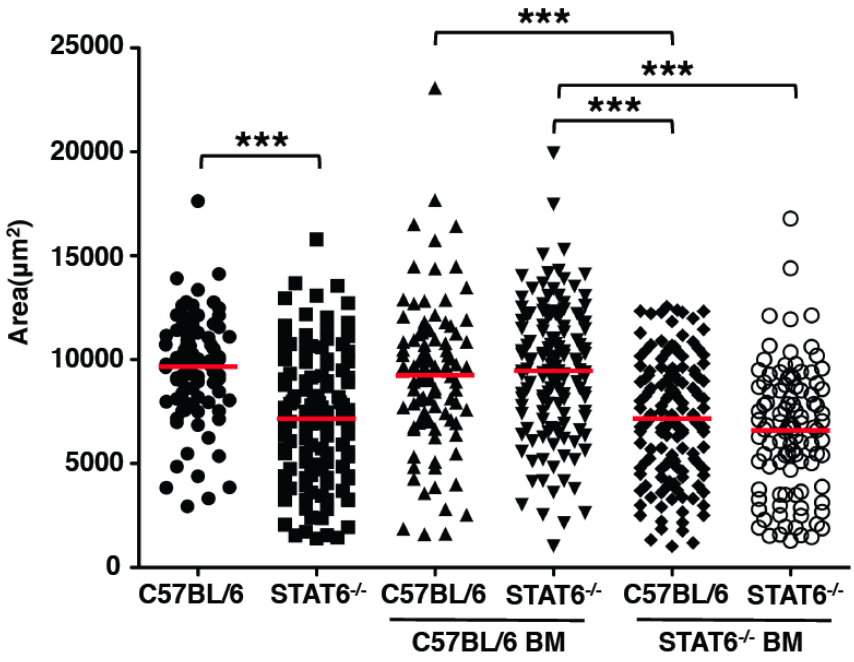


Figure 4.2. STAT6 signaling in bone marrow-derived cells is essential for parasite growth. B6.SJL and STAT6$^{-/-}$ mice were reconstituted with bone marrow cells from B6.SJL or STAT6$^{-/-}$ mice for eight weeks, then infected by intravenous injection of 25,000 NBL. Areas of larvae were estimated, 13 days post injection. Each data set was collected from two experiments with similar results. n = 4 mice. Significant differences were determined by ANOVA and Tukey's test. ***$p < 0.0001$.

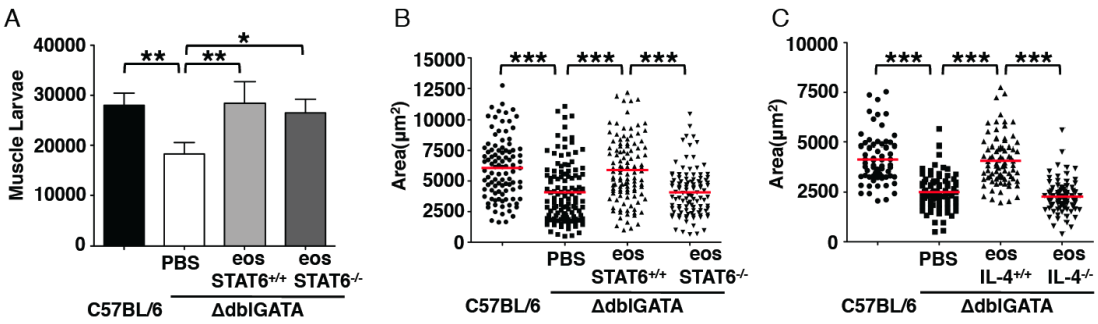


Figure 4.3. IL-4/STAT6 signaling in eosinophils is required for larval growth. (A) – (B), ΔdblGATA mice received PBS or 5×10^6 eosinophils from infected IL-5Tg$^+$ or IL-5Tg$^+$/STAT6$^{-/-}$ mice every 48 h from 5 – 9 dpi. **(A)** Total body larval burdens in muscle, 28 dpi. (B) Body size (area) of larvae, 17 dpi. **(C)** ΔdblGATA mice received PBS or 5×10^6 eosinophils from infected IL-5Tg$^+$ or IL-5Tg$^+$/IL-4$^{-/-}$ mice every 48 h from 5 – 9 dpi. Body size (area) of larvae, 17 dpi. Each data set was collected from two experiments with similar results. Values represent mean ± SD; n = 4 mice. Significant differences were determined by ANOVA and Tukey's test. *p < 0.05, **p < 0.001, ***p < 0.0001.

larval growth was normal in ILC2-ablated Rag2$^{-/-}\gamma c^{-/-}$ mice **(Fig. 4.4. B)** and furthermore, that recruitment of eosinophils to infected muscle was normal in the absence of ILC2 (14). Thus, eosinophils regulated larval growth in an innate context that was independent of ILC2.

Identification of transcriptional changes induced by NBL infection

To further investigate the mechanism of eosinophil-mediated larval growth in skeletal muscle, we performed whole genome microarray analysis to identify genes whose expression is altered in diaphragms during infection. We compared uninfected with infected Rag1$^{-/-}$ mice at 2 time points following intravenous infection with NBL. 434 genes that were differentially expressed in response to infection were identified. Hierarchical clustering revealed four distinct clusters of co-regulated genes **(Fig. 4.5. A).** Cluster 1 includes genes that are induced at 2 dpi but which are more strongly upregulated later in infection. Functional enrichment analysis, carried out using DAVID, revealed that this cluster included genes involved in antigen presentation, acute immune activation and cellular remodeling **(Fig. 4.5. B)**. Cluster 2 was comprised of genes specifically upregulated only at 7 dpi and largely involved in wound healing, cellular remodeling, and growth factor binding, consistent with substantial muscle cell hypertrophy and initiation of a nurse cell transformation by this time point **(Fig. 4.5. B)**. Cluster 2 also included collagen IV, a major component of the nurse cell capsule. In contrast, cluster 3 is an early response profile that incorporates genes robustly expressed at 2 dpi, but not at 7 dpi. This gene set was

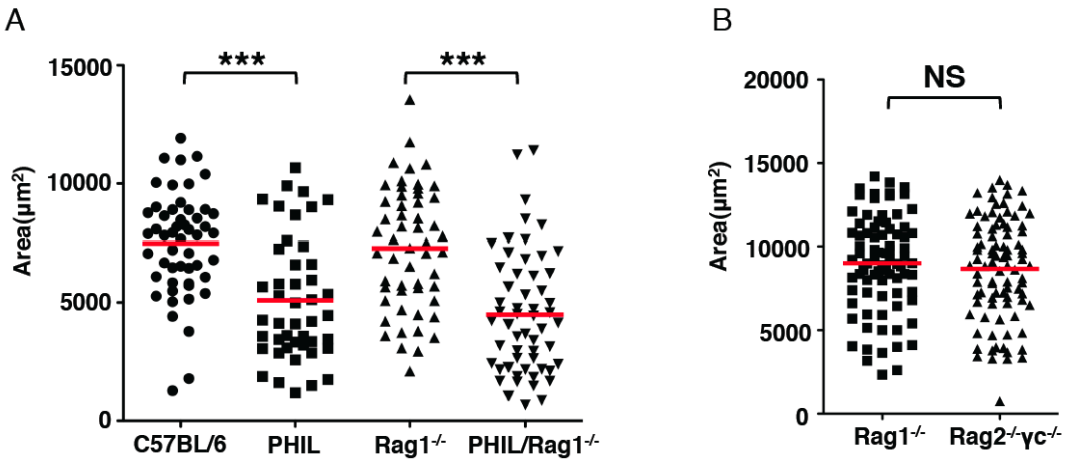


Figure 4.4. Eosinophils promote larval growth in an innate context. (A) – (B), C57BL/6, PHIL, Rag1$^{-/-}$, PHIL/Rag1$^{-/-}$ and Rag2$^{-/-}$γc$^{-/-}$ mice were injected with 25,000 NBL intravenously. **(A)** Body size (area) of larvae recovered from indicated strains 13 days post injection. **(B)** Body size (area) of larvae recovered from Rag1$^{-/-}$ and Rag2$^{-/-}$ γc$^{-/-}$ mice, 13 days post injection. Each data set was collected from two experiments with similar results. Values represent mean ± SD; n = 3 - 4 mice. Significant differences were determined by Student's *t* test or ANOVA and Tukey's test. ***p < 0.0001.

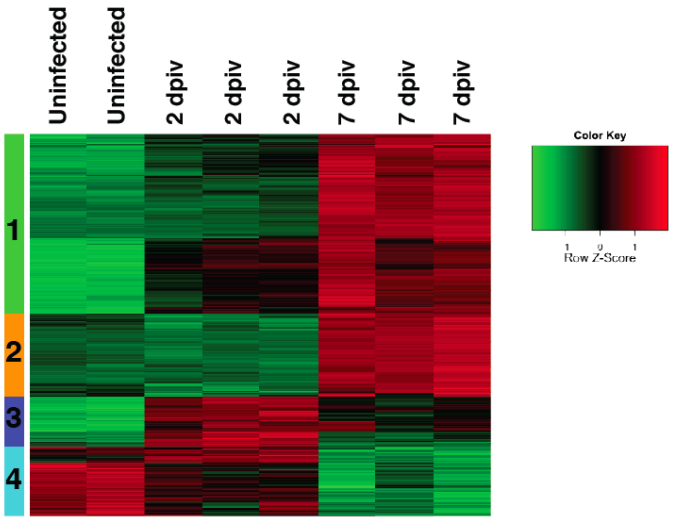
highly enriched for STAT1-inducible targets, including the immune activated GTPases, Gbp2, 3 and 6, Igtp1, Igtp2 and Irgm **(Fig. 4.5. B)**. Finally, Cluster 4 is unique in that it represents genes that are repressed during infection, and is enriched for genes involved in striated muscle cell development (e.g. myosin; myh4 and myocyte enhancer factor; mef2c) as well as fatty acid metabolism **(Fig. 4.5. B)**. Thus, transcriptional changes indicated that infection of NBL induces the loss of myofibers that is observed in infected cells, a strong regenerative response with transient induction of STAT1-dependent genes, and a shift in the source of energy in infected tissue.

Evidence of enhanced glucose uptake in nurse cells

The downregulation of genes involved in fatty acid metabolism suggested a shift to glucose metabolism. Total Akt was detected in a variety of cell types in sections of tongues, while phospho-Akt^{ser473} was specifically detected at high levels in nurse cells **(Fig. 4.6. A)**, consistent with active glucose metabolism in nurse cells. Furthermore, intracellular GLUT4 was dramatically increased and reorganized to be associated with vesicle-like structures in nurse cells, also supporting active transport of glucose **(Fig. 4.6. B)**. These results indicated that the nurse cell is an active metabolic complex that serves as a nutrient-rich site for the development of muscle larvae.

Figure 4.5. Regulation of gene expression in diaphragms of infected Rag1$^{-/-}$ mice. Microarray-based gene expression profiling of diaphragms of uninfected and infected Rag1$^{-/-}$ mice on 0, 2 and 7 days post injection. **(A)** Heatmap shows hierarchical clustering analysis 434 genes differentially regulated upon NBL infection (2-fold differentially expressed, $P \leq 0.05$). Four distinct clusters of co-regulated genes are indicated (colored bars left of heatmap). Color pattern represents row Z-score. **(B)** Functional enrichment results on 7 days post injection time point were carried out using DAVID.

A


Uninfected
Uninfected
2 dpiv
2 dpiv
2 dpiv
7 dpiv
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7 dpiv
1
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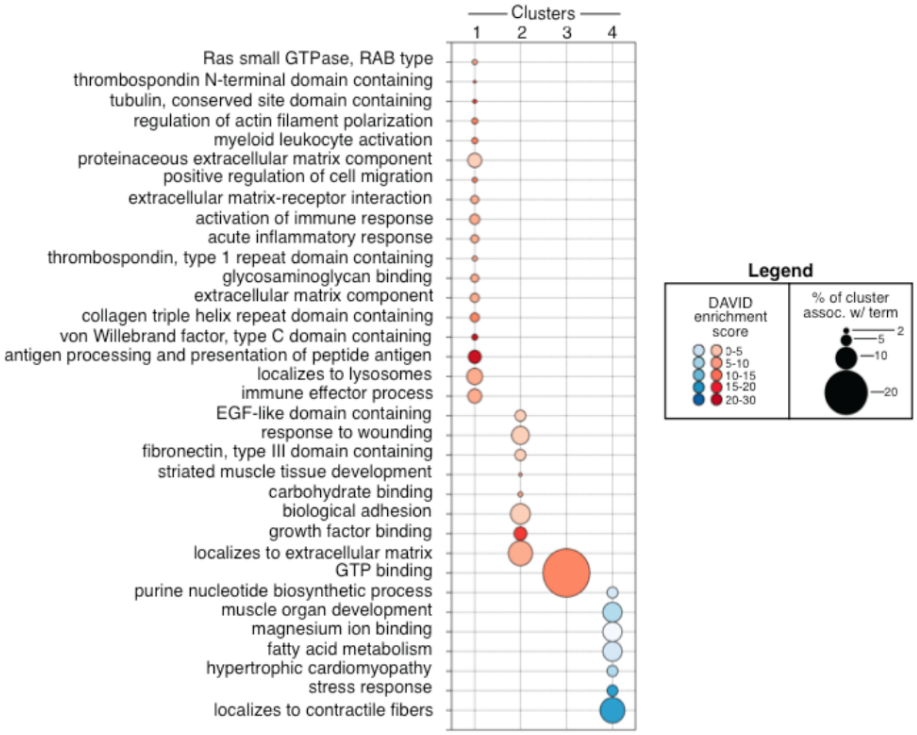
Color Key
Row Z-Score

B


Clusters
1
2
3
4
Ras small GTPase, RAB type
thrombospondin N-terminal domain containing
tubulin, conserved site domain containing
regulation of actin filament polarization
myeloid leukocyte activation
proteinaceous extracellular matrix component
positive regulation of cell migration
extracellular matrix-receptor interaction
activation of immune response
acute inflammatory response
thrombospondin, type 1 repeat domain containing
glycosaminoglycan binding
extracellular matrix component
collagen triple helix repeat domain containing
von Willebrand factor, type C domain containing
antigen processing and presentation of peptide antigen
localizes to lysosomes
immune effector process
EGF-like domain containing
response to wounding
fibronectin, type III domain containing
striated muscle tissue development
carbohydrate binding
biological adhesion
growth factor binding
localizes to extracellular matrix
GTP binding
purine nucleotide biosynthetic process
muscle organ development
magnesium ion binding
fatty acid metabolism
hypertrophic cardiomyopathy
stress response
localizes to contractile fibers
Legend
DAVID enrichment score
0-5
5-10
10-15
15-20
20-30
% of cluster assoc. w/ term
2
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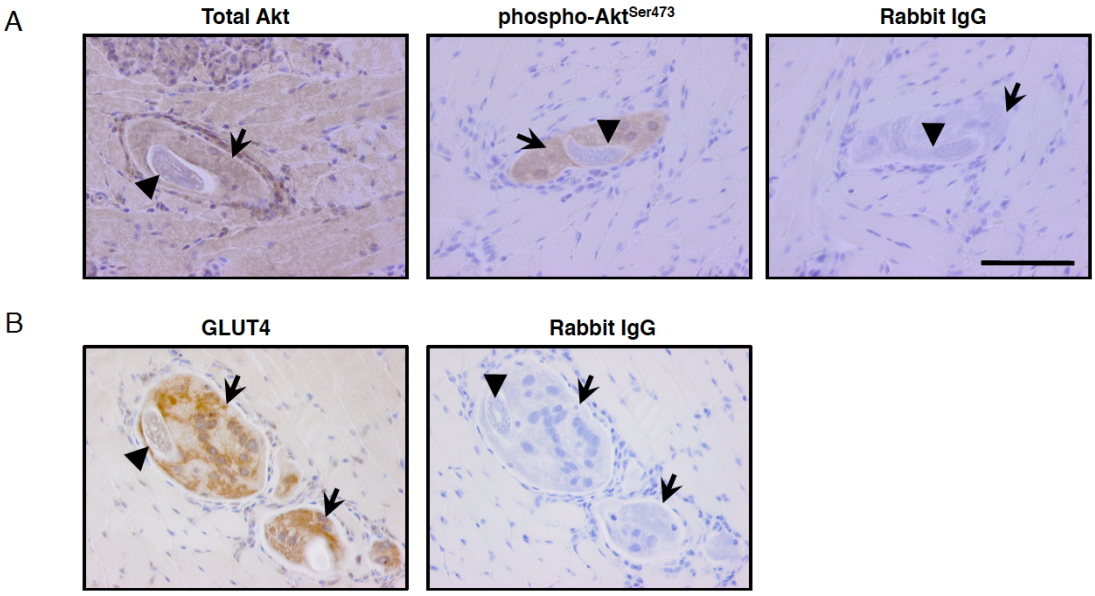

Figure 4.6. Nurse cells upregulate GLUT4 and phosphorylation of Akt. Detection of **(A)** Akt, phospho-Akt^{Ser473} and **(B)** GLUT4 in tongues collected from Rag1$^{-/-}$ mice infected by intravenous injection with NBL, 13 dpi. Arrows indicate nurse cells and arrowheads indicate muscle larvae. Scale bar = 100µm. n = 3 mice.

