REGULATION OF IMMUNE RESPONSE TO THE MUSCLE STAGE OF THE PARASITIC NEMATODE *TRICHINELLA SPIRALIS*

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REGULATION OF IMMUNE RESPONSE TO THE MUSCLE STAGE OF THE PARASITIC NEMATODE *TRICHINELLA SPIRALIS*

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The parasitic nematode *Trichinella spiralis* is a natural pathogen of rodents and humans, exhibits a broad host and geographic range, and has served as a valuable model for the study of mucosal immunity. Little is known, however, of the cellular immune response to the chronic and disseminated stage of the parasite that resides within individual, striated skeletal muscle cells. Despite a potent humoral response to muscle stage parasites, there is limited cellular infiltration of infected muscle. We hypothesized that suppression of inflammation was an active process mediated by T helper type 2 (TH2) responses that are characteristic of helminth infections. To address this hypothesis we conducted *in vivo* experiments using a synchronous model of infection that bypassed the intestine and allowed us to examine the immune response specifically to muscle-stage parasites. We utilized transgenic mice, adoptive transfer of specific cell populations, flow cytometry, cytokine analysis, histology and immunohistochemistry to characterize the cellular infiltrate that is recruited to infected muscle, identify molecules that modulate this inflammatory response, and finally, to delineate the mechanisms of effector T cell suppression during infection.

The results presented here show that the inflammatory response to muscle larvae is rich in macrophages and CD4 T cells, peaks in intensity at the completion of parasite maturation, and is rapidly down modulated

coincident with a shift to T helper type 2 (TH2)-driven response. Our studies in interleukin-10 deficient mice revealed a critical role for this cytokine in controlling inflammation during parasite development, but showed that it is not required during chronic infection. In the absence of IL-10, antigen-specific interferon-γ (IFN-γ) production and local synthesis of inducible nitric oxide synthase (iNOS) increased dramatically, while TH2 cytokines were unaffected. Our adoptive transfer studies showed that effector T cells were the critical source of IL-10 and are sufficient to protect mice from myositis. In contrast, we found that naturally-occurring regulatory T cells inhibit TH2 responses but do not influence local inflammation. Finally, we provide evidence that, in the absence of IL-10, the pluripotent cytokine transforming growth factor-β (TGF-β) is critical in limiting myositis and protecting parasites in the muscle.

BIOGRAPHICAL SKETCH

Daniel Patrick Beiting, the youngest of three children, was born in Chillicothe, Ohio on November 6, 1977 to Otto and Katherine Beiting. He grew up in the city of Wellston and attended grade school at Saint Peter and Paul Catholic School. At age 10, his family moved to a farm outside of town where his father grew wheat, corn, soybeans and raised cattle. Daniel attended high school in the neighboring city of Jackson, where his mother was a speech pathologist for the county school system. Upon graduation, he moved to Boone, North Carolina to pursue a degree in Exercise Physiology at Appalachian State University. Daniel arrived at Cornell University to take a position as a research technician in 1999 and began his graduate degree in 2001 in the laboratory of Judy Appleton.

This dissertation is dedicated to my parents, Katherine and Otto Beiting.

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CHAPTER ONE

Introduction and literature review

A. Immunity to helminth infections

1. Helminth-induced T helper type 2 (TH2) responses

Parasitic helminths include members of the phyla Nematoda (roundworms), Cestoda (flat worms) and Trematoda (flukes). It is widely accepted that helminth species induce polarized T helper type 2 (TH2) responses characterized by elevated levels of interleukin-4 (IL-4), IL-5 and IL-13, high titers of IgG1 and IgE, and expansion of specific cell subsets including basophils, mast cells and eosinophils. Uniformity of the immune response to helminths suggests a common mechanism of T cell polarization. Consistent with this notion, there is accumulating evidence that glycan moieties modifying surface and excreted/secreted (ES) helminth proteins are important inducers of TH2 responses. For example, surface proteins from eggs of the trematode *Schistosoma mansoni* are modified by lacto-N-fucopentaose III (LNFP-III), a sugar also found in human milk (37, 87). This glycan stimulates dendritic cells through toll-like receptor 4 (TLR4) (165, 166) to prime TH2 responses (40, 119, 120). Even glycoproteins of the free-living nematode *Caenorhabditis elegans* generate antigen-specific IL-4 producing T cells in lymph nodes of immunized mice (161), suggesting the presence of highly conserved TH2-inducing molecular signatures associated with helminth parasites.

2. Consequence of helminth-induced TH2 responses

It is clear that TH2 responses are protective against intestine-dwelling helminth parasites (41). IL-4 and IL-13 promote mastocytosis (39), smooth

muscle hypercontractility (1), goblet cell hyperplasia, and mucus secretion that are critical to the timely expulsion of nematodes from the gut (36, 172, 173).

Helminth parasites commonly have tissue migratory phases and occupy more than one anatomical niche during the life-cycle (114), and less is known about protective responses against parenteral stages of parasites, where the pathogen must often be destroyed rather than simply displaced from its habitat. For example, during *S. mansoni* infection, cercariae penetrate the skin of the host, develop into schistosomulae and migrate through the dermis to blood vessels (106, 182). Vaccination of mice with irradiated cercariae provides resistance to a challenge infection. Interestingly, immunity is elicited by vaccines that induce either TH1 or TH2 responses (5, 19, 69), and is amplified by a strong, but mixed TH1/TH2 response (69).

There is evidence that TH1 responses may be effective in killing larval stages of parasites (139, 169). When eggs of the tapeworm *Taenia solium* are ingested, oncospheres hatch in the intestine and migrate to muscle where they establish chronic infection as cysticerci. Studies in a murine model of cysticercosis show that elevated TH2 responses during chronic infection correlate with increased parasite load in susceptible BALB/c mice (163, 168). Conversely, resistant C57BL/6 mice, mount a protective TH1 response that can be reversed by administration of neutralizing antibodies to IFN-γ (164) or gene deficiency in Signal Transducer and Activator of Transcription-4 (STAT4) (140). TH2 responses also have the potential to kill parasites. For example, exaggerated TH2 cytokine production is associated with enhanced clearance of the filarial parasite *Litomosoides sigmodontis* from the thoracic cavity (162). In addition, gene deficiency in IL-4 is sufficient to confer susceptibility to *L. sigmodontis* in otherwise non-permissive C57BL/6 mice (93). Helminth-

induced TH2 responses include elevated levels of IL-5 that promote expansion and survival of eosinophils. There are conflicting reports in the literature regarding the importance of these cells in parasite killing (reviewed in (90)), but there is evidence that eosinophils may protect against reinfection (64, 94, 131), possibly by trapping larval stages at sites of inoculation (24).

B. Mechanisms of Immune suppression induced during helminth infection

1. Regulatory T cells in helminth infection

Chronic infection is a common feature of disease caused by parasitic helminths. Although the mechanisms that allow parasite persistence are complex, it is becoming clear that there is important interplay among certain host mediators, including T cells that exhibit regulatory properties, and suppressive cytokines such as IL-10 and transforming growth factor-β (TGF-β) (2, 88, 154).

The concept of suppressor T cells was supported by research conducted over 25 years ago (34, 48, 55), but lack of a definitive phenotypic marker confounded their study. In 1995, Sakaguchi and colleagues identified a population of small CD4+ T cells that constitutively express the IL-2 receptor alpha-chain (CD25) and suppress a range of autoimmune diseases (Treg cells, (145)). More recently, the transcription factor Foxp3 was identified as a definitive Treg marker (45, 70) that is required for suppressor cell development (46). Identification of Foxp3 and CD25 as Treg markers was critical in directing mechanistic studies of these cells, and has led to the current appreciation of a distinct, thymically-derived Treg population that is

CD4+CD25+Foxp3+ and comprises 5-10% of the CD4 T cell compartment (76) ("naturally occurring" Tregs). Tregs are functionally defined by their ability to limit the activity of effector T cells. In this context, a variety of T cell subsets would qualify as being "regulatory" based on surface expression of inhibitory molecules such as cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) (133) or Glucocorticoid-induced tumor necrosis factor-related protein (GITR) (79), or secretion of any number of regulatory cytokines, particularly IL-10 and TGF-β. Even conventional TH2 and TH1 cells are potent suppressors of immune function by virtue of their cytokine profiles that inhibit the opposing phenotype (112).