Discussion:

Parasitic worms are highly adapted to their hosts in ways that allow them to evade and even utilize host immune responses for their own purposes. Previous studies showed that the development of schistosomes require host $CD4^+$ T cells (2, 3). Other reports show that filarial parasites regulate their development and reproduction in response to the induction of eosinophilia (8) and that survival and growth of *T. spiralis* requires the presence of eosinophils (4, 6, 14). We have reported previously that eosinophils promote muscle larvae survival by initiating $CD4^+IL\text{-}10^+$ responses that inhibit local nitric oxide production (4, 6, 14, 22). Results from previous studies indicated that eosinophil support of larval growth appeared to be regulated by a distinct, yet undefined mechanism (14).

Our results demonstrate that eosinophil-derived IL-4 is essential for the normal development of muscle larvae. Recent studies have linked eosinophils to skeletal muscle regeneration, showing that eosinophils are the dominant IL-4 producing cells in response to muscle damage, and that IL-4 further activates the regenerative responses (10). Gene expression consistent with muscle regeneration feature in the response to NBL invasion. We speculate that eosinophils contribute to IL-4 dependent muscle regeneration in response to injury caused by migratory NBL. A previous report suggested that IL-4 secreted by regenerating myofibers stimulates $IL\text{-}4R\alpha^+$ myoblast fusion and muscle fiber growth (23). However, our chimera experiments indicated STAT6 signaling in skeletal muscle cells is not essential for larval growth. Thus, myoblast fusion mediated muscle regeneration is unlikely to be important for nurse cell formation and larval growth. STAT6 signaling in bone marrow derived cells was

required for larval growth. Relevant to this finding is the report that IL-4Rα^{+} fibro/adipocyte progenitors (FAP) respond to eosinophil-derived IL-4 and support myogenesis (10). The role of FAP and other bone marrow-derived cells in the response to eosinophil-derived IL-4 during *T. spiralis* infection merits further investigation.

After invading muscle cells, NBL establish an intracellular infection. Intracellular infection could trigger an innate defense mechanism, autophagy, which causes elimination of intracellular microbes (24). Autophagy is also involved in maintaining skeletal muscle mass and regulating glucose homeostasis in muscle cells (25, 26). Moreover, autophagic elimination of intracellular pathogens can be inhibited by Th2 cytokines, such as IL-4 and IL-13 (24). In addition to being a potent Th2 activator, helminth products can induce autophagy (27). As we observed reduced numbers of Th2 cells in eosinophil-ablated mice, we speculated that *T. spiralis* might induce the recruitment of IL-4 secreting eosinophils to sites of infection to prevent destruction of NBL by autophagy. However, gene expression array analysis did not show any significant regulation of autophagy-related genes (for example, LC3a and b). Furthermore, we examined developing nurse cells by transmission electron microscopy, and no formation of an autophagosome membrane was observed in infected muscle cells (Huang, Appleton, and Russell, data not shown). Thus, autophagy is not likely to play a role in regulating larval growth in skeletal muscle.

T. spiralis larvae store large quantities of glycogen. In the absence of exogenous glucose, under aerobic or anaerobic conditions, larvae rapidly break down endogenous stores of glycogen (28). Importantly, reduced larval glycogen correlates

with the impaired infectivity (29). Indeed, we found that mature larvae surviving in eosinophil-ablated mice were less infectious than larvae from WT mice (6), indicating a reduction of glycogen storage in muscle larvae when eosinophils are absent. In addition, it has been reported that nurse cells transport greater quantities of glucose than skeletal muscle cells (30). Other reports show that infection of *T. spiralis* induces host hypoglycemia and suggest that this is the result of an increase in glucose uptake by infected muscle cells via up-regulation of insulin signaling pathway (31). Recent reports demonstrated that IL-4 producing eosinophils are capable of promoting insulin sensitivity in adipose tissues (9, 12). The engagement of IL-4 with its receptor activates PI3K/Akt pathway and further promotes insulin stimulated glucose uptake in skeletal muscle via GLUT4 translocation (32). We therefore speculate that eosinophil-derived IL-4 promotes muscle larval growth by enhancing glucose uptake in infected muscle cells.

Inflammation has been shown to attenuate insulin sensitivity in multiple tissues (33, 34) IFN-γ attenuates insulin sensitivity via sustained activation of the STAT1 pathway (35). Our array analysis revealed the activation of the STAT1 pathway (Cluster 3). Furthermore, activation of STAT1 appears to be sustained in eosinophil-ablated mice (data not shown). The results support a model that eosinophil-derived IL-4 limits STAT1 signaling, and enhances muscle cell glucose uptake, which in turn promote larval growth. Alternatively, STAT6 signaling in bone marrow-derived cells is essential for larval growth. Thus, it is possible that bone marrow-derived cells, other than eosinophils, are also involved in suppressing local inflammation. Bone marrow-derived, IL-4Rα^{+} CD11b^{+} myeloid effector cells are capable of limiting excessive

inflammation via IL-4 induced STAT6 signaling (36). Such cells provide another target for eosinophil-derived IL-4 to prevent excessive inflammation and promote glucose uptake in skeletal muscle cells.

Our results uncover a novel function of eosinophils in helminth infections revealing an unexpected mechanism in which parasitic worms co-opt innate immunity and host metabolism to facilitate their life cycle. By targeting and manipulating helminth metabolism, our finding implicates new strategic approaches for development of novel treatments for helminth infections.

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CHAPTER 5

Summary and Future directions

Eosinophilia is a hallmark of the host immune response to helminth infection. The creation of two eosinophil deficient mouse models has transformed the study of eosinophils (1, 2). We applied two mouse models, PHIL and ΔdblGATA, towards investigating the roles of eosinophils in infection by the parasitic nematode *Trichinella spiralis*.

Previously, we showed that in the absence of eosinophils, *T. spiralis* muscle larvae die in a large numbers via a nitric oxide (NO)-mediated killing mechanism (3, 4). The death of larvae is associated with reduced $CD4^{+}IL\text{-}4^{+}$ T cells, classical activation of local macrophages (M1), and increased production of NO by M1 macrophages and neutrophils. Larval growth is also impaired in eosinophil-ablated mice, and restoring eosinophils improves both larval survival and growth (4). In this dissertation, I investigated the mechanisms underlying eosinophil-dependent larval survival and growth. I also investigated the role of eosinophils in secondary infection of *T. spiralis*. I found a distinct influence of eosinophils in each of these three contexts **(Fig. 5.1)**.

Our previous studies have shown that $CD4^{+}IL\text{-}10^{+}$ T cells are responsible for limiting production of NO in muscle (5, 6). The numbers of muscle larvae are reduced in $IL\text{-}10^{-/-}$ mice (7) and we addressed whether eosinophils induce $CD4^{+}IL\text{-}10^{+}$ response. We first confirmed the importance of NO in killing by infecting $Arg1^{flox/flox}$;Tie2cre mice, in which Arginase 1 is knocked out in myeloid cells (8). The reduced muscle larvae burdens correlated with the increased NO production, confirming a role for NO producing myeloid cells in larval clearance. Adoptive

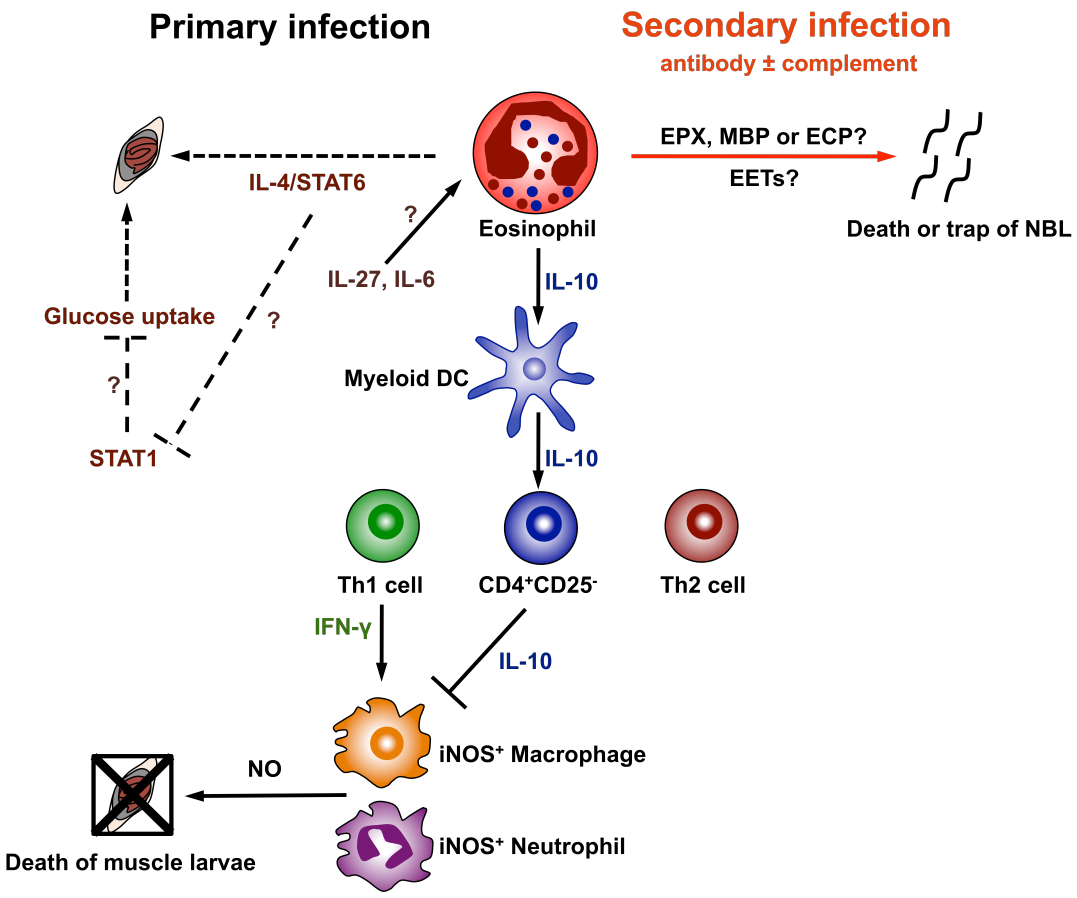

Figure 5.1. Functional versatility of eosinophils in *Trichinella* infection. In primary infection, *Trichinella* induces a mixed Th1 and Th2 immune response associated with eosinophilia in skeletal muscle. By secreting IL-10, eosinophils promote the expansion of IL-10 producing myeloid DCs, and enhance the production of IL-10 by $CD4^{+}CD25^{-}$ T cells. The production of NO by IFN-γ activated $iNOS^{+}$ macrophages and neutrophils is suppressed by IL-10, thereby promoting survival of muscle larvae. In secondary infection, eosinophils cooperate with antibodies and/or complement to interfere with migratory NBL. Eosinophil peroxidase (EPX), major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil extracellular traps (EETs) may be involved in killing or impeding of NBL. Intrinsic STAT6 signaling of eosinophils and eosinophil-derived IL-4 promote larval growth independently of adaptive immunity. Eosinophils may suppress activation of STAT1, which could attenuate insulin sensitivity and impair glucose uptake in infected muscle cells. Dashed lines indicate potential mechanisms that are unproven.

transfer of CD4+IL-10$^{-/-}$ T cells to eosinophil-ablated mice improved larvae burdens, indicating that eosinophils promote larval survival by regulating T cell responses.

Eosinophils may regulate T cell responses either by acting as antigen presenting cells or by influencing functions of dendritic cells (9-12). We observed improved larval survival and growth by adoptive transfer of MHCII$^{-/-}$ eosinophils to ΔdblGATA mice, indicating antigen presentation by eosinophils is dispensable in this process. In contrast, the numbers of CD11b^{+} myeloid dendritic cells are reduced in the absence of eosinophils, and transfer of eosinophils restored this population. Moreover, transfer of bone marrow-derived myeloid dendritic cells improved larval survival and the numbers of CD4^{+}IL-10^{+} T cells. Production of IL-10 intrinsic to myeloid dendritic cells is essential for development of CD4^{+}IL-10^{+} T cells. Future studies will determine how eosinophils influence myeloid dendritic cells, specifically, how eosinophils promote expansion of IL-10^{+} myeloid dendritic cells and shift their cytokine profile.

Eosinophils were rapidly and specifically recruited to sites of infection in response to entry by NBL. Early recruitment is required for larval survival, indicating that eosinophils engage local immune responses at the initiation of muscle infection. Levels of IL-5, KC and RANTES in serum were increased, suggesting that those mediators are responsible for recruitment of eosinophils, although we did not discover the cellular source of those mediators. Type 2 innate lymphoid cells (ILC2) are not involved in this process, as larval survival and recruitment of eosinophils to muscle are normal in ILC2-ablated mice. We speculate that endothelial cells and/or tissue

resident mast cells may be important for this rapid release of mediators (13-16). Future study will examine eosinophil recruitment in mast cell deficient mice.

Eosinophil-derived IL-4 is not required for larval survival; however, *T. spiralis* infection induces production of IL-10 in eosinophils, which is required for $CD4^{+}IL\text{-}10^{+}$ T cell response and larval survival. IL-10 can promote dendritic cell maturation (17, 18). Future study will determine whether eosinophil-derived IL-10 is required for the recruitment and IL-10 production in myeloid dendritic cells.

The mechanism that induces eosinophils to make IL-10 remains unclear. IL-6 and IL-27 have been shown to promote IL-10 responses in T cells (17, 19-23). Results of our pilot study conducted in $IL\text{-}27R^{-/-}$ mice supported only a partial role for IL-27R signaling in promoting IL-10 production in eosinophils. Further investigation of the roles of IL-27 and IL-6 in *T. spiralis* infection is merited.

We went on to investigate the role of eosinophils in secondary infection by *T. spiralis*. Previous studies by others using either anti-IL-5 antibody or $IL\text{-}5^{-/-}$ mice to deplete eosinophils yielded contradictory results (24, 25). Our results demonstrated that eosinophils are dispensable for intestinal immunity against either primary or secondary *T. spiralis* infection. In contrast, eosinophils are required for preventing the accumulation of muscle larvae in secondary infection, an impact that is opposite to that observed in primary infection. Although we observed impaired memory Th2 responses in eosinophil-ablated mice, memory responses were not to be essential for protection in muscle. Nevertheless, eosinophil-derived IL-25 has been reported to influence memory cells (26) and *T. spiralis* infection affords an opportunity to explore this mechanism.