Mechanistic studies of Tregs have, for the most part, been limited to autoimmune disease models where these cells suppress TH1-mediated pathology by mechanisms that include IL-10 (9, 101) and surface-bound or secreted TGF-β (117). Much less is known of how these cells may facilitate chronic infection by parasitic helminths. Peripheral blood mononuclear cells (PBMCs) from humans infected with the filarial parasite *Onchocerca volvulus* produce little IL-2 or IL-4, but abundant IL-10 and TGF-β, and suppress other T cells *in vitro* (31, 147). In a mouse model of filariasis, Treg cells suppress TH2 responses by an IL-10-independent mechanism. *In vivo* administration of anti-CD25 and anti-GITR reverses this suppression, resulting in elevated TH2 cytokines and enhanced parasite clearance (162). In contrast, during *S. mansoni* infection, Treg cells, effector T cells and innate cells all contribute IL-10 that limits TH1, but not TH2, responses during during egg deposition (66, 105).

2. The influence of IL-10 on cells of the immune system

IL-10 is a 36-kDa homodimer (110) that was first identified as a product of TH2 cells and was named cytokine synthesis inhibitory factor (CSIF) (42) because of its ability to inhibit cytokine production by TH1 cells. It is now well established that IL-10 interferes with T cell function both directly, through inhibition of T cell IL-2 production (26, 158); and indirectly, by inhibiting production of cytokines (43, 122), nitric oxide (23, 50), MHCII (135), and co-stimulatory molecules (30) in macrophages. Unlike other cytokines, transcription of the IL-10 gene is controlled by the ubiquitously and constitutively expressed Sp1 and Sp3 transcription factors (170). These DNA binding proteins also regulate essential "housekeeping" functions that include DNA methylation (16) and cell cycle progression (56). A large number of cell types have been reported to synthesize IL-10 (25, 42, 61, 84, 109, 116). The 3'-untranslated region of IL-10 mRNA includes multiple copies of AUUUA destabilizing motifs that allow posttranscriptional regulation of the cytokine (129). Thus, although IL-10 is readily transcribed in a variety of cell types, protein production is controlled at the mRNA level. Once synthesized and secreted, IL-10 binds to the high-affinity type 1 receptor (67) and subsequently associates with a low-affinity type 2 receptor through which it signals (91). Suppression of antigen presenting cells by IL-10 is mediated mainly through activation of the transcription factor STAT3 (183) and requires *de novo* protein synthesis (14). The mechanisms by which IL-10 signaling results in suppression are not fully understood, but activation of suppressor of cytokine signaling-3 (SOCS3) has been shown to be partially responsible for the effects of IL-10 (12, 183).

During helminth infection, IL-10 has been shown to be critical in limiting inflammation and tissue damage. For example, IL-10 deficient mice are unable to control infection by the intestinal helminth *Trichuris muris*, resulting in delayed expulsion, increased inflammation, and death due to sepsis (149). During infection with *S. mansoni*, eggs that are normally bound for excretion in the feces can become lodged in the liver and intestine. A macrophage-rich infiltrate sequesters eggs, providing a barrier between the irritant and the host tissue (104, 143, 171). This response, although protective in design, often leads to significant fibrosis, tissue destruction and sometimes death due to portal hypertension (121). Velupillai and collegues demonstrated that LNFP-III from *S. mansoni* eggs induced peritoneal B-1 cells to produce IL-10, but not IL-4 (175, 176). Additional studies have shown that IL-10, produced by cells of both the innate and adaptive immune system, serves as a critical regulator of the pathogenic immune response to *S. mansoni* eggs (66, 68, 186). At the onset of egg deposition, limiting TH1 responses is critical in inducing granuloma formation and protecting the liver parenchyma from toxic effects of the eggs (124); however, uncontrolled or exaggerated TH2 responses are also detrimental, leading to increased fibrosis and impaired liver function (68). Recently, IL-10 has also been identified as a major product of Treg cells (60, 128) as well as a subset of B cells that modulate intestinal inflammation (109) and allergic responses (102). Taken together, these observations support a role for IL-10 as a key regulator of inflammation through down-modulation of cellular immune responses.

3. The effects of TGF-β on cells of the immune system

TGF-β was originally identified as a soluble factor in the supernatants of virus transformed cells that promoted anchorage-independent cell growth in soft-agar (7). Subsequent studies revealed that this molecule could be produced by T lymphocytes and had potent anti-proliferative properties *in vitro* (86). Targeted disruption of the TGF-β gene in mice results in massive lymphoproliferation and death by three weeks of age (151). Unlike IL-10, TGF-β is regulated by extensive posttranslational modification. TGF-β is synthesized as an inactive pre-pro-protein (126). After cleavage of the signal peptide (pre-domain), the large pro-domain is removed by a furin convertase in the golgi (33). Liberated pro-domains dimerize, forming a latency-associated protein (LAP), and associate with dimers of mature TGF-β to form a biologically inactive molecule (51) termed the small latent complex (SLC). The SLC is either directly secreted or first modified by the binding of a latent TGF-β-binding protein (LTBP) to form a macromolecule termed the large latent complex (LLC). The LTBP is believed to be important in targeting TGF-β to the extracellular matrix, thereby generating a reservoir of inactive cytokine that can be rapidly mobilized during immune responses (6). Both the LAP and LTBP must be proteolytically removed to allow binding of TGF-β to its receptor, and signaling via intracellular Smad molecules.

TGF-β inhibits T cell proliferation by blocking IL-2 gene transcription (15) and, independent of this property, also inhibits the differentiation of both CD4+ and CD8+ T cells to acquire effector function (132, 144, 157). This suppression affects only naïve cells (99), and seems to have the strongest influence on TH2 cells. Naïve cells are rendered unresponsive to TGF-β in the presence of IFN-γ (144). Recent data shows that TGF-β drives the expansion

of Tregs *in vivo* (71) and, as a surface-bound molecule, may facilitate contact-mediated suppression by this cell type (117). Tregs have been shown to produce both IL-10 and TGF-β (89) and there is evidence that IL-10 potentiates the activity of TGF-β by inducing the upregulation of TGF-β-receptor type II on activated T cells (21). The immune modulatory properties of TGF-β, combined with its upregulation during nematode infections (31, 147), implicates this molecule as an important player in chronic infection.

TGF-β has a wide range of biological activities and is found in nematodes as well as higher order mammals. Free-living nematodes arrest development and enter a dauer stage under conditions of stress, allowing long-term survival in the environment even when nutrients are limited. This process is regulated in part by nematode TGF-β (136). It has long been recognized that the dauer stage of free-living nematodes is similar to the infective stage of many parasitic nematodes (177). Orthologues of mammalian TGF-β, have been identified in the filarial parasites *Brugia malayi* (52) and *B. pahangi* (53, 54), as well as the parasitic nematodes *Ancylostoma caninum* (47), *Strongyloides ratti* and *Parastronglyloides trichosuri* (22). TGF-β synthesized by *B. malayi* can bind and signal through the mammalian TGF-β receptor (52). This raises the possibility that host/parasite TGF-β-like molecules and signaling pathways may be exploited to direct parasite development and/or manipulate the host immune response (8).

4. Nematode induced changes in antigen presenting cell populations

The monocyte/macrophage lineage is a heterogeneous cell population with tremendous flexibility in phenotype and function. Macrophages that

engage bacterial ligands through TLRs and other pattern recognition receptors produce proinflammatory cytokines IL-12, TNF-α, IL-1 and IL-6, and demonstrate elevated expression of MHCII and costimulatory molecules. These classically activated macrophages (113) mediate effective killing of phagocytosed bacteria through the metabolism of L-arginine by inducible nitric oxide synthase (iNOS) to generate reactive nitrogen intermediates. Conversely, IL-4 and IL-13 produced during nematode infections are key signals in the alternative activation of macrophages (aaMA) (141, 152), characterized by the production of arginase, a competitive inhibitor of iNOS; and proline an essential component for collagen synthesis in the process of tissue fibrosis (65).

Recent studies have shown that parasite products directly promote the acquisition of the aaMA phenotype. Thioredoxin peroxidase released by *Fasciola hepatica* stimulates macrophages to produce high levels of IL-10 and prostoglandin E_2 *in vitro*, and induces the expansion of arginase1+Fizz1+ cells *in vivo* (32). Similarly, *S. mansoni* LNFP-III expands a macrophage population that produces IL-10 and TGF-β, yet suppression of T cell proliferation by these aaMA required IFN-γ and nitric oxide, with only a minor dependency on IL-10 (Terrazas, 2001 #1135). aaMA recruited during *B. malayi* infection inhibit cell proliferation by a contact-dependant mechanism (98), and promote TH2 differentiation of naïve T cells *in vivo* (97). Similarly, macrophages isolated from *S. mansoni* granulomas produce anti-inflammatory cytokines IL-10 and IL-1 receptor antagonist, and fail to upregulate surface MHCII or costimulatory molecules. *In vitro*, these cells render egg-specific TH1 cells unresponsive to restimulation, while retaining the ability to stimulate a TH2 response (44). The importance of aaMA in immunity is only beginning to be examined *in vivo*.

Mice with macrophage/neutrophil-specific deletion of the IL-4 receptor alpha-chain show impaired aaMA generation, and develop extensive intestinal lesions during *S. mansoni* egg deposition (63).

It is important to consider that as nematodes migrate through tissue, they disrupt and likely liberate extracellular matrix (ECM) components. Host proteins released during tissue damage also activate macrophages and dendritic cells. Tissue trauma has been shown to expand arginase-producing macrophages that inhibit CD3/CD28-mediated T cell proliferation and IL-2 production. This suppression could be reversed with the addition of arginase antagonists or extra L-arginine to cultures (100). Heparan sulfate, a major component of ECM, induces a TLR4-dependent upregulation of CD80, CD86, CD40 and MHCII on the surface of dendritic cells (80). Moreover, the extra domain A of fibronectin stimulates the human monocyte/macrophage cell line THP-1 to produce MMP-9, and induces splenocytes to produce IL-10, both in an TLR4-dependent manner (118).

There is little information about the influence of ECM components on the immune response to tissue-migrating parasites. Studies with heparan sulfate-containing proteoglycans (HSPG) purified from *S. mansoni* granulomas showed that these molecules promote GM-CSF and IL-3-dependent growth and differentiation of the myeloid cell line FDC-P1 (4, 77). The proteoglycan syndecan-1 anchors cells to the ECM (146) and, when shed from the cell surface, limits TH2 responses in a model of lung allergy induced by *Aspergillus* antigen (187). The ability of syndecan-1 and its heparan sulfate chains to directly modulate antigen presenting cells holds particular interest in the context of this dissertation, as we have shown that syndecan-1 is synthesized by muscle cells infected with *Trichinella spiralis.*

C. Using helminths to suppress autoimmune and allergic responses

Early epidemiological studies revealed a correlation between exposure to microbial agents and reduced risk for developing allergy and autoimmune diseases (153). Experimental evidence now supports this so-called "hygiene hypothesis," and clinical trials have demonstrated that helminth infection can suppress the symptoms of Crohn's disease (155) and ulcerative colitis (156). The role of IL-10 in mediating this protection is controversial. The fact that IL-10 deficient mice spontaneously develop colitis is a clear indication of importance of this cytokine in limiting intestinal pathology (92). Moreover, tapeworm infection in mice provides protection from chemically-induced colitis, an effect that is reversible by *in vivo* neutralization of IL-10 (73). In contrast, suppression of experimental colitis by infection with *Heligmosomoides polygyrus* occurs independently of IL-10 (35). T cells residing in the lamina propria of *H. polygyrus* infected mice upregulate TLR4 and respond to LPS by producing TGF-β (74), providing a direct link between helminth infection and altered T cell activity resulting in suppression.

Chronic nematode infection is also associated with suppression of T cell responses to allergens (11, 174). Eosinophilic inflammation and airway hypersensitivity are nearly abolished in a mouse ovalbumin sensitivity model when extracts of the nematode *Ascaris suum* were co-administered during allergen challenge (96). It has been suggested that helminth-induced IgE may saturate mast cell Fc receptors, thereby preventing sensitization by allergen-specific IgE, yet experimental evidence does not support this hypothesis (107). Again, the role for IL-10 in mediating tolerance to allergens has been investigated, yielding contradictory results. Neutralization of IL-10 activity *in vivo* abrogates helminth-mediated suppression of anaphylaxis (102) and food

allergy in mice (11). In a recent and detailed study of helminth-mediated suppression of airway inflammation, *H. polygyrus* was shown to induce expansion of Treg cells that produce IL-10 and TGF-β. While Tregs were necessary and sufficient to transfer protection from sensitivity to dust-mite allergen, Treg-derived IL-10 was not required for this effect (184). It is important in the context of this dissertation that TGF-β can regulate allergic airway disease (59, 167), and may be a major mediator of helminth-induced immune suppression.

D. *Trichinella spiralis* muscle infection as a model to study helminth-mediated immune suppression

1. Establishment of muscle infection

Trichinella spiralis is a zoonotic pathogen that has evolved to infect a wide range of mammalian hosts. Transmission occurs when muscle contaminated with infective, first-stage larvae is consumed, the parasite is released from muscle by digestive enzymes in the host stomach and subsequently invades the epithelium of the small intestine, where it matures, mates and reproduces. Eggs hatch *in utero* and newborn larvae (NBL) are released by female worms in the epithelium, migrate to the lamina propria, enter mesenteric venules, transit the liver and disseminate throughout the host (180, 181). Muscle infection is initiated when NBL extravasate from blood vessels and invade individual, terminally differentiated muscle cells (myotubes) (28). Over a period of 20 days (28), modification of the infected myotube by the parasite induces reentry into the cell cycle (78), remodeling of the cytoplasmic matrix (29), synthesis of a collagen capsule (137), and

formation of a capillary rete around the altered cell (72). Genetic reprogramming during the early stages of nurse cell formation reflects these structural changes: muscle-specific transcripts fall to near undetectable levels (78), vascular endothelial growth factor (VEGF) genes are activated (18), and collagen transcripts are increased (127). These dramatic morphological and biochemical changes in the host cell provide a supportive, long-term, intracellular habitat for the larva, constituting a structure referred to as a nurse cell (130) (Figure 1.1). Once the parasite completes development in the muscle, larvae remain infectious for years (49).

2. Cellular response during muscle infection

Research on muscle infection with *T. spiralis* has focused on elucidating the series of changes that the host muscle cell undergoes following infection. Although early histologic studies of infected muscle revealed a focus of inflammation surrounding individual infected muscle cells (179), the kinetics and composition of the infiltrate were ill-defined. Similar to a granuloma, the focal infiltrate surrounding individual nurse cells is rich in macrophages. An immunohistochemical study of masseter muscle from orally infected mice revealed a peak of the Infiltrating macrophages, making up approximately 50% of the infiltrate, at 21 dpi. By 28 dpi the infiltrate had receeded and macrophages comprised only 20% of the infiltrate (82). These cells were also observed in the cytoplasm of the nurse cell (83).