Transfer of eosinophils to previously infected ΔdblGATA mice conferred protection against secondary infection. Moreover, transfer of eosinophils and immune serum to naïve mice was protective. These results indicated that eosinophils, and most likely antibodies, are required for protection. Further investigation is needed to determine which antibody isotype is essential, and whether complement is involved.

The mechanism of eosinophil-mediated protection remains to be determined. Although MBP and EPX are capable of killing *T. spiralis* in vitro (27-29), results of pilot studies indicated that $MBP^{-/-}$ and $EPX^{-/-}$ mice were similar to WT mice in resisting secondary infection. Other granule proteins, such as ECP may be important (30). In addition, an alternate, less direct mechanism may be at work. Recent studies indicate that antibodies are capable of cooperating with basophils or alternatively activated macrophages to impede the mobilization of larvae (31, 32). Thus, it is possible that eosinophils trap NBL in the presence of antibodies to prevent colonization of muscle. This hypothesis needs to be addressed. Furthermore, extracellular traps released by neutrophils and macrophages are capable of killing parasitic larvae (33). Eosinophils can also be activated to released extracellular traps that are comprised of DNA fibers and eosinophil granule proteins (34). Therefore, eosinophil-derived extracellular traps could be involved in secondary infection of *T. spiralis*. All of these hypotheses are testable *in vitro* or *in vivo*.

The last part of the dissertation addressed the mechanism of eosinophils in promoting muscle larvae growth, a distinct mechanism that is independent of eosinophil-derived IL-10. Results of larval growth inhibition and killing of *T. spiralis* in knockout mice have been summarized in **Table 5.1**. Our results indicate that

eosinophils promote larval growth independently of adaptive immunity. Eosinophil-derived IL-4 and intrinsic STAT6 signaling are necessary to the mechanism. Adoptive transfer of IL-$4^{-/-}$ or $STAT6^{-/-}$ eosinophils to PHIL/$Rag1^{-/-}$ mice will confirm the dependence of growth on eosinophil-derived IL-4. Microarray analysis supported a model in which eosinophil-derived IL-4 enhances insulin sensitivity and the glucose uptake of nurse cells. Thus, further study will compare the activation of insulin signaling pathway between infected muscles of $Rag1^{-/-}$ and PHIL/$Rag1^{-/-}$ mice. Sustained activation of that STAT1 pathway was observed in eosinophil-ablated mice, suggesting that excessive inflammation may attenuate the insulin sensitivity in nurse cells, which in turn impairs larval growth. Future work will confirm the microarray data, and adoptive transfer experiments will be performed to confirm that eosinophil-derived IL-4 is capable of inhibiting activation of STAT1 and improving insulin sensitivity in infected muscle cells.

The mouse model has proven to be a powerful tool for advancing our understanding of the function of eosinophils. Nevertheless, extrapolation of our findings to human disease requires care because there are differences between human and mouse eosinophils (35). A recently developed humanized mouse model may prove useful for functional study of human eosinophils (36). In summary, the data presented in this dissertation provide evidence that at different stages of infection, a particular parasitic worm may be dependent upon, susceptible to, or unaffected by effector functions of eosinophils. Studying of helminth infections in mice is invaluable to advancing our understanding of the dependency of a parasite on its host, and will lead to the new therapeutics and prophylactic approaches to disease control.

Strain	Growth inhibition	Killing
PHIL	52%	75%
ΔdblGATA	33%	50%
IL-10$^{-/-}$	NS	50%
Arg1^{Tie2Cre}	NS	44%
IL-13$^{-/-}$	NS	NS
IL-4$^{-/-}$	32%	NS
STAT6$^{-/-}$	27%	NS
Rag1$^{-/-}$	NS	NS
Rag2$^{-/-}$γc$^{-/-}$	NS	NS
PHIL/Rag1$^{-/-}$	35%	N/A

Table 5.1. Results of larval growth inhibition and killing of *T. spiralis* in knockout mice. NS = not significant; all other values $p < 0.05$ or less compared to control mice. N/A = not available.

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APPENDIX A

The TLR2 agonist Pam3CSK4 as an immune adjuvant to treat experimental cutaneous leishmaniasis in mice[*]

[*] Lu Huang, Meleana Hinchman and Susana Mendez.

Abstract

Cutaneous leishmaniasis caused by *Leishmania major* is an emergent, uncontrolled public health problem and there is no vaccine. A promising prophylactic approach has been immunotherapy with Toll-like receptor (TLR) agonists to enhance parasite-specific immune responses. We have reported that vaccination of C57BL/6 mice with live *L. major* plus the TLR9 agonist CpG DNA prevents lesion development and confers immunity to reinfection. Our current study aims to investigate whether other TLR agonists can be used in immunotherapy against cutaneous leishmaniasis. We have found that live *L. major* plus the TLR2 agonist Pam3CSK4 also reduces pathology in both genetically resistant (C57BL/6) and susceptible (BALB/c) mouse strains. The addition of Pam3CSK4 activates dermal dendritic cells and macrophages to produce greater amounts of proinflammatory cytokines in both strains of mice. Th1 and Th17 responses were enhanced by vaccination in C57BL/6 mice; however, Th17 cells were unchanged in BALB/c mice. Another immunological difference between the two strains was the sustained influx of IL-17^{+} neutrophils found in the vaccination site only in BALB/c mice. Our data demonstrate that the mechanism behind vaccination with TLR agonists may be very different depending upon the immunological background of the host. This needs to be taken into account for the rational development of successful vaccines against the disease. This is also the first report showing efficacy of TLR2 immunotherapy in susceptible strains of mice using a cutaneous leishmaniasis model.

Introduction

The prevalence of cutaneous leishmaniasis due to *Leishmania major*, a chronic disease leading to disfigurement and social stigmatization, is estimated to be at 2 million cases (1). Recent data, however, demonstrate that this number is greatly underestimated (2). Current drugs are inadequate due to toxicity, resistance, and cost. A significant amount of work focused on prophylactic vaccine approaches have been tested in mice (*Mus musculus*), a species chosen because wild rodents are natural hosts for *L. major* (3). This has included the use of attenuated parasites, parasite extracts and leishmanial antigens. Although all these vaccine have yielded promising results in rodent models (4), they have failed when tested in primates or humans (5). Inoculation of virulent *L. major* (leishmanization), practiced in endemic areas for millennia, has been the only strategy that has ever provided protection in humans, possibly because it mimics a natural infection, parasite persistence, and concomitant immunity. Leishmanization was widely applied, but because of exacerbated skin disease reported in rare cases (6), the vaccine was discontinued. However, the traditional practice of leishmanization has made a comeback in certain endemic regions, given that it is the only vaccine with proven efficacy in humans.

Efforts to improve the safety of leishmanization have included the addition of killed parasites or immune adjuvants to reduce the size and duration of lesions (6). Our particular approach to a safer leishmanization has been to use Toll-like receptor (TLR) agonists. TLRs are a family of 11 transmembrane proteins that specifically recognize different pathogens (7). The therapeutic effects of TLR activation in immunotherapy are associated with the expression of high levels of IL-12 and IFN-γ. In particular the

use of TLR agonists as immune adjuvants in leishmaniasis have yielded promising results As examples, the TLR7 agonist Aldara™ showed anti-leishmanial activity in experimental models and in clinical studies of cutaneous leishmaniasis in combination with conventional therapy (8, 9). CpG DNA, a TLR9 agonist, has been extensively tested and has shown wide prophylactic and therapeutic anti-leishmania potential (10-13). For the last past years, we have investigated a leishmanization approach consisting on the inoculation of live parasites along with CpG DNA (Lm/CpG). We showed that Lm/CpG prevents vaccinal lesions (an undesired effect of live vaccination) in C57BL/6 mice while achieving parasite persistence and immunity (14, 15). Mechanistically, we found that Lm/CpG causes activation of dermal dendritic cells (DCs) to produce IL-6 (15), and IL-2, activation of NK cells (16), and expansion of Th17 responses (17).

Mice have remained the major model for testing the efficacy of vaccines against cutaneous disease. Resistance or susceptibility to *L. major* in this species is dependent on the type of $CD4^+$ helper T cell (Th) subset that is induced. Healing in resistant mice (*i.e.* C57BL/6) is associated with the development of IFN-γ-producing Th1 cells. In contrast, susceptibility (*e.g.* in BALB/c mice) is mediated by an early IL-4 production that promotes the development and expansion of Th2 cells (18). Contrasting with these highly polarized responses in mice, human infection data show that a mixed Th1/Th2 response is more typically observed (19). Hence, we propose that prospective prophylactic strategies must be evaluated in both Th1 and Th2 models of disease.

The aim of this study is to determine whether TLR agonists other than CpG DNA could be use in immunotherapy to treat cutaneous leishmaniasis. Here, we have found that in C57BL/6 mice, *L. major* infection upregulates the expression of TLR2 in bone-marrow-derived dendritic cells. This contrasts with our data obtained using BALB/c mice, where there is no change in the expression of TLR2 in the same cell type. Interestingly however, TLR2 agonist Pam3CSK4 treatment of infected cells from both strains of mice results in an enhanced proinflammatory response. Because TLR2 agonists have been proposed as adjuvants of vaccine in other models (20-22), we investigated the use of Pam3CSK4 as an immune adjuvant using our leishmanization model. We have also found that vaccination with live *L. major* plus Pam3CSK4 completely prevents lesion development and decreases parasite burdens in susceptible (BALB/c) and resistant (C57BL/6) strains of mice. In both cases, dermal dendritic cells and macrophages express greater amounts of pro-inflammatory cytokines. Both Th1 and Th17 responses were enhanced in C57BL/6 mice, conversely, Th17 responses were not enhanced in BALB/c mice in the presence of Pam3CSK4. However, neutrophils responses were enhanced and sustained in the susceptible mice.

Materials and Methods

Mice

Six-week-old C57BL/6 and BALB/c mice were purchased from Taconic and The Jackson Laboratory, respectively. All mice were maintained in the Baker Institute for Animal Health animal care facility under pathogen-free conditions. All animal experiments were performed according to federal guidelines and institutional policies.

Parasites

L. major clone V1 (MHOM/IL/80/Friedlin) promastigotes were grown at 26 °C in medium 199 supplemented with 20% heat-inactivated fetal calf serum (FCS) (Gemini, Sacramento, CA), 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, 40 mM HEPES, 0.1 mM adenine (in 50 mM HEPES), and 5 mg/ml hemin (in 50% triethanolamine).

Infection protocol and vaccine preparation

Infective-stage promastigotes (metacyclics) of *L. major* were isolated from stationary cultures (4-5 days old) by Ficoll enrichment as described before(23). Mice were vaccinated intradermally in both ears with 10^6 *L. major* promastigotes alone or mixed with 50 μg (in DMEM) of a single TLR2 agonist, the synthetic triacylated lipopeptide Pam3CSK4 (InvivoGen, San Diego, CA), using a 27G needle in a volume of 10 ml.

Parasite titration

Parasite loads in the ears were determined as described before (24). Briefly, the ear sheets were separated and deposited in DMEM containing Liberase CI enzyme blend (0.5 mg/ml) for 60 min at 37 °C. The sheets were then dissociated using a handheld tissue homogenizer. The homogenates were filtered using a 70-mm cell strainer (BD Falcon, San Jose, CA) to produce single cell suspensions and serially diluted in a 96-well flat-bottom microtiter plates containing biphasic medium prepared using 50 mL Novy-MacNeal-Nicolle (NNN) medium containing 20% of defibrinated rabbit blood overlaid with 100 mL M199. The number of viable parasites in each ear was estimated by limiting dilution from the highest dilution at which promastigotes could be grown out after 7 days of incubation at 26 °C. Parasite numbers were also determined in the local draining lymph node (submandibular). Lymph nodes were mechanically dissociated and parasite load was determined by limiting dilution as described above.

Preparation of soluble Leishmania antigen

Thirty mL of stationary phase cultures (4-6 days old) were taken in a 50-ml tube and centrifuged at 3,500 rpm for 15 min at CT. The resulting pellets were washed three times with cold 0.02 M PBS (pH 7.2) subjected to three cycles of freezing and thawing, and centrifuged at 10,000 rpm for 20 min. Supernatant was collected, and protein estimation was done by BCA assay following the manufacturer's recommendations. Protein samples were stored at -80 °C until use.

Cell analysis from ears and draining lymph nodes

Single-cell suspensions from the ear dermis were obtained as described above. For the analysis of surface markers and intracytoplasmic staining for cytokines, single cell suspensions obtained from ears and draining lymph nodes (as described above) were stimulated overnight with 25 mg/mL soluble *Leishmania* antigen, 5 ng/mL IL-2 and 10 mg/mL anti-CD28, and then cultured with brefeldin A at 10 ng/ml for 6 h and then fixed in 4% paraformaldehyde (24). Prior to staining, cells were incubated with an anti-Fcg III/II receptor antibody and 10% normal mouse serum in PBS containing 0.1% BSA, 0.01% NaN_3. Cells were permeabilized and stained for the surface markers CD4 (clone RM4-5), CD11c (clone N418), Ly-6G (Gr-1, clone 1A8) and F4/80 (clone BM8), for the cytokines IL-6 (clone MP5-20F3), IL-12/IL-23p40 (clone C17.8), IL-4 (clone 11B11), IL-10 (clone JES5-16E3), IL-17A (clone TC11-18H10.1) and IFN-γ (clone XMG1.2). Incubations were carried out for 30 min on ice. For each sample, at least 50,000 cells were analyzed. The data were collected and analyzed using CellQuest software and a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

Bone marrow-derived dendritic cells and macrophage culture

Bone marrow-derived dendritic cells (BMDDCs) were generated as described (25). In brief, bone marrow cells from C57BL/6 mice or BALB/c mice were isolated by flushing femurs and tibiae with RPMI 1640. After treatment with ACK buffer to lyse red blood cells, bone marrow cells were cultured in complete RPMI 1640 supplemented with 20 ng/ml recombinant murine granulocyte-macrophage colony-

stimulating factor (GM-CSF). Fresh cell culture medium was added on day 3 and day 6. After 9 days, floating cells were used as immature BMDDCs.

Bone marrow-derived macrophages (BMDMs) were generated as also as described before (25). Briefly, bone marrow cells from C57BL/6 mice or BALB/c mice were isolated as described above and cultured in complete DMEM supplemented with 20% L-929-conditioned medium, which contains granulocyte colony-stimulating factor (G-CSF). Fresh cell culture medium was added on day 5. After 7 days, BMDMs were ready to use.

In vitro macrophage and dendritic cell infection and surface TLR2 expression analysis

Infective-stage promastigotes (metacyclics) of *L. major* from Ficoll enrichment were washed three times in PBS, resuspended at 20×10^6/ml in PBS, and incubated with 5 mM 5 (6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) for 15 min at 37 °C. Depending on the experiment, BMDDCs or BMDMs were infected for 18 h with unlabeled or CFSE-labeled parasites at a cell/parasite ratio of 1:5. Cells were also treated with 0.5 mg/ml of the TLR2 agonist Pam3CSK4, either at the time of infection, or at 18 h post infection.

Eighteen hours post infection, supernatants were collected for cytokines analysis and cells were harvested for surface TLR2 expression analysis. To determine surface TLR2 expression, free parasites were washed away from BMDDCs culture by washing three times with cold PBS. Cells were then harvested, incubated with an anti-

Fcg III/II receptor antibody and 10% normal mouse serum in PBS, and then stained for expression of the surface markers CD11c (clone N418) and TLR2 (clone 6C2).

Cytokine measurements

Cytokine IL-12p40/p70 in the supernatants from *in vitro* stimulation was measured by c sandwich ELISA as described (24). All antibodies were purchased from BD Bioscience.

RNA extraction and real-time PCR analysis

Total RNA from BMDDCs uninfected or infected with *L. major* was extracted using TRIzol reagent. Reverse transcription of the RNA (1 mg) was performed using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Real-time PCR was performed in the Applied Biosystems 7500 real-time PCR system. The reaction was performed using the FAST SYBR Green master mix (Applied Biosystems, Carlsbad, CA). β-actin was used as internal control for each sample. The primers used were as follows: TLR2 forward, 5'-CTCTGTCATGTGATGCTTCTG-3'; TLR2 reverse, 5'-ATGTTACCCCCAGTGTCTGG-3'; β-actin forward, 5'-GCTCCGGCATGTGCAA-3'; β-actin reverse, 5'-AGGATCTTCATGAGGTAGT-3'.