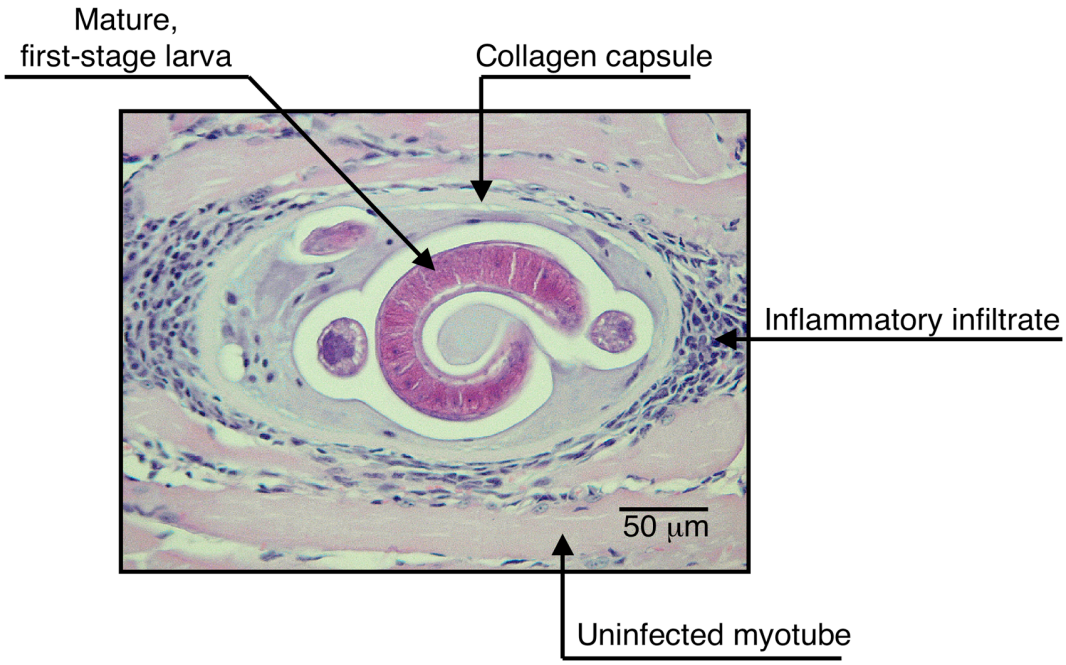

Figure 1.1 The *T. spiralis* nurse cell. Formalin-fixed, paraffin-embedded diaphragm from a C57BL/6 mouse at 20 days post-infection was stained with hematoxylin and eosin. Relevant features of the nurse cell are indicated by arrows.

The phenotypic and functional attributes of muscle infiltrating macrophages have not been described. Macrophages recovered from the peritoneum of mice orally infected with *T. spiralis* were able to inhibit the proliferation of EL-4 tumor cells *in vitro*, and failed to control growth and proliferation of the intracellular pathogen *Toxoplasma gondii* (185). More recently, the gene encoding Ym1, a protein chemotactic for eosinophils and associated with alternative activation, was found to be upregulated in peritoneal macrophages from *T. spiralis* infected mice at 15 dpi, a time coincident with establishment of parasites in the muscle (20). Taken together, these data suggest that *T. spiralis* infection elicits an alternative activation of macrophages, but the consequence of macrophage activity on the outcome of muscle infection is not understood.

T. spiralis muscle larvae secrete a complex mixture of glycoproteins and, although it is tempting to speculate that these molecules may influence the activity of infiltrating leukocytes, there is no direct evidence that such effects occur. Proteomic analysis of excreted/secreted (ES) products has identified numerous serine proteases (115, 138, 142); however, this class of molecules is not generally associated with alteration of macrophage or T cell activity. In contrast, a molecule with structural and functional homology to mammalian macrophage migration-inhibitory factor (MIF) has been characterized in secreted products from *T. spiralis*, *T. muris* and *B. pahangi* (123, 125, 160). The main function of mammalian MIF is generally understood to be pro-inflammatory. Neutralization of this cytokine prevents proliferation and IL-2 production *in vitro* (10) and rescues mice from septic shock *in vivo* (17). Interestingly, when injected into mice, recombinant *B. malayi* MIF potently induced Ym1 gene expression in macrophages and elicited

recruitment of eosinophils to the peritoneal cavity (38). The potential for *T. spiralis* MIF to influence the composition and activity of the cellular infiltrate surrounding infected myotubes needs to be examined.

A recent and extensive analysis of expressed sequence tags (ESTs) from the different life-stages of *T. spiralis* has provided insight into possible mechanisms of parasite persistence and immune evasion in muscle. EST clusters encoding serine proteinase inhibitors (serpins) and cysteine proteinase inhibitors (cystatins) were widely represented in NBL but not mature muscle larvae or adult parasites (108). *B. malayi and O. vulvulus* also secrete cystatins, and the recombinant form of these molecules inhibits antigen presentation on MHCII (103), suppresses proliferation of PBMC and elicits IL-10 production (148). Finally, several EST clusters encoding antioxidants were found primarily in mature *T. spiralis* muscle larvae and not in NBL (108). This is supported by *in vitro* evidence that only NBL are highly susceptible to oxidative killing (85) and suggests that larvae could be destroyed in muscle if infiltrating cells release reactive oxygen intermediates during parasite development.

3. Systemic responses during muscle infection

The antibody response to *T. spiralis* infection has been studied extensively in orally infected mice. Almond and Parkhouse documented a dramatic increase in IgG1 and IgG2, specific for muscle larva surface proteins, during chronic muscle infection in mice (3). 80% of IgG1 specific for larval antigens recognizes a single shared epitope (27), now known to be the highly immunogenic sugar, tyvelose (134). In addition to IgG, *T. spiralis* infection

also induces high levels of parasite-specific IgE (188) that bind to tyvelosylated surface antigens on encysted larvae (57, 58). The dominance of parasite-specific IgG1 and IgE during chronic *T. spiralis* infection is consistent with a polarized TH2 response during the muscle stage of infection, (IL-4 drives isotype switching to IgG1 and IgE (81, 150, 159)); however, oral infection complicates any firm conclusion regarding the isotype response specifically to muscle stage parasites.

T cell deficient (nude) mice fail to form a focal inflammatory response to the nurse cell, identifying these cells as the coordinators of the cellular response to muscle infection (179). Little is known however of the mechanisms by which T cells regulate inflammation during *T. spiralis* muscle infection. Cytokine responses during intestinal infection with *T. spiralis* are well documented (75, 178), yet cytokine secretion profiles of cells recovered from draining nodes during muscle infection are poorly studied. NBL injected into thigh muscle of BALB/c mice activate poplitial lymph node cells that produce IL-4 when stimulated *ex vivo* with the mitogen concanavalin A (95). PBMC recovered from human subjects over a year after a recorded outbreak of trichinosis produced significant quantities of IFN-γ, IL-10 and IL-5, and retained the ability to proliferate in response to somatic antigens from muscle larvae for as long as three years after infection (111). Collectively, these data suggest that *T. spiralis* induces a mixed response, but a more thorough examination of the T cell response to muscle infection is needed.

The influences of IL-10, TGF-β and Treg cells on the inflammatory and cytokine response to muscle stage *T. spiralis* have not been investigated previously. A study by Helmby et al. demonstrated that IL-10 limited both TH1 and TH2 responses during intestinal *T. spiralis* infection (62). Enhanced IFN-γ

levels promoted killing of migrating NBL, an effect that could be reversed with neutralizing antibody (62). In addition, IL-10 protects mice from the development of necrotic lesions induced by NBL as they transit the liver on their way to skeletal muscle (13). Collectively, these data suggest that IL-10 induced during *T. spiralis* infection may support parasite survival while protecting host tissue from detrimental inflammation.

5. The T. spiralis model of immune modulation

Unregulated inflammation underlies a wide range of serious illnesses including allergy, Crohn's disease, rheumatoid arthritis and multiple sclerosis. Reducing inflammation has been a strategy for developing treatments for these diseases. Inflammation of skeletal muscle (myositis) in patients afflicted with myasthenia gravis, muscular dystrophy or polymyositis compromises tissue integrity and exacerbates disease symptoms. The muscle phase of *T. spiralis* infection is characterized by a tight regulation of the host inflammatory response, providing insight into regulatory mechanisms that prevent excessive myositis.