Statistical analysis

All comparisons of non-normally distributed continuous data were analyzed with Student *t*-test, Mann-Whitney test or ANOVA with Tukey's post hoc test for multiple means using GraphPad Prism 5 (San Diego, CA).

Results

L. major-infected bone marrow derived dendritic cells upregulate TLR2 expression only in C57BL/6 mice

Because it has been suggested that TLR2 interacts with *L. major* and triggers the host immune response against the parasite (26-28), we investigated changes in TLR2 expression in *L. major*-infected bone marrow-derived DCs. We carried out these experiments using both resistant (C57BL/6) and susceptible (BALB/c) mouse strains. **Fig. A1 A** shows that TLR2 mRNA expression was upregulated 9-fold in infected DCs derived from C57BL/6 mice. However, this upregulation was not observed in infected DCs from BALB/c mice. To confirm the genetic results, we then determined TLR2 protein expression in both infected and uninfected cells by flow cytometry. **Fig. A1 B** demonstrates that, in C57BL/6 mice, TLR2 was expressed in 43% of cells from uninfected cultures. The mean intensity of fluorescence (MFI) for the receptor was 145.1. TLR2 cell expression was increased to 76% in the infected cultures; the MFI also increased to 280. To establish whether those changes in TLR2 expression were a direct consequence of infection, we employed CFSE-labeled parasites to directly track the infected cells. A parasite:cell ratio of 5:1 resulted in the infection of more than 60% of the cells in culture **(Fig. A2 A).** Seventy percent of DCs from C57BL/6 mice containing fluorescent parasites expressed TLR2; in contrast, its expression was reduced to 19% in uninfected cell cultures **(Fig. A2 B).** As before, the MFI for TLR2 expression increased in the infected cells (from 158.4 to 213.7).

Interestingly, TLR2 expression in uninfected cells from BALB/c mice was significantly higher (>80%), and infection with *L. major* did not significantly increase

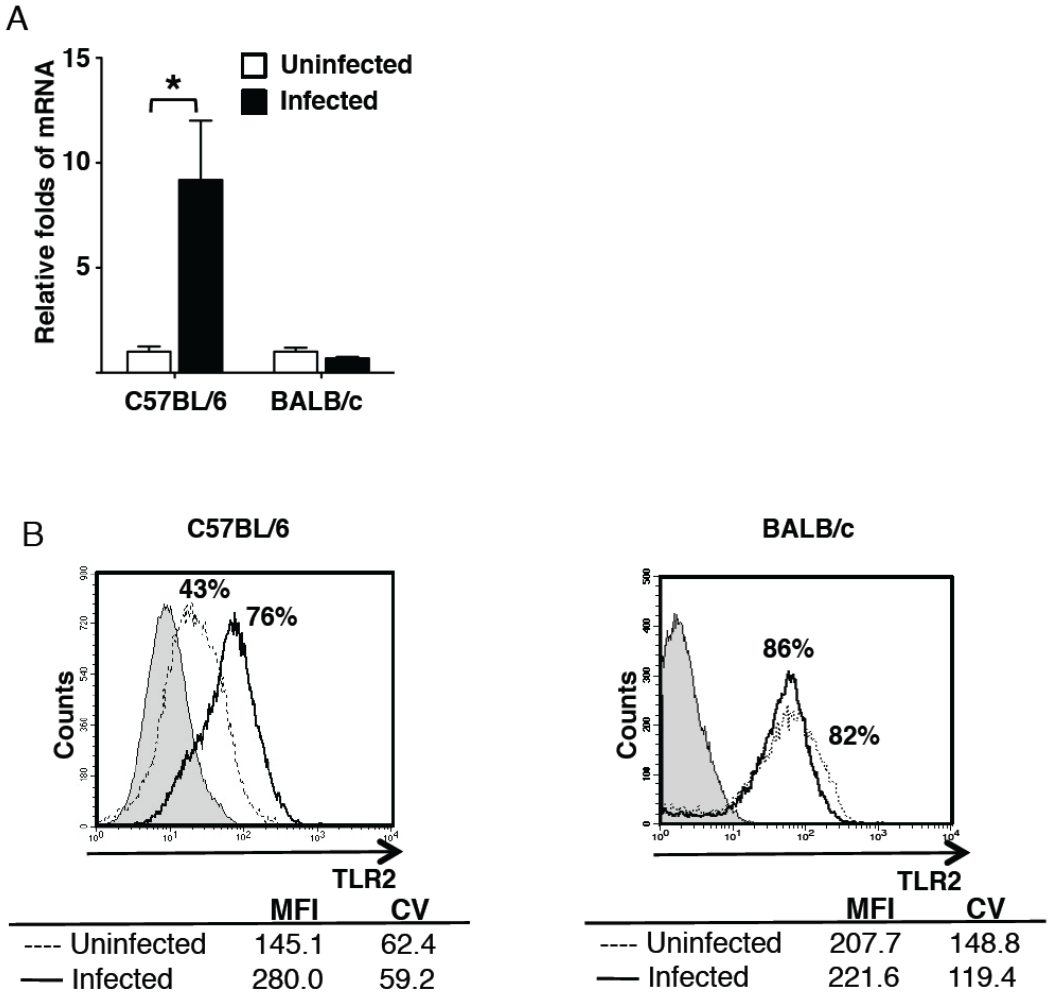

Figure A1. *L. major*-infected BMDDCs have higher expression of TLR2 only in C57BL/6 mice. (A). TLR2 mRNA transcript level measured by real-time PCR analysis post infection. Data are normalized to β-actin, and show mean ± SEM (n = 5 different experiments). Surface TLR2 expression of BMDDCs from **(B)** C57BL/6 and **(C)** BALB/c measured by flow cytometry. Grey histogram, unstained; dotted line, uninfected; solid line, *L. major*-infected cells. Mean fluorescence intensity (MFI) and Coefficient of Variation (CV) values are include in the figure. Data are representative of n = 3 experiments with similar results. Asterisks indicate statistically significant differences following Student *t*-test between the two groups; *$p<0.05$.

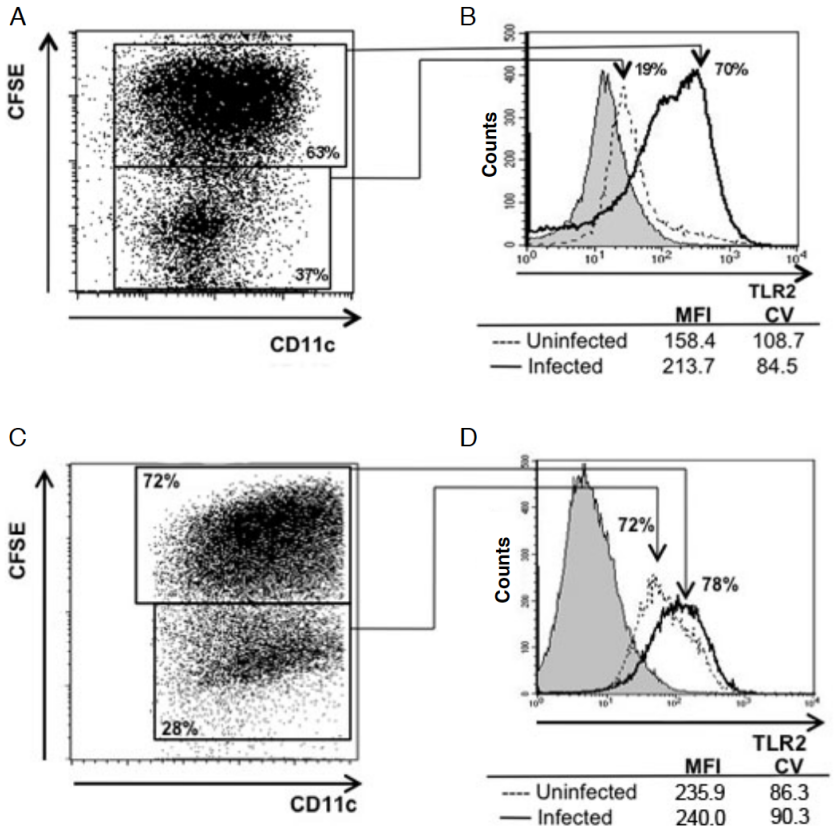

Figure A2. Infected BMDDCs upregulate TLR2 expression in the C57BL/6 mouse. Flow cytometry plots of bone marrow-derived CD11c$^+$ DCs from **(A)** C57BL/6 and **(C)** BALB/c mice infected with CFSE-labeled *L. major* at a cell/parasite ratio of 1:5. Numbers indicate relative frequencies of infected (double positive) and uninfected (single positive) cells. Surface TLR2 expression in the infected, double positive (solid line) and in the uninfected, single positive (dotted line) populations from **(B)** C57BL/6 and **(D)** BALB/c mice. An unstained sample is included as a grey histogram. MFI and CV values are also shown. Data are representative of n = 3 experiments with similar results.

the receptor expression **(Fig. A1 C).** MFI values for TLR2 did not significantly change either. As expected, infection did not significantly change the already elevated expression of the receptor **(Fig. A2 D).**

The TLR2 agonist Pam3CSK4 increases the ability of *L. major*-infected BMDDCs and BMDMs to secrete the pro-inflammatory IL-12

Next we determined whether the upregulation of TLR2 expression would result in an enhanced response to TLR2 stimulation. We infected BMDDCs from either C57BL/6 or BALB/c with *L. major* and treated them with the TLR2 agonist Pam3CSK4, either at the time of infection, or later. The production of IL-12 was measured in culture supernatants 24 h post stimulation. **Fig. A3 A** shows that, as expected, uninfected DCs produce IL-12 in response to Pam3CSK4. This cytokine response was significantly greater than what was secreted following infection ($P<0.001$). Interestingly, IL-12 production was enhanced in *L. major*-infected cells treated with the TLR2 agonist, irrespective of when it was added to the cultures (at the time of after infection). We also determined the effect of agonist treatment in infected BMDMs. In this population, addition of the TLR agonist alone did not significantly increase cytokine production **(Fig. A3 B)** when compared to infected cells, conversely, Pam3CSK4 treatment enhanced IL-12 production in macrophages when added at the time of infection ($P<0.02$), or after infection ($P<0.001$). These results indicate that infected cells that display upregulation of TLR2 become more responsive to the effect of agonist treatment, enhancing proinflammatory responses.

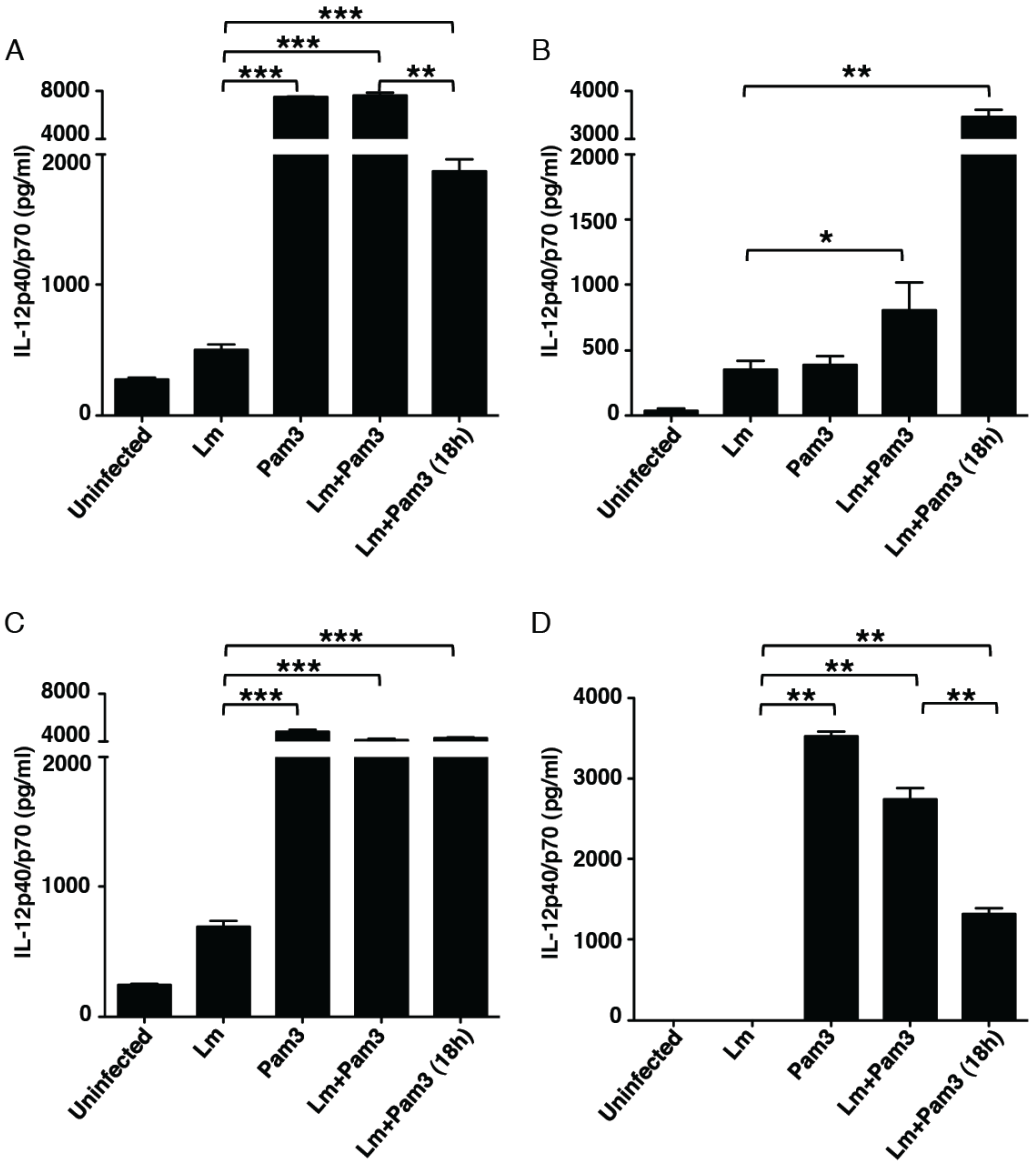

Figure A3. Pam3CSK4 increases secretion of IL-12 in *L. major* infected BMDDCs and BMDMs. IL-12 production measured by ELISA 24 h post stimulation in culture supernatants from BMDDCs and BMDMs infected or not with *L. major* (Lm, 1 cell : 5 parasites). Infected cultures were treated with 0.5 mg/ml of Pam3CSK4 at the time of the infection (Lm + Pam3) or after the infection (Lm + Pam3 (18h)). BMDDCs data are shown in (**A)** C57BL/6 and (**C)** BALB/c. BMDM data are shown in (**B)** C57BL/6 and (**D)** BALB/c. Data show mean ± SEM (n = 5 independent experiments). Asterisks indicate statistically significant differences between the two groups. Significant differences were determined by ANOVA and Tukey's test. *$p < 0.05$, **$p<0.01$, ***$p<0.001$.

Pam3CSK4 treatment at the time of *L. major* infection prevented the development of lesions and decreased parasite burden in mice

Our in vitro data strongly suggested that the pro-inflammatory properties of Pam3CSK4 could enhance anti-leishmanial immunity *in vivo*. In order to determine the outcomes of Pam3CSK4 treatment, we tested our hypothesis by using two different strains of mice. We infected C57BL/6 mice (Th1-biased, self-healing disease) and BALB/c mice (Th2 biased, progressive disease) in the ears with a suspension of 10^6 *L. major* parasites containing or not 50 μg Pam3CSK4. We monitored lesion development and determined parasite burden in ears at early (day 2) and late (days 42 for C57BL/6 mice and 70 for BALB/c mice) time points. **Fig. A4 A** and **A4 B** show that all mice treated with Pam3CSK4 developed either small or no lesions when compared with mice infected with parasites alone ($P < 0.001$).

Parasite burden data from ears and lymph nodes of C57BL/6 mice **(Fig. A4 C and A4 D**, **respectively)** revealed no differences between the two experimental groups at the early time point (day 2). This suggests that treatment with the TLR2 agonist did not interfere with parasite establishment in these mice. In contrast, parasite burden was significantly decreased in both ears and lymph nodes of C57BL/6 at day 42 ($P < 0.001$ and $P < 0.01$, respectively).

On the other hand, the establishment of *L. major* infection in ears and draining lymph nodes of BALB/c mice was dramatically compromised at the early time point, as burdens were significantly decreased in both sites at day 2. At day 70, parasite burdens were still significantly lower in the ears of mice treated with Pam3CSK4, but no differences were detected in lymph node burdens. These results suggest that, while

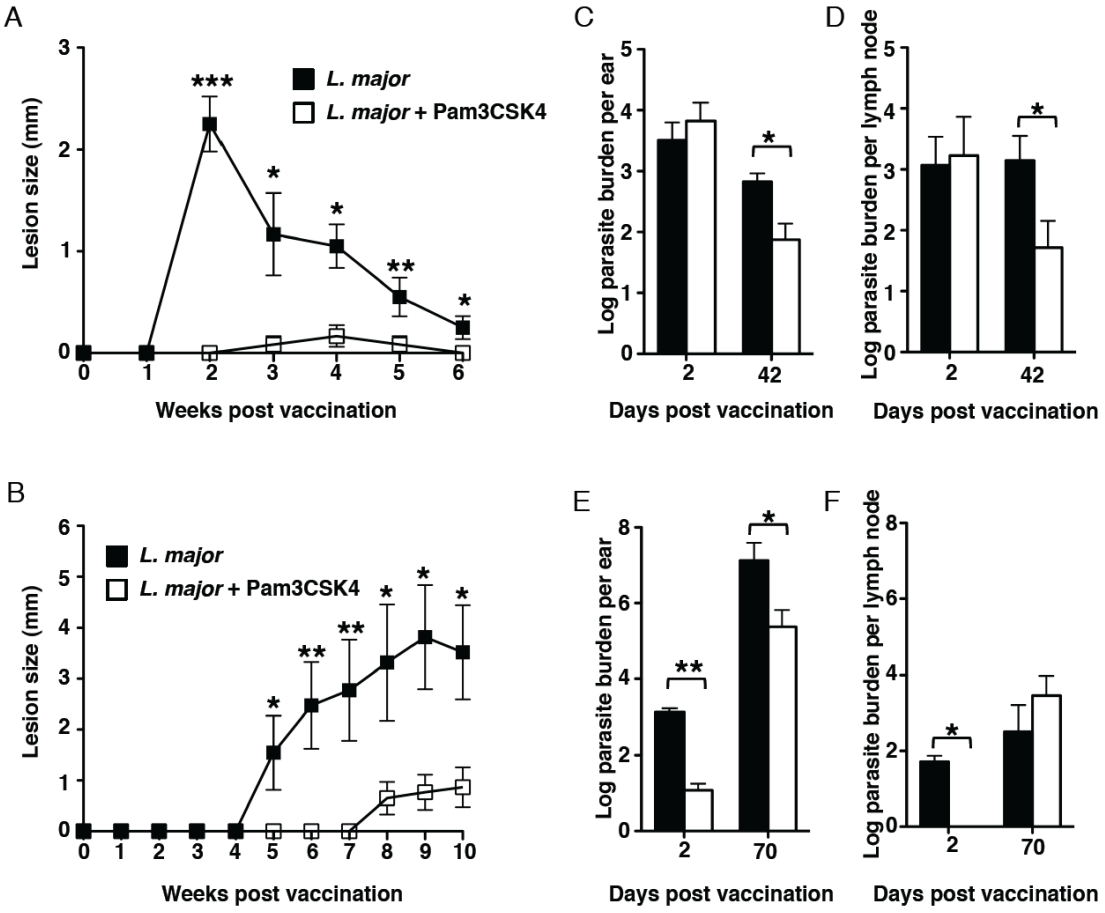

Figure A4. Vaccination with *L. major* + Pam3CSK4 decreased lesion size and parasites burden in mice. Mice were injected in the ear dermis with 10^6 *L. major* alone or in combination with 50 µg Pam3CSK4. Figure shows lesion size (mm) in **(A)** C57BL/6 and **(B)** BALB/c mice expressed as mean ± SEM (n = 12 ears/group). Asterisks indicate statistically significant differences following Student *t*-test between the two groups at each time point; **p* < 0.05. Parasites burden shown in **C** and **E** represent data from ears of C57BL/6 and BALB/c mice respectively. Parasites burden shown in **D** and **F** represent data from submandibular lymph nodes of C57BL/6 and BALB/c mice respectively. Data were collected at day 2 (both strains), 42 (C57BL/6) and 70 (BALB/c mice) post vaccination, and are expressed as log geometric mean ± SD, n = 6 ears or lymph nodes per group. Data are representative of two to three experiments with similar results. Asterisks indicate statistically significant differences following Mann-Whitney analysis between the two groups at each time point; **p* < 0.05, ** *p* < 0.001.

Pam3CSK4 prevents the development of pathology in both mouse strains; the kinetics and the mechanism whereby pathology is prevented may be different.

Pam3CSK4 increases the expression of dermal proinflammatory cytokines

We have shown that vaccination with *L. major* and CpG DNA increased the early proinflammatory cytokine production in the dermis of C57BL/6 mice (15). To determine if this activation mechanism caused by the TLR9 agonist is shared with other TLR ligands, we investigated the expression of the proinflammatory cytokines IL-12 and IL-6 at 48 h post vaccination, in both dermal DCs (expressing CD11c) and macrophages (expressing F4/80). As shown in **Table A1**, the total numbers of cells positive for IL-12 and IL-6 staining was significantly increased at 48 h in all mice vaccinated with parasites plus Pam3CSK4, irrespective of the mouse strain. Cytoplasmic IL-10 staining did not show significant differences among any of the groups, and the level of expression was very low (>2%) in all cases (not shown). This demonstrates that vaccination with live parasites and TLR2 agonists also induces the early initiation of a proinflammatory response at the vaccination site.

Vaccination with *L. major* plus Pam3CSK4 induces the expansion of Th1 and Th17 cells in C57BL/6

Our results have revealed so far that both resistant and susceptible mouse strains are protected against the development of lesions by Pam3CSK4 treatment. Our previous work with live parasites and CpG DNA revealed that Th1, but particularly

Table A1. Cytokine expression at 48 h post vaccination in dermal cells of mice vaccinated with *L. major* alone or in combination with 50 µg Pam3CSK4. Data show average ± standard deviation of the percentage of cytokine expressing cells (n = 3). P values obtained from comparing both groups are included in the table.

		L. major	*L. major*+Pam3CSK4	P values
C57BL/6				
CD11c$^+$ DC	IL-12	7.3 ± 4.0	21.4 ± 3.9	P=0.005
	IL-6	5.8 ± 2.6	15.7 ± 6.2	P=0.06
F4/80$^+$ Macrophages	IL-12	11.4 ± 1.2	32.3 ± 4.8	P=0.001
	IL-6	5.7 ± 2.5	11.0 ± 1.2	P=0.03
BALB/c				
CD11c$^+$ DC	IL-12	5.1 ± 1.3	11.3 ± 2.9	P=0.006
	IL-6	4.5 ± 2.3	11.3 ± 2.7	P=0.02
F4/80$^+$ Macrophages	IL-12	4.4 ± 1.2	13.1 ± 3.3	P=0.01
	IL-6	6.1 ± 3.0	18.8 ± 2.4	P=0.004

Th17 responses were required to control vaccinal pathology in the C57BL/6 mouse (17). However, the immune response of the BALB/c mice to this vaccine has remained uncharacterized. To investigate whether the CpG DNA-induced Th17 cell expansion is shared with other TLR agonists, we determined the absolute numbers of cytokine producing CD4 T cells in the ear draining lymph nodes of the vaccinated mice at early and late time points as described before. **Fig. A5 A** shows that both Th17 and Th1 responses are enhanced by vaccination with a mixture of live parasites and TLR2 agonist in resistant mice. This enhanced CD4 response was similar to what was described in our previous work using CpG DNA (17). Conversely, *L. major* plus Pam3CSK4 vaccination did not enhance Th17 responses in the BALB/c strain, although the number of Th1 IFN-γ expressing cells was higher in the *L. major* plus Pam3CSK4-vaccinated group **(Fig. A5 B)**; the immune response in these mice was dominated by Th1 cells, as opposed of what was found in the mice vaccinated with *L. major* alone. An interesting finding was that the immune response to *Leishmania* switched from a Th2 into a Th1 in the vaccinated mice. This trend continued throughout the course of the infection, as demonstrated by the data obtained in the latest time point **(Fig. A5 C and A5 D)**. These data suggest that the mechanisms underlying protection are different between both strains of mice.

Pam3CSK4 induces neutrophil influx as well as a rapid IL-17 production to the site of vaccination

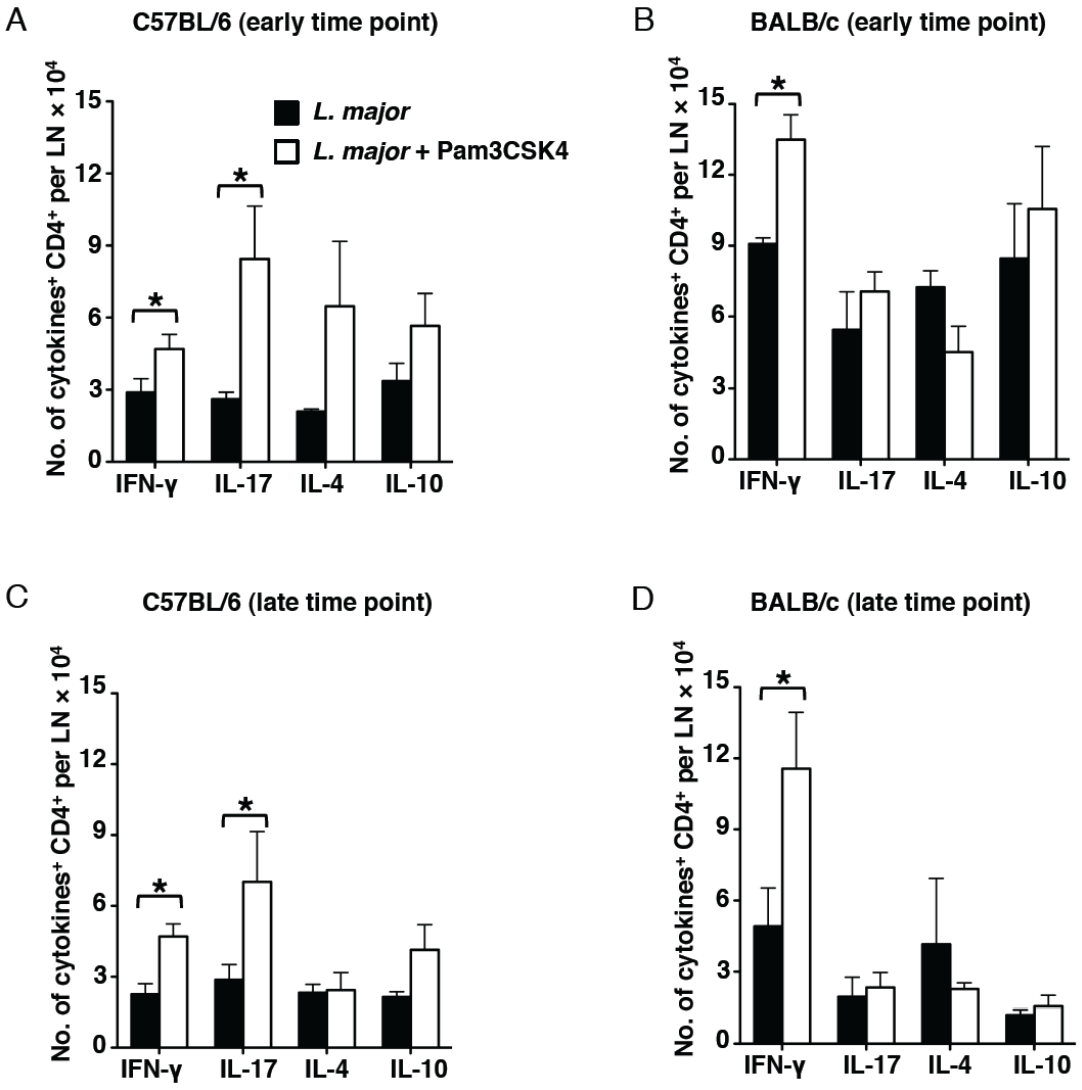

Figure A5. Vaccination with *L. major* plus Pam3CSK4 increased the number of Th1 and Th17 cells in C57BL/6 mice but only Th1 in BALB/c mice. Absolute numbers of IFN-γ (Th1 cells), IL-17 (Th17 cells), IL-4 and IL-10 producing $CD4^+$ T cells shows in **(A)** and **(C), (B)** and **(D)** in submandibular lymph nodes of C57BL/6 and BALB/c mice respectively. Recovered lymph node cells were restimulated with 25 mg/mL soluble *Leishmania* antigen, 5 ng/mL IL-2 and 10 mg/mL anti-CD28 overnight before performing cytokine staining. Data were collected at day 2 (both strains), 42 (C57BL/6) and 70 (BALB/c mice) post vaccination, and are expressed as mean ± SEM, n = 6 lymph nodes per group. Data are representative of two to three experiments with similar results. Asterisks indicate statistically significant differences following Mann-Whitney analysis between the two groups at each time point; $*p < 0.05$.

We have reported that, in C57BL/6 mice, vaccination with live parasites and CpG DNA increased the influx of neutrophils to the vaccination site early after vaccination (17). **Fig. A6 A and B** show that shortly after vaccination, neutrophils are significantly increased in both C57BL/6 and BALB/c, although their numbers were dramatically decreased at the later time point in the resistant mouse. Conversely, and despite the lack of pathology, neutrophil numbers remained high in the skin of vaccinated BALB/c mice. Because other authors have reported increasing amounts of IL-17 production by neutrophils in infected BALB/c (29), we assessed the ability of neutrophils produce IL-17 following vaccination. Although neutrophils infiltrate the vaccination site immediately after vaccination in in both mouse strains, there was a greater number of IL-17 producing neutrophils in BALB/c mice **(Fig. A6 C and A6 D)**. Moreover, neutrophils from BALB/c mice treated only with Pam3CSK4 also produce large amount of IL-17, indicating a specific effect of Pam3CSK4 on neutrophils in susceptible mice.