Despite a potent and persistent B cell response during muscle infection, the inflammatory response to the *T. spiralis* nurse cell remains limited, suggesting that parasite and/or host factors suppress local T cell responses in order to limit local inflammation. *T. spiralis* infection of host muscle affords a relevant model to study the mechanisms by which nematodes modulate the host cellular response during chronic infection. The studies presented in this dissertation address the mechanism of suppression by using a synchronous model of infection, allowing us to limit our

analysis of the immune response to muscle stage parasites. Using this method, we are able to take a controlled, mechanistic approach to understanding how IL-10, TGF-β and Treg cells influence the balance of parasitism and cell-mediated immune responses.

E. Objectives and organization of dissertation

The overall goal of this study was identify host factors that limit the inflammatory response to muscle stage *Trichinella spiralis*. We hypothesized that IL-10 and TH2 cytokines would contribute to control of local inflammation. To address this hypothesis, we first used histopathology, immunohistochemistry, flow cytometry and ELISA to evaluate the cellular and humoral response to infection in wild-type and IL-10 deficient mice (Chapter 2). In the process of carrying out these studies we made the incidental observation that parasitized muscle cells produce the proteoglycan syndecan-1 and fail to export it to the cell membrane (Chapter 3). This was a departure from our IL-10 work, but provided us with the unique opportunity to examine, for the first time, the importance of a specific nurse cell protein in parasitism and the host immune response to *T. spiralis*. Finally, we expanded our investigation of T cell suppression during muscle infection by evaluating the interplay among IL-10, TGF-β, Treg cells and TH2 responses on local inflammation and parasite survival (Chapter 4). The dissertation closes with a discussion of the results obtained in this study (Chapter 5).

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CHAPTER TWO

IL-10 limits local and body cavity inflammation during infection with muscle stage *Trichinella spiralis*[*]

[*] Daniel P. Beiting, Susan K. Bliss, Donald H. Schlafer, Victoria L. Roberts, Judith A. Appleton. IL-10 limits local and body cavity inflammation during infection with muscle stage *Trichinella spiralis*. Infection and Immunity 2004; 72(6): 3129-3137

Summary

The aim of this study was to characterize cellular responses to muscle stage *T. spiralis*. From its intracellular habitat in muscle, *T. spiralis* secretes potent glycoprotein antigens that elicit a strong systemic host immune response. Despite the magnitude and prolonged nature of this response, nurse cells are rarely destroyed by infiltrating cells. We tested the hypothesis that the anti-inflammatory cytokine interleukin-10 (IL-10) moderates cellular responses to muscle stage parasites. *Trichinella* larvae colonize the diaphragm in large numbers, prompting us to evaluate regional responses in body cavities in addition to local responses in muscle.

Mice deficient in IL-10 demonstrated an exaggerated inflammatory response around nurse cells and in the pleural cavity. The effect of IL-10 was most evident 20 days following muscle infection. The increased intensity of the response in IL-10 deficient mice did not affect parasite establishment or survival. Between 20 and 50 days post-infection, the inflammatory response was diminished in both wild-type and IL-10 deficient mice. Muscle infection also elicited an antibody response, characterized initially by mixed isotypes directed at somatic larval antigens and changing to an IgG1-dominated response directed at tyvelose-bearing excreted/secreted antigens. We conclude that IL-10 limits local and regional inflammation during the early stages of muscle infection, but that chronic inflammation is controlled by an IL-10-independent mechanism that is coincident with a TH2 response.

Introduction

Infection by the parasitic nematode *Trichinella spiralis* occurs when meat contaminated with infective, first-stage larvae is consumed and the

parasite is released from muscle by digestive enzymes in the host stomach. *T. spiralis* invades the epithelium of the small intestine where it matures, mates and reproduces (21). Newborn first-stage larvae (NBL) are released in the epithelium, migrate to the lamina propria and enter capillaries (5). Larvae travel via the bloodstream, eventually entering skeletal muscle where each larva invades a single, terminally differentiated muscle cell (myotube) (19). Over a period of 20 days (19) the parasite modifies the infected myotube by inducing reentry into the cell cycle (34), remodeling of the cytoplasmic matrix (19), synthesis of a collagen capsule (47), and formation of a capillary rete around the altered cell (33). These dramatic morphological and biochemical changes in the host cell provide a suitable long-term habitat for the larva, constituting a structure called the nurse cell (44). Although an individual NBL will infect any striated muscle cell, the diaphragm is a preferred site of infection in rodents (51).

Research on muscle stage *T. spiralis* has focused on elucidating the series of changes that the host muscle cell undergoes following infection (4, 11, 20, 35, 42). The host response to this phase of the infection is not well characterized. Early histologic studies of infected muscle revealed a very limited focus of inflammation surrounding chronically infected muscle cells (24), but the composition and dynamics of the infiltrate remain ill-defined.

The immune system sequesters persistent sources of antigen by establishment of a granulomatous barrier (37, 48, 54). Infections with *Schistosoma mansoni* or *Mycobacterium spp.* are characterized by disease resulting from chronic granulomatous responses to these highly immunogenic pathogens. From its intracellular habitat, *T. spiralis* secretes potent glycoprotein antigens that elicit a strong, systemic host immune response (45),

yet local cellular infiltrates are limited. As a first step toward understanding this modulation, we examined the influence of interleukin-10 (IL-10) during synchronized muscle infections of C57BL/6J (WT) mice and B6.129P2-*IL10*^{tm1Cgn} (IL-10 -/-) mice. Interleukin-10 was first identified as a product of T helper 2 (TH2) cells and was named cytokine synthesis inhibitory factor (CSIF) (26) because of its ability to inhibit cytokine production by T helper 1 (TH1) cells. It is now well established that IL-10 interferes with T cell function indirectly by inhibiting production of cytokines (27, 41), nitric oxide (14, 29), class II MHC (46) and co-stimulatory molecules (23) by macrophages. More recently, IL-10 has been identified as a major product of regulatory T cells (30, 43) as well as a subset of B cells that modulate intestinal inflammation (39).

In this report we describe histochemical, immunohistochemical and flow cytometric findings that identify the constituents and characterize the architecture of the local cellular infiltrate as well as the cellular response in the pleural and peritoneal cavities. In order to avoid any complicating influences of IL-10 deficiency on the intestinal stages of infection, we established synchronous muscle infections by intravenous injection of newborn *T. spiralis* larvae. Our findings reveal a role for IL-10 in limiting inflammatory responses during the early stages of muscle infection by *T. spiralis*. We also provide evidence that sustained control of inflammation during chronic muscle infection is independent of IL-10 and accompanied by a shift to a TH2 response following completion of parasite development in the muscle.

Materials and Methods

Rats and mice

Adult AO strain rats were produced and maintained in the James A. Baker Institute vivarium. Eight-week old C57BL/6J (WT) and B6.129P2-*IL10*^{tmlCgn} (IL-10 -/-) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). IL-10 -/- mice had been back-crossed 10 times onto a C57BL/6J background. Mice were maintained in a Bioclean isolation unit (Lab Products Inc, Seaford, DE), fed autoclaved, pelleted ration (5K67; Jackson Laboratories, Bar Harbor, ME) and acidified water (pH 3). All rodents were housed in the James A. Baker Institute vivarium in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care.

Parasite and antigens

T. spiralis (pig strain) infectious larvae were recovered from muscles of irradiated AO rats by digestion with 1% pepsin in acidified water (13). Rats had been infected at least 28 days prior to collection of larvae. For recovery of adult worms, rats were lightly sedated with ether and inoculated by gavage with 6000 infectious larvae suspended in 0.3 to 0.8 ml of 2% nutrient broth/0.6% gelatin. Six days post-inoculation, infected rats were killed by CO_2 inhalation. Intestines were removed, flushed with saline, opened and incubated for two hours in saline containing antibiotics (200 IU/ml penicillin, 200 μg/ml streptomycin, and 50 μg/ml gentamicin). Adult worms were recovered on a sterile, 75 μm sieve, washed two times with sterile saline containing antibiotics, and cultured for 24 hours in MEM containing 30% FCS, antibiotics and 2mM L-glutamine. Newborn larvae were separated from adult

worms with a sterile, 75 μm sieve. Larvae were washed two times by gentle centrifugation in serum-free MEM.

Excretory/secretory antigen (ESA) was obtained from overnight cultures of muscle larvae as described previously (2). Somatic antigens from muscle larvae were prepared from whole worm homogenate as described previously (3).

Experimental Design

Mice were administered a single intravenous injection (lateral tail vein) of 16,000-26,000 NBL suspended in 0.25 ml serum-free MEM. Infection by this route bypasses the intestinal phase of infection, eliminating the intestinal immune response as a confounding variable. In addition, I.V. injection of NBL results in synchronous development of nurse cells and the host response to muscle infection. Mice were killed by CO_2 inhalation at the times indicated in each experiment.

Total muscle burden estimates and distribution of larvae following parenteral infection

Carcasses of infected mice were skinned, eviscerated, minced and digested in separate flasks containing 200 ml of 1% pepsin-HCL. A minimum of 100 mature L1 were counted from each digestion flask, and total parasite burden was estimated by extrapolation. In some experiments, anatomic distribution of larvae was determined by digesting the head, diaphragm, upper and lower body separately.

Histology

Diaphragm and tongue were fixed in 10% neutral buffered formaldehyde solution for a minimum of 48 hours before embedding in paraffin. Five μm sections were mounted on glass slides and stained with hematoxylin and eosin (H & E).

Immunohistochemistry

Diaphragm and tongue were embedded in Tissue Freezing Medium (TFM; Electron Microscopy Sciences, Ft Washington, PA) and snap-frozen on dry ice. Eight μm sections were prepared (Cryocut 1800; Reichert-Jung, Buffalo, NY), mounted on glass slides and stored at –80 degrees Celsius. Slides were warmed to room temperature and fixed in ice-cold, 100% ethanol for 10 min. All incubations were carried out at room temperature (RT) for 30 min unless otherwise noted. Slides were washed 3 times for a total of 5 min in phosphate-buffered saline (PBS) following each incubation. In order to block endogenous peroxidase activity, slides were immersed in 0.3% H_2O_2 and 1% NaN_3 for 15 min. As a general blocking step, sections were incubated in 10% bovine serum albumin (BSA; Sigma Chemical Co, St. Louis, MO). Rat antibodies were prepared in 10% BSA. Biotinylated rabbit anti-rat antibody (Vectastain® Elite ABC Kit; Vector Labs, Burlingame, CA) was diluted to 3 μg/ml in 10% normal mouse serum (NMS) and 10% normal rabbit serum. Following incubation with the Avidin-Biotin Complex reagent (Vectastain®), sections were incubated for 10 min with metal enhanced diaminobenzidine substrate (DAB; Pearce, Rockford, IL), counter-stained with Gill's #2 hematoxylin (Vector Labs, Burlingame, CA) for 2 min, rinsed in tap water and mounted with Glycergel (DAKO Corporation, Carpinteria, CA). Preparations

were examined and photographed on an Olympus BX51 microscope fitted with an Olympus DP-12 digital camera system (Olympus, Melville, NY).

Antibodies

Unless otherwise indicated, all antibodies were purchased from BD Pharmingen (San Diego, CA). Immunohistochemistry experiments employed rat monoclonal antibodies specific for mouse CD8β (clone 53-5.8), CD45R/B220 (clone RA3-6B2) and CD45 (clone 30-F11). Monoclonal antibodies specific for CD4 (clone GK1.5; ATCC, Rockville, MD) and MHCII (clone M5/114.15.2; ATCC, Rockville, MD) were purified from hybridoma supernatants by affinity chromatography (protein G-Sepharose; Pharmacia biotech, Uppsala, Sweden) as described previously (8). Flow cytometric analyses employed Cy-Chrome™-conjugated anti-TCR β-chain (clone H57-597), allophycocyanin (APC)-conjugated anti-CD45R/B220 (clone RA3-6B2) and phycoerythrin (PE)-conjugated CD11b (clone M1/70). Specificity of binding was ensured by inclusion IgG2a conjugated to Cy-Chrome™, APC, or PE. ELISA experiments utilized rat monoclonal antibodies specific for mouse IgG1 (clone A85-1), IgG2a (clone R19-15), IgG2b (clone R12-3), IgG3 (clone R40-82) and IgM (clone R6-60.2). Isotype-specific antibodies were detected with HRP-conjugated mouse monoclonal antibody directed against rat Ig kappa light chain (clone G16-510E3).

Preparation of single cell suspensions from diaphragm and cavity exudates.

The diaphragm was removed and washed with PBS. Adherent and infiltrating cells were recovered by mincing the tissue and digesting for 15 min at 37°C in collagenase I (Sigma Chemical Co., St. Louis, MO). Undigested

muscle was manually dispersed on a stainless steel tea strainer using a 12cc syringe pestle. Cell preparations were pooled from 3 mice and passed through 70 μm and 40 μm sieves (Falcon, Oxnard, CA). Erythrocytes were lysed with Gey's solution for 5 min on ice. Remaining cells were washed two times with 0.25% BSA in PBS (BSA/PBS), and viable cells were counted following dilution in trypan blue. A ratio was calculated using total pooled cell number from IL-10 -/- mice compared to WT mice. Experiments were repeated 3 times for uninfected and 100 days post-infection (dpi), and 4 times for 20 dpi. Means were determined for each sampling period and evaluated by Fisher's protected least significant difference analysis (alpha level of 0.05).

Pleural exudates were obtained by lavage with 2.5 ml of BSA/PBS. The thorax was gently massaged before fluid was recovered with a 3cc syringe affixed with a 22 gauge, 1-inch needle. Peritoneal exudates were collected by lavage with 10 ml of BSA/PBS. Fluid was recovered with a 12cc syringe affixed with an 18 gauge, 1-inch needle. Cell preparations from individual mice were prepared as described above. Mean cell numbers were calculated for groups of 4-5 mice.

Flow Cytometry

Cells were treated with 10% NMS in PBS for 20 min on ice, stained with fluorescent antibodies for 12 min at room temperature, washed once with PBS and analyzed with a FACSCaliber® Flow Cytometer (Becton Dickenson, Mansfield, MA). For data acquisition, ten thousand events were collected within a gate that included mononuclear leukocytes. Phenotypic analysis was carried out on a gate of the lymphocyte population using CellQuest Software (BD Pharmingen).

Enzyme-Linked Immunosorbent Assay (ELISA)

Parasite-specific immunoglobulins in sera were measured by ELISA. Unless otherwise indicated, incubations were carried out for 1 hour at RT. Polyvinyl 96-well plates were coated with either somatic antigen (5 μg/ml) or ESA (1 μg/ml) overnight at 4 degrees Celsius. Sera from individual mice were serially diluted 3-fold and bound antibody was detected with rat monoclonal antibodies to IgG1, IgG2a, IgG2b, IgG3 and IgM (1 μg/ml), followed by HRP-conjugated mouse anti-rat kappa chain (1 μg/ml). Plates were developed for 30 minutes with TMB peroxidase substrate (KPL Laboratories, Gaithersburg, MD) and stopped with 1M H_3PO_4. Absorbance (450 nm) was measured with an ELISA plate reader (Bio-Tek Instruments, Winooski, VT). Titers for each isotype were defined as the reciprocal of the last dilution at which the absorbance was greater than 0.1 units. Geometric mean titers were calculated from groups of four mice.

Results

Anatomic distribution and morphologic development of nurse cells.

The anatomic distribution of muscle larvae was similar in WT and IL-10 -/- mice. Mice were killed between 8 and 20 weeks post-infection. In WT mice (n=7), the diaphragm yielded 460±400 larvae, while the head, upper body and lower body each yielded 1039±797, 4314±2894 and 2532±1850 larvae, respectively. In IL-10 -/- mice (n=10), the diaphragm yielded 395±512 larvae, while the head, upper body and lower body each yielded 987±512, 3103±1453 and 2290±1408 larvae, respectively. This distribution is similar to that described in orally infected mice of various strains (51).