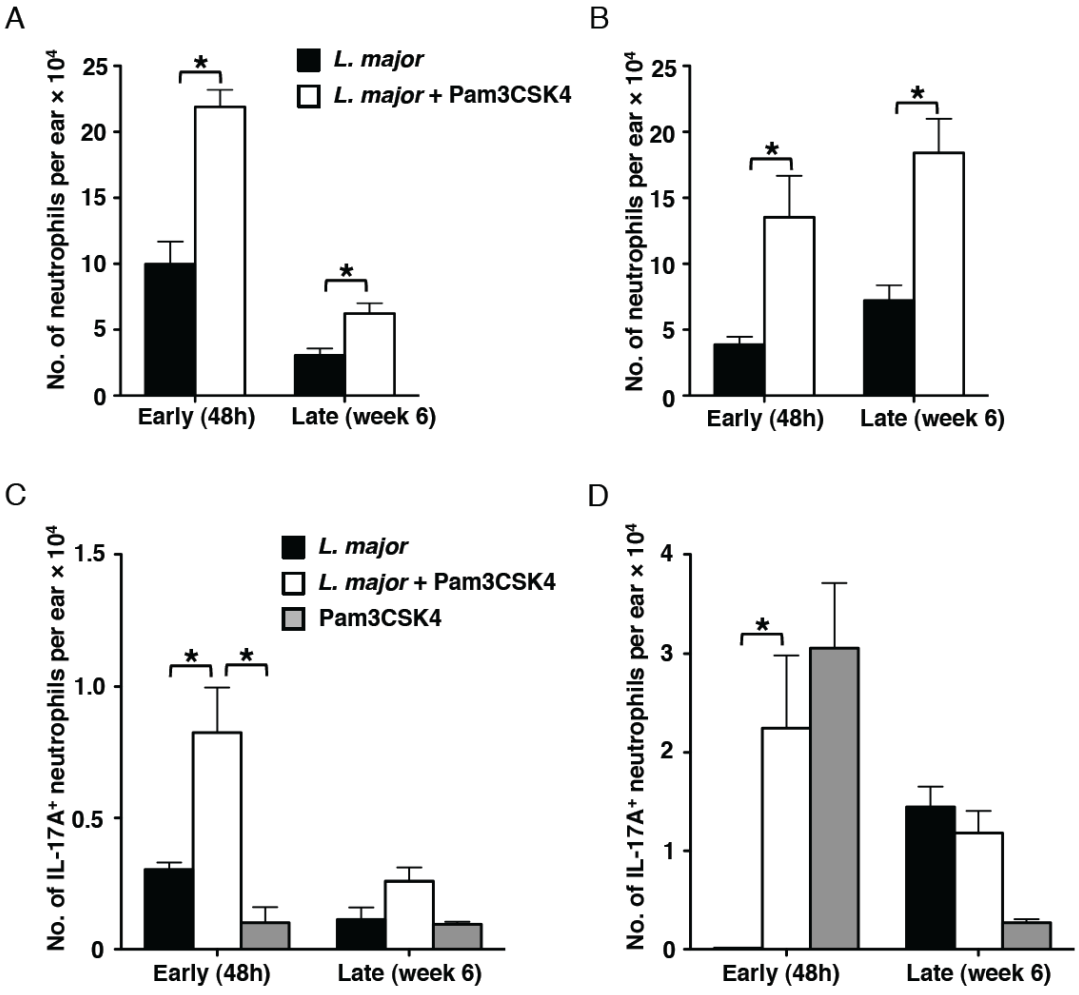

Figure A6. BALB/c mice showed sustained infiltration of neutrophils and rapid production of IL-17 after vaccination with *L. major* plus Pam3CSK4. Absolute numbers of Ly-6G$^+$ neutrophils shows in **(A)** C57BL/6 and **(B)** BALB/c mice ears. Absolute numbers of dermal IL-17$^+$ Ly-6G$^+$ neutrophils determined by flow cytometry in **(C)** C57BL/6 and **(D)** BALB/c mice. Data were collected at day 2, 42 (C57BL/6) and 70 (BALB/c mice) post vaccination, and are expressed as mean ± SEM, n = 6 ears per group. Recovered ear cells were retimulated with 25 mg/mL soluble *Leishmania* antigen, 5 ng/mL IL-2 and 10 mg/mL anti-CD28 overnight before performing cytokine staining for IL-17. Data are representative of two to three experiments with similar results. Asterisks indicate statistically significant differences following Mann-Whitney analysis between the two groups at each time point; *$p < 0.05$.

Discussion

To date, there is no vaccine against cutaneous leishmaniasis. The major impediment in vaccine design has been the failure in translating data from animal models to human disease, as well as the lack of understanding of how protective immune responses and immunological memory are generated and maintained (30). In this paper, we have discovered that vaccination with live parasites in the presence of the TLR2 agonist Pam3CSK4 protects susceptible and resistant mice against the development of leishmaniasis, albeit the underlying immunological mechanisms appear to be completely different.

Our work has focused on understanding how live vaccination immunity is modulated by the addition of TLR agonists. In particular, we have extensively characterized the immune responses of the C57BL/6 mouse to vaccination with live parasites plus the TLR9 agonist CpG DNA (14-17, 31, 32). We employed this mouse strain because, unlike the susceptible BALB/c that succumb to systemic disease by *L. major*, infection of C57BL/6 replicates all clinical features of human cutaneous leishmaniasis: self-healing lesions (33, 34), chronicity (35) and concomitant immunity (36).

The first objective of this study was to validate the immunological mechanism of protection behind live vaccination with CpG DNA, and to investigate whether this mechanism is shared with other TLR agonists. We chose TLR2 because this is the most promiscuous TLR receptor, being able to recognize the most diverse set of pathogen-associated molecular patterns (PAMP). Furthermore, lipophosphoglycan (LPG), a PAMP in *Leishmania*, has been shown to bind to TLR2 and activate NF-κB

translocation in a TLR2-dependent manner. This ligation upregulates TLR2 expression and eventually promotes the production of IFN-γ and TNF-α in NK cells (26, 27). Moreover, TLR2 is widely express among human leukocytes, what will ensure a very intense response following receptor ligation. And most importantly, we have shown that *L. major*-infected cells become more sensitive to TLR2 stimulation and increase their proinflammatory response. Our data demonstrate that vaccination with live parasites plus the TLR2 agonist Pam3CSK4 completely protected mice against the development of lesions, suggesting that TLR2 stimulation also results in enhanced anti-leishmanial immunity. Unexpectedly, we have found that expression of TLR in DCs is different between the two strains of mice. This result is similar with the previous report that expression levels of TLR2, TLR4, TLR5 and TLR6 in naïve splenic DCs are higher in BALB/c mice than in C57BL6 mice (37). The reactivity of DCs in both strains of mice is also different upon TLR ligand stimulation. Take together, our data suggest that difference in both expression pattern and reactivity of TLRs may be associated with susceptibility and resistance to *L. major* infection in C57BL/6 and BALB/c mice.

The second objective of our work was to compare the immunological events associated with protection following vaccination of both genetically susceptible and resistant mouse strains, which are characterized by extreme Th2 or Th1 polarization, respectively. The immune responses to cutaneous leishmaniasis in humans lack the strong polarity found in mouse models. Epidemiological data from patients with localized cutaneous leishmaniasis seem to confirm the Th1/Th2 dichotomy shown in mice. However, patients with diffuse cutaneous leishmaniasis display a more

predominant Th2 cytokine response. Moreover, patient with mucosal leishmaniasis show a mixture of Th1 and Th2 cytokines (38). Thus, the comparative study of the mouse models is important to be able to predict how, and whether, vaccine efficacy studies that employ TLR2 agonists would translate to human vaccines.

Our data showing that live vaccination with parasites plus Pam3CSK4 protects both C57BL/6 mice and BALB/c against lesions are very promising, and point towards the feasibility of the use of TLR2 agonists as immune adjuvants against leishmaniasis. However, our studies have also revealed that the mechanism underlying protection is very different between the two mouse strains. C57BL/6 mice, as we demonstrated before, develop a strong Th17 response following vaccination with TLR agonists. However, this effector population did not expand in vaccinated BALB/c mice; in these animals, protection was caused by enhanced Th1 responses.

Another remarkable vaccination difference between the two strains was the sustained IL-17^{+} neutrophil influx in BALB/c, but not in C57BL/6 mice. We still do not have a mechanistic explanation to explain these differences. The role of the neutrophil is leishmaniasis is not well understood because it varies depending on the species of *Leishmania* and the animal models employed. As a few examples, studies in the C57BL/6 mice have shown that neutrophils may help infection by harboring parasites (39). But conversely, others have revealed that neutrophils contribute to parasite killing (40). Moreover, neutrophil influx has been associated with resistance or susceptibility in *L. amazonensis* murine models (41). Finally, neutrophils appear to be required for protective responses in *L. braziliensis* (42). Our data also uncovered an interesting outcome of vaccination which is at early time point, neutrophils in BALB/c

mice that vaccinated with live parasites plus Pam3CSK4 produce large amount of IL-17, which may be due to the specific effect of Pam3CSK4 in this cell type only in the susceptible strain. Some studies have already shown the distinct phenotypes of neutrophils expressing different TLRs in both resistant and susceptible mice during *L. major* infection (43). Differential expression of TLRs by neutrophils may cause the diverse responses to TLRs agonist and thus influence the development of *L. major* specific immune response in our vaccination. We plan to define the function of neutrophils as well as the role of IL-17 in future studies.

Our findings are relevant because they reveal the complexity and the difficulty to achieve vaccine protection: by exclusively taking into account the C57BL/6 data, we would have concluded that enhancing Th17 response is necessary to protect against leishmanial challenge. However, enhancing Th17 responses may not to work individuals displaying Th2 or mixed Th1/Th2 responses. Understanding the factors that regulate parasite persistence and its role in maintenance of immunologic memory in cutaneous leishmaniasis is critical for proper design and development of effective vaccine and vaccination strategies against the disease, and may explain why vaccination strategies have not translated very well from the mouse to the human.

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APPENDIX B

A Th-17 inducing live vaccine comprised of *Leishmania major* and CpG DNA does not induce memory Th17 cell establishment in the skin of vaccinated mice[*]

[*]Lu Huang, Weishan Huang, Avery August and Susana Mendez

Abstract

Cutaneous leishmaniasis caused by *Leishmania major* is a neglected, emerging parasitic disease with no vaccine. We have previously demonstrated that intradermal vaccination of the resistant C57BL/6 mice with live parasites plus CpG DNA is highly effective as it eliminates disease pathology and provides long-term immunity. We have also reported that this strategy induces the generation of $CD4^+$ Th17 cells in the dermis of the vaccinated animals, which in turn causes parasite killing and contributes to disease prevention in the skin. Because Th17 cells have been associated with several skin immune disorders, we investigated the establishment of memory Th17 cells in the skin of mice immunized with the anti-leishmanial vaccine. Our studies have demonstrated that dermal memory $CD4^+$ T cells isolated from the skin and lymph nodes of the vaccinated mice produce small amounts of IFN-γ, but no IL-17. Revaccination of these mice with the live anti-leishmanial vaccine or inoculation of other antigens (*i.e.* OVA) did not induce the expansion of IL-17 effector memory $CD4^+$ T cells either. Our results demonstrate that Th17 cells induced by the vaccination of *L. major* plus CpG DNA are a transient cell population that involves in the early protection against infection but do not generate memory in the chronic site. This suggests that this is a safe approach to vaccination that can be applied to individuals prone to skin immune disorders involving IL-17 production.

Introduction

The leishmaniases are a group of emerging, uncontrolled vector-borne parasitic diseases prevalent worldwide. The disease is endemic in 88 countries; 350 million people are at risk and 12 million people are affected (1). Recent data suggest that these numbers are greatly underestimated. Although the disease can be treated, current drugs are inadequate due to toxicity, resistance, cost and adverse effects. There is no vaccine. Thus, there is a clear need for both prophylactic and therapeutic control measures. During the past decade, our research has focused on studying the mechanism of action of leishmanization (live vaccination), the only strategy that has ever provided lifelong protection (2), by using a murine model of cutaneous disease (C57BL/6 mice and *Leishmania major*). Our vaccination approach (immunization of mice with live *L. major* parasites along with CpG DNA) reduced or completely eliminated disease in C57BL/6 mice without compromising long-term protection (3, 4). We have previously demonstrated that this live vaccine enhances recruitment of IFN-γ-producing $CD4^+$ T cells (4), decreases expansion of dermal regulatory T cells (5), causes activation of dermal NK cells (6), and most interestingly, promotes the local expansion of T helper (Th)-17 cells (7).

Th17 cells are subset of $CD4^+$ T cells that preferentially produce IL-17, IL-21, and IL-22 (8). Multiple evidence has accumulated for a role of Th17 cells in the protective response against fungi and yeast (9, 10), extracellular bacteria (reviewed in (11)), viruses (12, 13), and protozoa (14-16). However, and despite the beneficial functions of this Th cell subset in protective immunity against pathogens, IL-17 is also associated with human immune pathology. For example, IL-17-expressing T-cell

clones are reported in synovial tissues from rheumatoid arthritis patients (17, 18), and in the cerebrospinal fluid of multiple sclerosis patients (19). Moreover, increased IL-17 levels are observed in sera and inflamed mucosae from patients suffering from Crohn's disease or ulcerative colitis (20).

Our intradermal vaccination model has demonstrated that IL-17 may be an important modulator of *Leishmania* growth and pathology in the skin, particularly in the context of vaccination with CpG DNA (7). However, as with other human immune pathologies, recent findings have suggested that Th17 cells profoundly participate in the pathogenesis of certain skin disorders, such as psoriasis or contact hypersensitivity (21). Hence, and before proposing a vaccination strategy that may enhance dermal Th17 expansion, we must rule out that the manipulation of dermal T-cell differentiation by our *L. major*/CpG DNA vaccine may result in the establishment of potentially pathogenic T cell lineages in the skin (*i.e.* activated Th17 lymphocytes) that could possibly enhance inflammation in susceptible individuals.

In this report, we investigate the establishment of $CD4^+$ memory cells in the skin of C57BL/6 mice vaccinated with *L. major*/CpG DNA, and their ability to produce cytokines once the vaccinated mice reach a chronic, immune stage. We also determined the effect of revaccination on the ability of resident memory T cells to produce the inflammatory cytokines IFN-γ and IL-17. Our data revealed that Th17 producing memory T cells do not establish at the chronic site of Lm/CpG vaccinated mice, and that revaccination of these mice leads to an increase in IFN-γ, but not IL-17 production, by memory $CD4^+$ T cells.

Materials and Methods

Mice

Six-week-old C57BL/6 mice were purchased from Taconic (Germantown, NY). All mice were maintained in the Baker Institute for Animal Health animal care facility under pathogen-free conditions. All animal experiments were performed according to federal guidelines and institutional policies (animal protocol 2008-177). All efforts were made to minimize suffering. All animal work has been conducted according to relevant national and international guidelines. The work described in the manuscript has been approved by Cornell's IACUC committee (Protocol no. 2010-0035).

Parasites

L. major clone V1 (MHOM/IL/80/Friedlin) promastigotes were grown at 26 °C in medium 199 supplemented with 20% heat-inactivated fetal calf serum (Gemini, Sacramento, CA), 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, 40 mM HEPES, 0.1 mM adenine (in 50 mM HEPES) and 5 mg/ml hemin (in 50% triethanolamine).

Infection protocol and vaccine preparation

Infective-stage promastigotes of *L. major* were isolated from stationary cultures (4-5 days old) by Ficoll enrichment as described (22). Mice were vaccinated intradermally in ears with 10^4 *L. major* promastigotes with or without 50 mg CpG DNA (5' TCC ATG ACG TTC CTG ACG TT-3', IDT, Coralville, IA), using a 27G

needle in a volume of 10 ml. The evolution of the lesions was monitored by measuring the diameter of the induration using a vernier caliper.

Parasite titration

Parasite loads in the ears were determined as described before (23). Briefly, the ear sheets were separated and deposited in DMEM containing Liberase CI enzyme blend (0.5 mg/ml) for 60 min at 37 °C. The sheets were then dissociated using a handheld tissue homogenizer. The homogenates were filtered using a 70-mm cell strainer (BD Falcon, San Jose, CA) to produce single cell suspensions and serially diluted in a 96-well flat-bottom microtiter plates containing biphasic medium prepared using 50 mL Novy-MacNeal-Nicolle (NNN) medium containing 20% of defibrinated rabbit blood overlaid with 100 mL medium 199. The number of viable parasites in each ear was estimated by limiting dilution from the highest dilution at which promastigotes could be grown out after 7 days of incubation at 26 °C. Parasite numbers were also determined in the local draining lymph node (submandibular). Lymph nodes were mechanically dissociated and parasite load was determined by limiting dilution as described above.