In general, the development of nurse cells in WT and IL-10 -/- mice followed a progression reported previously in mice of various strains (19). Five days after inoculation, occasional basophilic myotubes, each containing a single immature larva in an elongate or serpentine orientation, were observed in diaphragm (Figure 2.1A), tongue and hind leg muscles (data not shown). Larvae grew approximately 2-fold between 5 and 10 dpi and began to assume a coiled conformation. Significant hypertrophy of infected cells and the initiation of a surrounding capsule were observed by 10 dpi (Figure 2.1B). At 20 dpi, infected myotubes were surrounded by thick collagen capsules, had lost all striations, had shortened significantly and were approximately 5 times the diameter of adjacent, uninfected myotubes (Figure 2.1C). Larvae were mature at 20 dpi, as evidenced by their resistance to pepsin digestion. The microscopic appearances of larvae and infected cells were not altered between 20 and 55 dpi (Figure 2.1D).

Development of the inflammatory response in T. spiralis infected muscle.

At 5 dpi, hypertrophy of infected muscle cell nuclei was evident, together with a minimal infiltrate consisting primarily of small lymphocytic foci (Figure 2.1A). By 10 dpi the inflammatory infiltrate had increased markedly, and infected myotubes were cuffed by infiltrating cells (Figure 2.1B). Infiltrates were comprised largely of mononuclear, fusiform to ovoid cells with ample cytoplasm, interpreted to be macrophages. At 20 dpi, cuffing was more prominent and the intensity of the inflammatory infiltrate had peaked (Figure 2.1C). Eosinophils, scattered plasma cells and numerous large lymphoblasts were observed, however, macrophages were dominant. Focal areas of necrosis were observed in scattered, dense areas of infiltration.

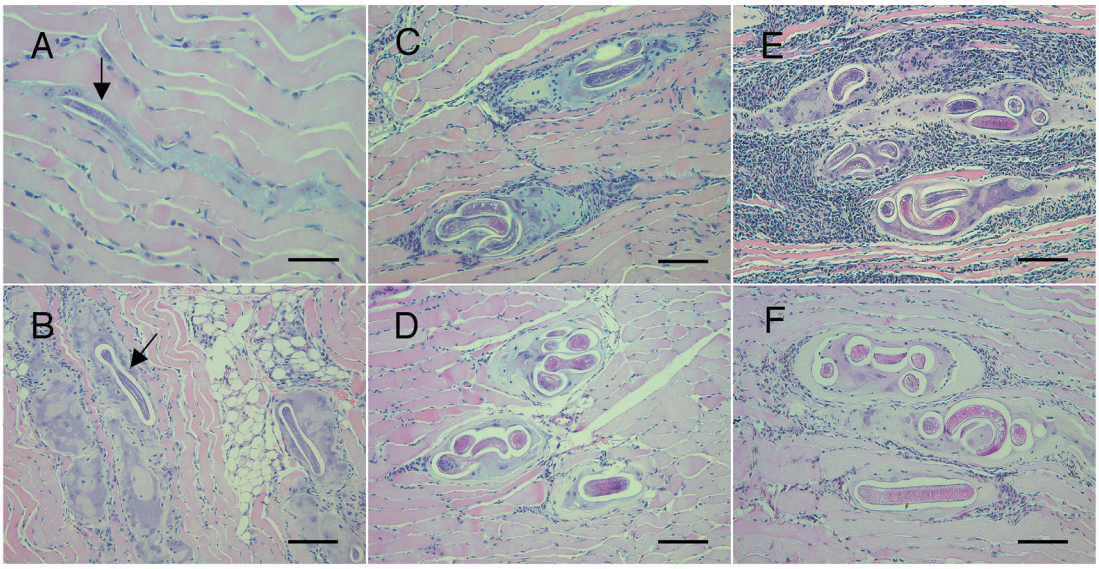


Figure 2.1 IL-10 inhibits inflammation during parasite development in muscle. Diaphragms were collected from synchronously infected WT (A-D) and IL-10-/- (E, F) mice, sectioned and stained with H & E. (A) 5 dpi, WT; infected myotubes are basophilic and demonstrate nuclear hypertrophy. (B) 10 dpi, WT; cellular infiltrates are evident around infected, hypertrophied muscle cells. (C) 20 dpi, WT; focal infiltrates cuff mature nurse cells. (D) 55 dpi, WT; attenuated focal infiltrates. (E) 20 dpi, IL-10-/-; intense focal infiltration by inflammatory cells. (F) 55 dpi, IL-10 -/-; limited focal infiltrates. Arrows denote the location of the *T. spiralis* larva within the muscle cell (A, B). Scale bars = 50 μm (A), 100 μm (B-F).

In some instances, infiltrating cells appeared to have entered the nurse cell (Figure 2.1C). The volume of the cellular infiltrate began to decline as early as 23 dpi (data not shown), continuing until 55 dpi when only residual infiltrates remained closely associated with the collagen capsules of infected cells (Figure 2.1).

Influence of IL-10 on the inflammatory response in infected muscle.

Comparison of muscles collected 5 dpi from WT and IL-10 -/- mice revealed no apparent differences in either the composition or the magnitude of inflammation (data not shown). By 10 dpi, there was a marked increase in the number of inflammatory cells both surrounding individual nurse cells and in the interstitia of IL-10 -/- versus WT mice (data not shown). The most dramatic differences, however, were apparent at 20 dpi (Figure 2.1C, E). In IL-10 -/- mice (Figure 2.1E) there was complete effacement of the interstitial area surrounding some nurse cells, as well as degeneration of several uninfected myotubes incarcerated by infiltrating cells. Nevertheless, by 55 dpi, inflammation in both groups of mice had resolved (Figure 2.1D, F). Composition of infiltrates was similar in the two strains, with a predominance of macrophages and smaller numbers of lymphocytes and eosinophils.

Despite the exaggerated response in the IL-10 -/- mice, nurse cell integrity was uncompromised. This was confirmed by assessing whole body larval burdens. At 20 dpi, we recovered 13,402 ± 2638 larvae from WT mice (n=7), compared to 13,862 ± 4016 larvae recovered from IL-10 -/- mice (n=9). Muscle larvae burdens remained similar at 55 dpi (WT (n=3); 20,826±5230, IL-10 -/- (n=4); 17,790±4472). Worms recovered from IL-10 -/- mice were motile and morphologically identical to WT counterparts (normal cuticle structure,

coiled conformation, and full stichocyte (secretory organ) development). Although infectivity of muscle larvae recovered from IL-10 -/- mice was not directly evaluated, larvae survived pepsin-HCL digestion, a key criterion in evaluation of physical maturity and infectivity for *T. spiralis.* In addition, muscle larvae in both groups of mice induced high titers of tyvelose-specific antibodies, confirming the secretion/excretion of tyvelose-bearing glycoproteins, a hallmark of infective first-stage larvae. Mice in both groups maintained normal body weight throughout the course of the experiments (data not shown).