Analysis of dermal lymphocytes

Single-cell suspensions from the ear dermis were obtained as described above. For the analysis of surface markers and intracytoplasmic staining for cytokines, cells were stimulated overnight with 25 mg/mL soluble *Leishmania* antigen (SLA), 5 ng/ml IL-2 and 10 mg/ml anti-CD28, cultured with brefeldin A at 10 ng/ml for 6 h and then

fixed in 2 % paraformaldehyde (23). Prior to staining, cells were incubated with an anti-FcgIII/II receptor antibody and 10% normal mouse serum in PBS containing 0.1% BSA, 0.01% NaN_3. Cells were permeabilized and stained for the surface markers CD4 (clone GK1.5), TCR-β (clone H57-597), CD44 (clone IM7), CD62L (clone MEL-14), and for the cytokines IFN-γ (XMG1.2) and IL-17A (clone eBio17B7). All antibodies were purchased from BD Bioscience (San Jose, CA) or eBioscience (San Diego, CA). Incubations were carried out for 30 min on ice. For each sample, at least 100,000 cells were analyzed. The data were collected and analyzed using BD FACSDiva or FlowJo software and a LSRII flow cytometer (BD Bioscience, San Jose, CA).

Statistical analysis

All comparisons of non-normally distributed continuous data were analyzed with Student's T test, Mann-Whitney U test or ANOVA using GraphPad Prism (San Diego, CA).

Results

Parasite persistence is achieved following live vaccination with L. major in the presence or absence of CpG DNA

As previously reported, we vaccinated C57BL/6 mice in one ear with *L. major* alone (10^4) or in combination with 50 mg CpG DNA, and monitored them for 3 months. As reported multiple times before (4, 5), mice vaccinated with the *L. major*/CpG DNA combination displayed a decrease in lesion size and accelerated healing compared with the animals vaccinated with parasites alone (**not shown**). After healing, the infection site of *L. major* becomes a chronic site characterized by parasite persistence in the absence of lesions (24). **Figure B1** shows that, as expected, chronicity was achieved in all vaccinated ears. The parasite burden after healing was significantly reduced in the group vaccinated with *L. major*/CpG DNA (P = 0.004). Interestingly, a small number of parasites could also be detected in the opposite, naïve ears in both groups (216 ± 125 *vs.* 270 ± 108 for *L. major* and *L. major*/CpG DNA, respectively; these numbers were not statistically different). Parasites were also present in the submandibular lymph nodes draining from the vaccinated ears, although they could not be detected in the opposite site. Again, there was a slight reduction in numbers in the group vaccinated with CpG DNA (P = 0.04)

$CD4^+$ memory T cells produce IFN-γ but not IL-17 in the skin and draining lymph nodes of chronic, vaccinated mice

We have previously reported that Th1, and particularly Th17 cells expand at the vaccination site of *L. major*/CpG DNA infection (7). We have not however

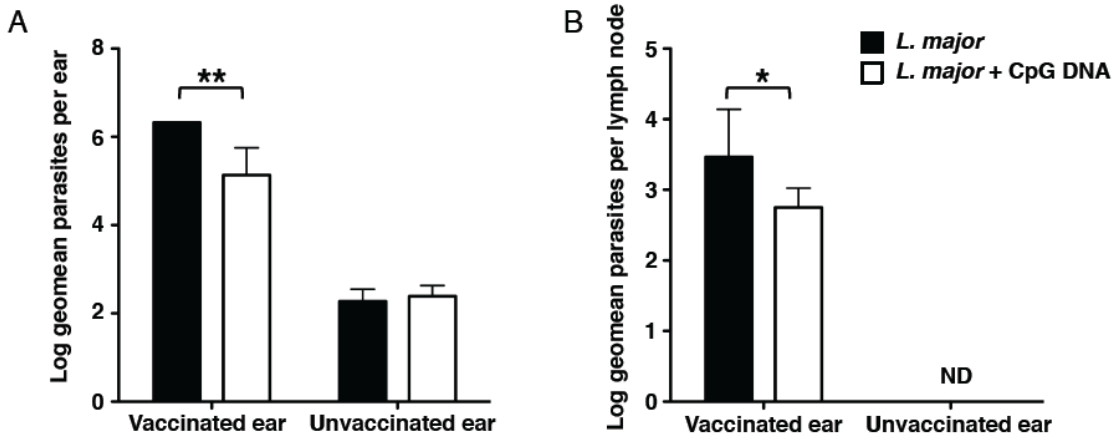


Figure B1. Live vaccination establishes a persistent infection in ears and draining lymph nodes. Mice were vaccinated in the ear dermis with 10^4 *L. major* promastigotes alone or in combination with 50 μg CpG DNA. Parasite burden (**A**) per ear and (**B**) per draining lymph node at 12 weeks post vaccination, expressed as geometric mean ± SD (n= three mice per group). **P = 0.004; *P = 0.04. N.D.= not detected.

investigated whether specific memory T cells are generated following the attrition of these populations during the acute phase of infection. We therefore studied the frequencies of tissue effector memory T cells (defined as $TCRb^{+}CD44^{+}CD62L^{low}$) in the ears and draining lymph nodes of chronic, vaccinated mice. In order to gain a comprehensive view of the function of these specific memory cells, we tested them for the production of IFN-γ and IL-17. **Figure B2** shows that memory cells preferentially produced IFN-γ in both the ears and the lymph nodes of the vaccinated mice. No IL-17^{+} $CD4^{+}$ memory cells were detectable in any of the tissues analyzed. Although the frequency of IFN-γ^{+} cells was slightly reduced in the *L. major*/CpG DNA group if compared with the mice vaccinated with *L. major* alone, the absolute number analysis revealed that cytokine positive cells were elevated in this group (2618.9 ± 211.3) when compared with the number found in the ears of mice vaccinated with L. major alone (1977.2 ± 162.0, P=0.04), No differences in frequencies or absolute numbers of cytokine positive cells were found in the lymph nodes (**not shown**).

Revaccination of vaccinated mice leads to an increase in IFN-γ, but not IL-17 production, by memory $CD4^{+}$ T cells

The objective of our next experiment was to elucidate whether revaccination of chronic vaccinated mice would cause expansion of cytokine positive $CD4^{+}$ T cells. Mice vaccinated with *L. major* alone in one ear were inoculated with 10^{4} parasites alone or with the CpG DNA in the opposite ear 20 weeks following the initial vaccination. Similarly, mice vaccinated originally with *L. major* /CpG DNA were

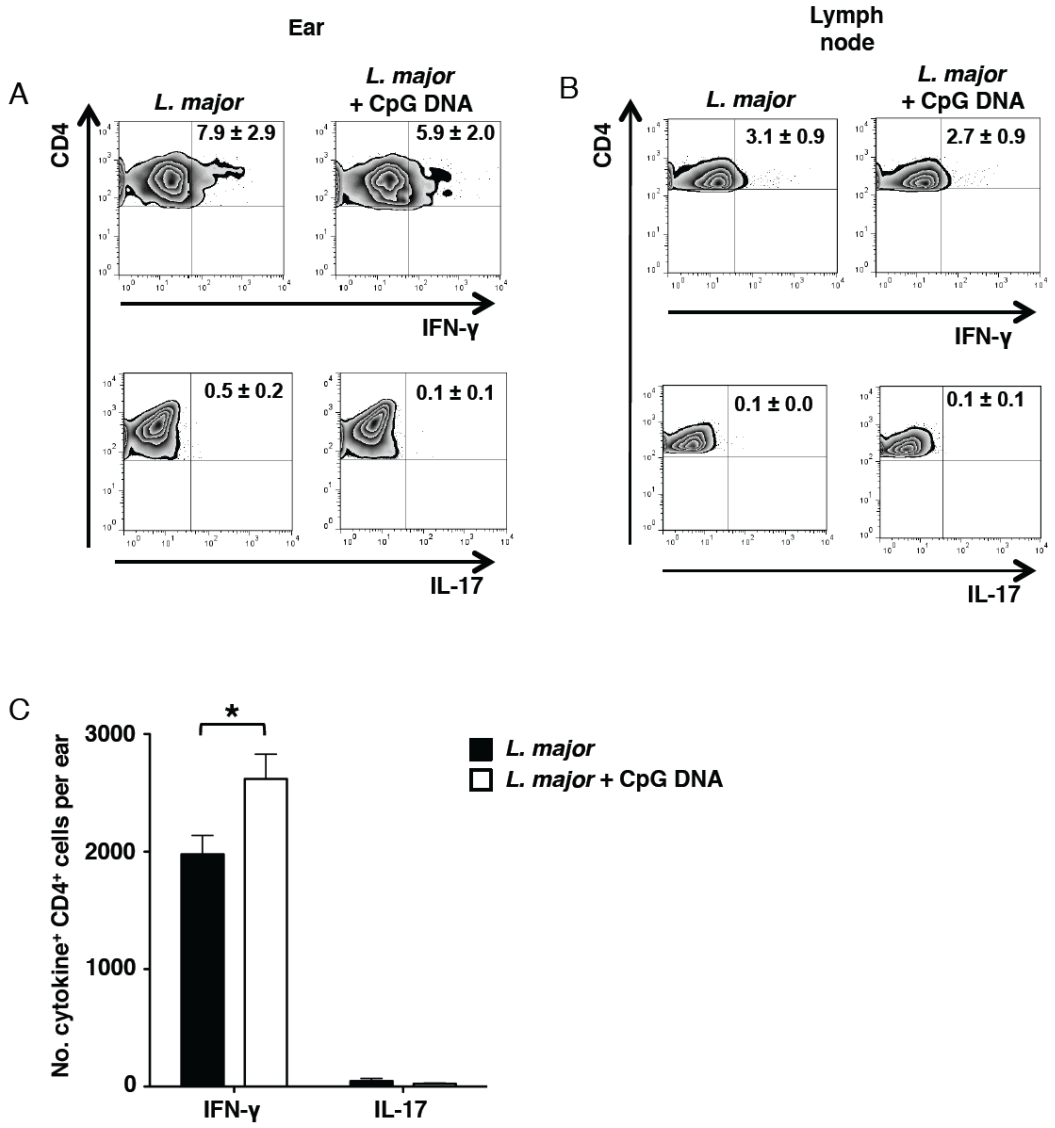
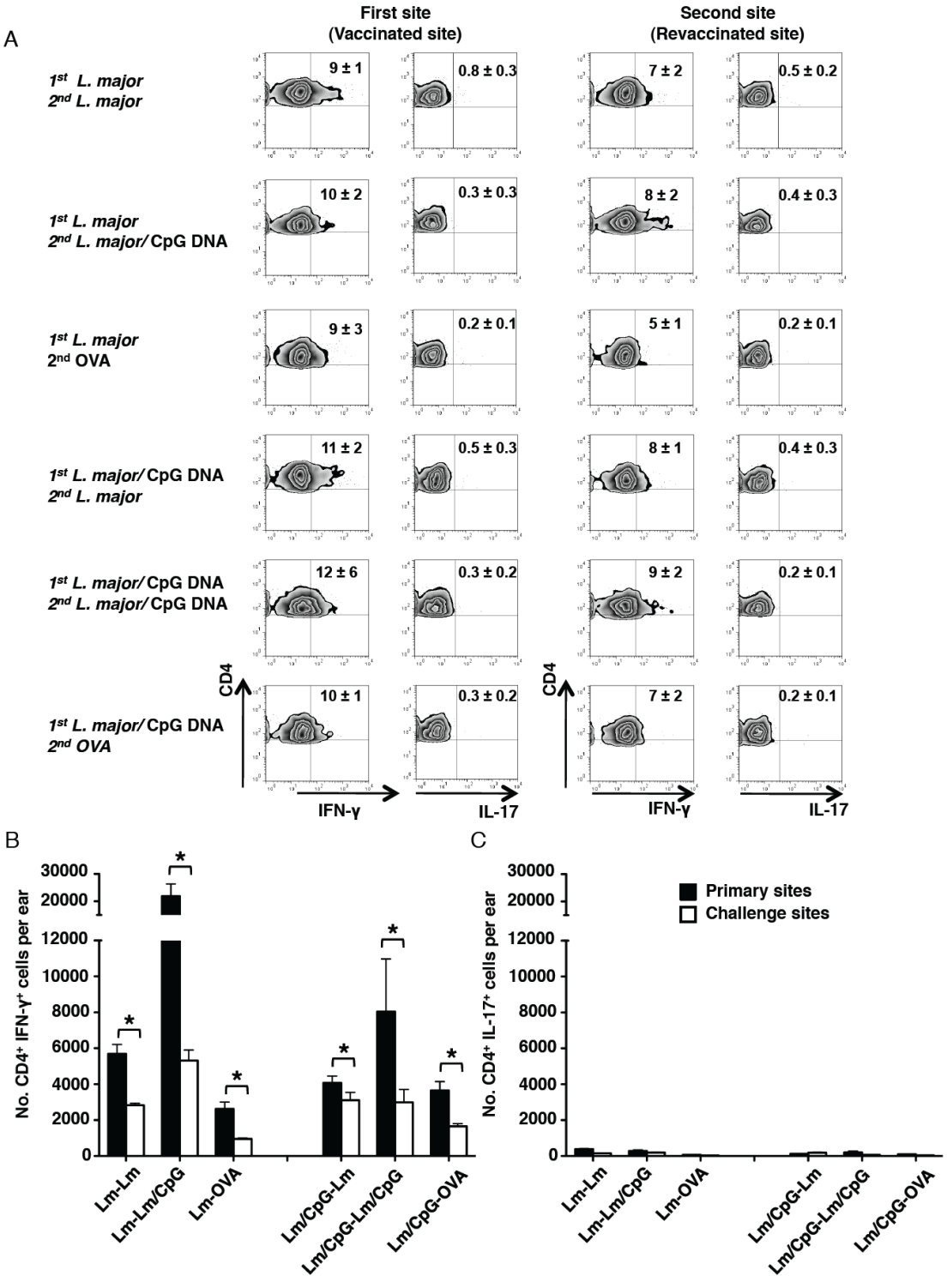


Figure B2. CD4$^+$ memory T cells of vaccinated mice produce IFN-γ, but not IL-17. Comparative analysis of IFN-γ and IL-17 expression by CD4$^+$ memory T cells extracted from ears and draining lymph nodes of mice vaccinated with 10^4 *L. major* alone or in combination with 50 μg CpG DNA. For each experiment, IFN-γ and IL-17 producing cells extracted from ears and draining lymph nodes were analyzed on gated cells by flow cytometry (n=3 mice per group). Flow cytometry plots showing the frequency of IFN-γ and IL-17 producing CD4$^+$ effector memory T cells (TCRβ$^+$CD44$^+$CD62L^{low}) in (**A**) ear and (**B**) draining nodes 12 weeks post vaccination. Data are expressed as mean ± SEM from three independent experiments. (**C**) Absolute numbers of IFN-γ and IL-17 producing CD4$^+$ memory T cells in vaccinated ears at 12 weeks post vaccination. Data are mean ± SEM from three independent experiments. *P = 0.04 (*L. major*/CpG DNA *versus L. major*).

inoculated in the opposite ear with either parasites alone or together with CpG DNA. The frequencies and absolute numbers of memory $CD4^+$ T cells producing IFN-γ or IL-17 were determined in the vaccinated (first site) and in the opposite ears (second site, revaccinated site) three days after the expansion. **Figure B3. A** shows that, three days after injection, the frequencies of IFN-γ^+ $CD4^+$ effector memory cells increased slightly (to *ca.* 10%). This frequency was higher than what previously found in the chronic, unchallenged stage (7.9 %, **Figure B1**), suggesting that the disturbance caused by revaccination in the chronic site led to the expansion, or new recruitment, of the IFN-γ^+ $CD4^+$ T cell population. This increase was detectable in both the vaccinated and the challenged sites, and took place irrespective of the vaccine type employed. The quantitative analysis of the absolute number revealed a 10-fold increase in the number of cytokine positive cells (**Figure B3. B**). This expansion was always greater in the primary site when compared with the revaccinated site, and was statistically significant. To determine if the enhanced response was *L. major* specific, we also inoculated some mice with an irrelevant antigen (i.e., ovalbumin (OVA)) as a control. Mice injected with OVA also displayed an increase in IFN-γ^+ cells, although the absolute numbers were significantly reduced compared to the mice challenged with live parasites, suggesting that reinfection/inoculation with a non-leishmanial antigen would not cause a significant increase in IFN-γ^+ cells. In contrast, the frequency of IL-17^+ effector memory cells did not change following revaccination, and in all cases remained below 1% (**Figure B3. C**). Analysis of the absolute numbers confirmed these conclusions and showed that although IL-17^+ cells can be detected in the ear, their numbers were very low and not different from the chronic state. Finally, there

Figure B3. Revaccination leads to IFN-γ, but not IL-17 production by $CD4^+$ effector memory T cells. Mice vaccinated with *L. major* alone or with the CpG DNA in one ear were inoculated with 10^4 parasites alone or with the CpG DNA or with 50 μg OVA in the opposite ear at 20 weeks post vaccination. Three days after injection, IFN-γ and IL-17 expression by $CD4^+$ memory T cells extracted from ears dermis were analyzed on gated cells by flow cytometry (three mice per group). (**A**) Flow cytometry plots showing the frequency of IFN-γ and IL-17 producing $CD4^+$ effector memory T cells ($TCR\beta^+CD44^+CD62L^{low}$) in both vaccinated and revaccinated ears. Data are expressed as mean ± SEM from three independent experiments. (**B**) and (**C**) Numbers of IFN-γ and IL-17 producing $CD4^+$ memory T cells in both vaccinated and revaccinated ears. Data are expressed as mean ± SEM from three independent experiments. *$P < 0.05$ (First site *versus* second site).


A
First site
(Vaccinated site)
Second site
(Revaccinated site)
1st L. major
2nd L. major
9 ± 1
0.8 ± 0.3
7 ± 2
0.5 ± 0.2
1st L. major
2nd L. major/ CpG DNA
10 ± 2
0.3 ± 0.3
8 ± 2
0.4 ± 0.3
1st L. major
2nd OVA
9 ± 3
0.2 ± 0.1
5 ± 1
0.2 ± 0.1
1st L. major/ CpG DNA
2nd L. major
11 ± 2
0.5 ± 0.3
8 ± 1
0.4 ± 0.3
1st L. major/ CpG DNA
2nd L. major/ CpG DNA
12 ± 6
0.3 ± 0.2
9 ± 2
0.2 ± 0.1
1st L. major/ CpG DNA
2nd OVA
10 ± 1
0.3 ± 0.2
7 ± 2
0.2 ± 0.1
CD4
IFN-γ
IL-17
B
No. CD4+ IFN-γ+ cells per ear
30000
20000
12000
10000
8000
6000
4000
2000
0
Lm-Lm
Lm-Lm/CpG
Lm-OVA
Lm/CpG-Lm
Lm/CpG-Lm/CpG
Lm/CpG-OVA
C
No. CD4+ IL-17+ cells per ear
Primary sites
Challenge sites

was no expansion of cytokine positive memory cells in the draining lymph nodes (**Fig. B5**).

Revaccinated mice were also monitored for the development of lesions for 14 weeks. A group of naïve mice challenged with *L. major* were included in the experiment as a control. As expected, naïve mice developed lesions following infection, which healed after 14 weeks. Also as expected all non-naïve mice (already vaccinated with *L. major* with or without CpG DNA) developed a transient inflammation that resolved spontaneously, but did not develop lesions in the challenged site (**Figure B4. A**). Parasite burden at day 3 after revaccination was determined in both the vaccinated and the challenged ears. **Figure B4. B** shows that revaccination did not dramatically expand the number of parasites detectable in ears.

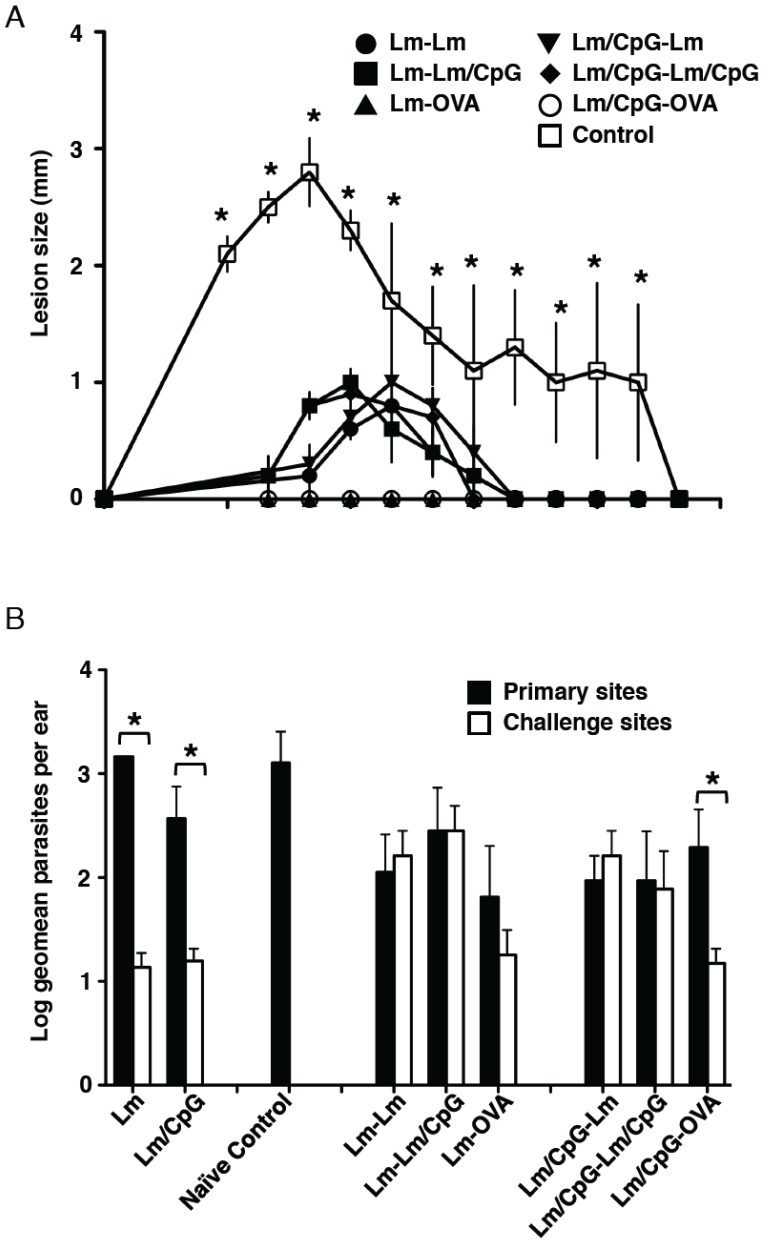
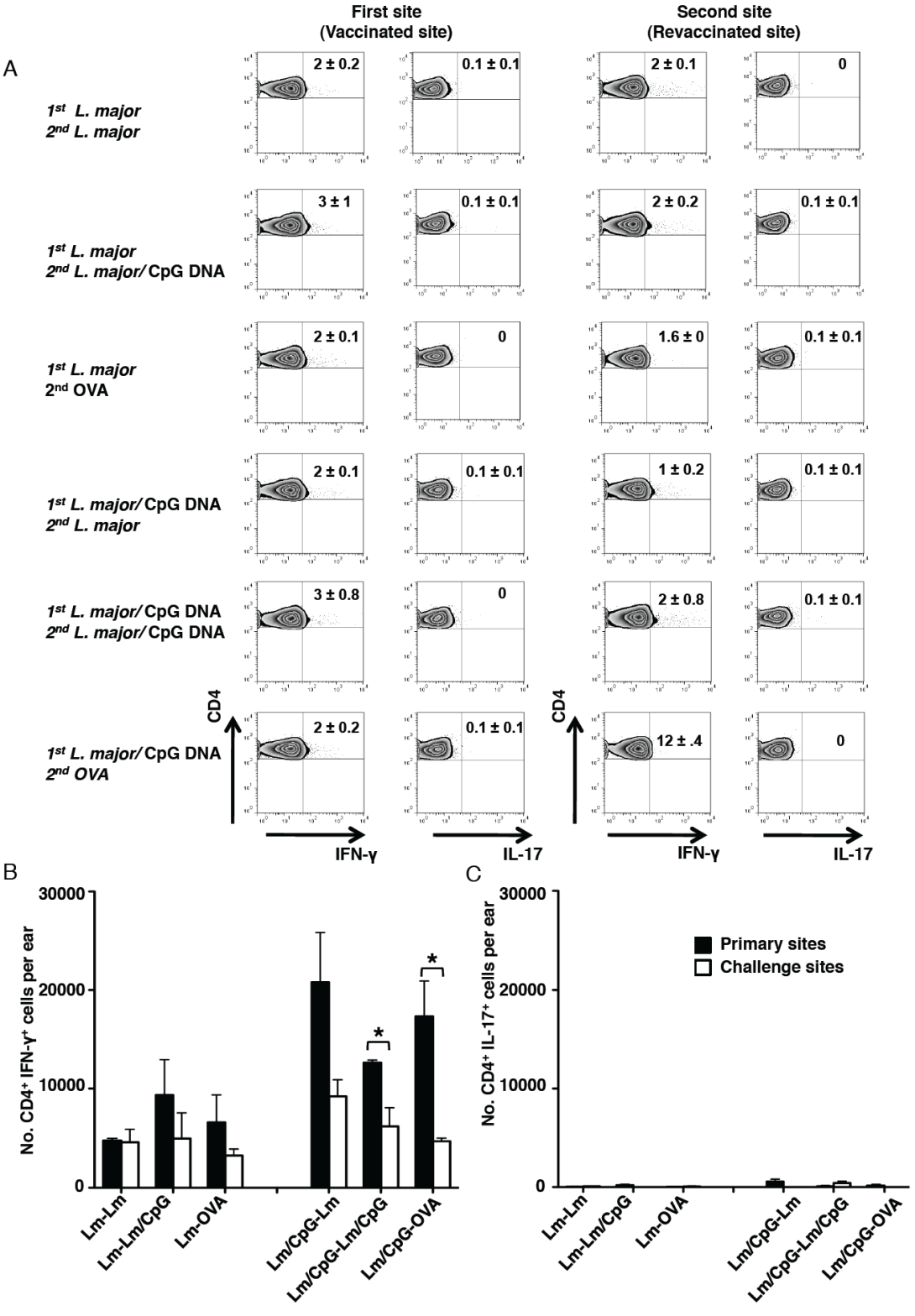


Figure B4. Revaccinated mice did not develop lesions or increase parasite numbers in ears. Mice were vaccinated with *L. major* alone or with the CpG DNA in one ear. At 20 weeks, vaccinated mice were inoculated with 10^4 parasites alone (Lm), with the CpG DNA (Lm/CpG), or with 50 μg OVA in the opposite ear. (**A**) Lesion size at challenge ear was followed up to 14 weeks. Data are expressed as mean ± SEM, three mice with n=6 ears per group. (**B**) Parasite numbers at day 3 after revaccination per ear, expressed as geometric mean ± SEM, six ears per group. * $P < 0.01$.

Figure B5. Frequency and numbers of IFN-γ and IL-17 producing $CD4^+$ memory T cells in lymph nodes. Mice vaccinated with *L. major* alone or with CpG DNA in one ear were inoculated with 10^4 parasites alone (Lm), with CpG DNA (Lm/CpG), or with 50 μg OVA in the opposite ear at 20 weeks post vaccination. Three days after injection, IFN-γ and IL-17 expression by $CD4^+$ memory T cells extracted from draining lymph nodes were analyzed on gated cells by flow cytometry (three mice per group). (**A**) Flow cytometry plots showing the frequency of IFN-γ and IL-17 producing $CD4^+$ effector memory T cells ($TCR\beta^+CD44^+CD62L^{low}$) in both vaccinated and revaccinated lymph nodes. Data are expressed as mean ± SEM from three independent experiments. (**B**) and (**C**) Numbers of IFN-γ and IL-17 producing $CD4^+$ memory T cells in both vaccinated and revaccinated lymph nodes. Data are expressed as mean ± SEM from three independent experiments. *$P < 0.05$ (First site versus Second site).


A
First site
(Vaccinated site)
Second site
(Revaccinated site)
1st L. major
2nd L. major
2 ± 0.2
0.1 ± 0.1
2 ± 0.1
0
1st L. major
2nd L. major/ CpG DNA
3 ± 1
0.1 ± 0.1
2 ± 0.2
0.1 ± 0.1
1st L. major
2nd OVA
2 ± 0.1
0
1.6 ± 0
0.1 ± 0.1
1st L. major/ CpG DNA
2nd L. major
2 ± 0.1
0.1 ± 0.1
1 ± 0.2
0.1 ± 0.1
1st L. major/ CpG DNA
2nd L. major/ CpG DNA
3 ± 0.8
0
2 ± 0.8
0.1 ± 0.1
1st L. major/ CpG DNA
2nd OVA
2 ± 0.2
0.1 ± 0.1
12 ± .4
0
CD4
IFN-γ
IL-17
B
No. CD4+ IFN-γ+ cells per ear
30000
20000
10000
0
Lm-Lm
Lm-Lm/CpG
Lm-OVA
Lm/CpG-Lm
Lm/CpG-Lm/CpG
Lm/CpG-OVA
*
*
C
No. CD4+ IL-17+ cells per ear
Primary sites
Challenge sites

Discussion

Compelling and accumulating evidence suggests that Th17 cells have evolved to mediate protective immunity against a variety of pathogens, primarily at mucosal sites. Our own data (7) and work by others (25-30) support the view that Th17 cells may be crucial players in the generation of vaccine-induced protective responses against pathogens. We recently published that intradermal vaccination of mice with a combination of *L. major* and CpG DNA modifies the immunological features of leishmanial infection to cause de novo expansion of Th17 cells in the skin (7). After our work was published, other authors have confirmed a role for Th17 cells in *L. major* infection (31) and as part of protective responses in vaccines against *L. infantum* (32). Th17 cells, however, have also been found to be present at the site of inflammation in some human inflammatory and autoimmune diseases (33). In particular, the presence of IL-17 in lesional skin in humans and data from mouse models suggest that IL-17 is pivotal in the pathogenesis of certain skin conditions such as psoriasis and contact hypersensitivity (21). Hence, it is paramount that we investigate whether our Th17 inducing live vaccine would establish a chronic population of this potentially pathogenic population in the skin.

In this report, we have demonstrated that our live vaccine adjuvanted with CpG DNA did not cause the establishment of memory Th17 cells in the vaccination site or the draining lymph nodes. We have also shown that revaccination with live vaccines containing *L. major*, or inoculation of other antigens such as OVA, did not cause a recall increase of Th17 cells either, confirming that Th17 responses are transient and rapidly controlled following its initial expansion. We have hypothesized that, in our

model, Th17 cells may be negatively regulated immediately after their expansion by a sustained vaccine-induced production of IL-2 to favor the expansion and survival or regulatory T cells (6, 7). How attrition of this population of inflammatory cells takes place, and the factors involved, deserve further investigation.

While no IL-17 producing cells were detected, we observed however an expansion of IFN-γ^+ $CD4^+$ memory T cells following revaccination with specific anti-leishmanial vaccines or inoculation of irrelevant antigens such OVA. We have already reported that the proportion of effector cells increased during the effector immune response (24), so this expansion may have been a consequence of a transient recall response to an injection in the skin. Moreover, it is likely that these IFN-γ producing, activated effector memory cells would also produce other cytokines such as IL-2, contributing to the negative regulation of Th17 development in the skin.

The potential for inflammatory and paradoxical complications should always be considered in the rational design of new vaccines, especially in the case of patients suffering other clinical conditions or comorbidities. The data shown here specifically demonstrate that potentially pathological Th17 cells are not chronically established at the site of *L. major* infection of vaccination, and it would be safe to use in patients predisposed to Th-17 mediated proinflammatory conditions.

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