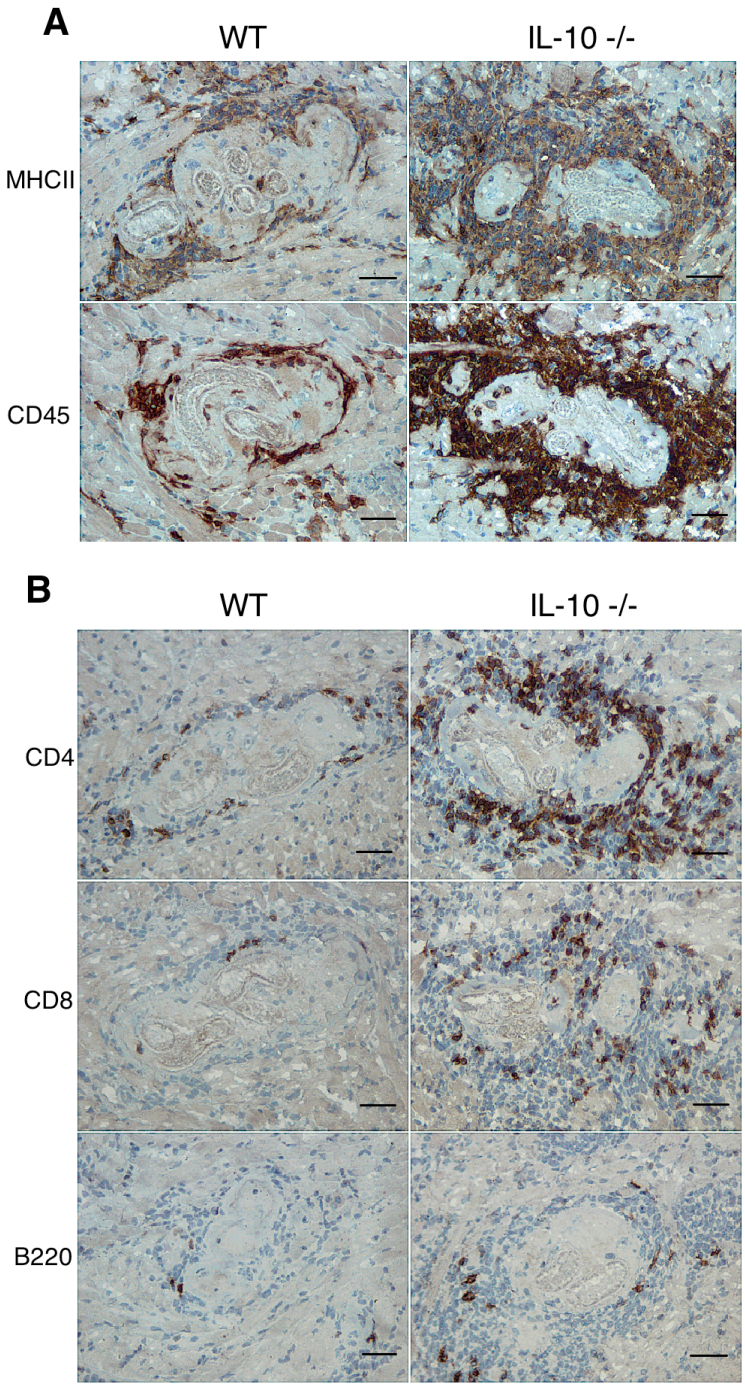
Composition of cellular infiltrates associated with nurse cells.

Despite the difference in intensity of inflammation in all tissues examined from WT and IL-10 -/- mice, the cellular composition of the infiltrates was similar: predominantly CDllb+ cells (data not shown), MHCII+ cells and CD45+ cells (Figure 2.2A), with fewer CD4+ cells, CD8+ cells and rare B220+ cells (Figure 2.2B). Small clusters of syndecan-1+ plasma cells were observed in proximity to nurse cells (data not shown). MHCII+ and CD45+ cells were mononuclear and they were presumed to be macrophages.

Expansion of lymphoid aggregates and nodules associated with superficial aspects of the diaphragm following infection with T. spiralis.

Appreciating the preference for infection of the diaphragm by *T. spiralis*, and the unique properties of this muscle with regard to lymph drainage, we explored the inflammatory response at this site in greater detail.

Figure 2.2 IL-10 influences intensity but not composition of local infiltrates. Sections of tongues recovered from WT and IL-10 -/- mice at 20 dpi were stained with antibodies. Panel A: Macrophages (MHCII+, CD45+) dominate the focal response around the nurse cell. Panel B: Infiltrating lymphocytes are largely CD4+, with fewer CD8+ T cells and rare B cells. Scale bars = 50 μm.

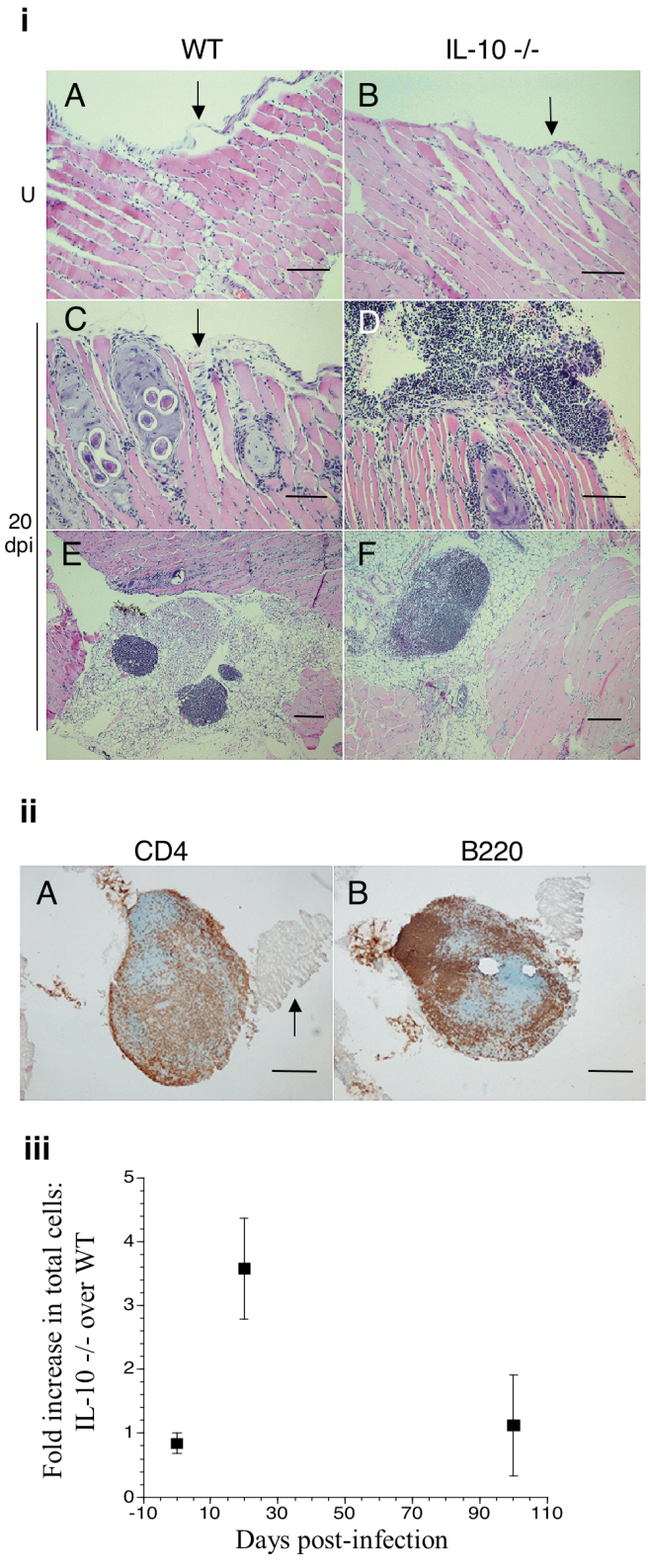

A
WT
IL-10 -/-
MHCII
CD45
B
WT
IL-10 -/-
CD4
CD8
B220

Diaphragms of uninfected WT and IL-10 -/- mice were covered with an intact and normal mesothelial layer (Fig 2.3i, panel A, B). There were occasional, small lymphocytic foci present in the associated adipose tissue as well as the mesothelium of uninfected WT and IL-10 -/- mice. The mesothelium of WT mice at 20 dpi had a mild cellular infiltrate extending into lymphatic lacunae (Fig 2.3i, panel C, arrow). Small lymphoid nodules present in uninfected mice were enlarged in infected WT mice (Fig 2.3E). Germinal centers were evident in the nodules of WT (Fig 2.3ii) and IL-10 -/- mice. IL-10 -/- mice had larger and more numerous lymphoid nodules (Fig 2.3i, panel F) together with a more pronounced cellular infiltrate of the mesothelium (Fig 2.3i, panel D). By 55 dpi, enlarged lymphoid nodules and infiltrates were no longer evident in either WT or IL-10 -/- mice (data not shown).

Quantification of cellular responses in the diaphragms of IL-10 -/- mice.

In order to quantify the inflammatory response, we recovered cells by digesting diaphragms with collagenase I. Figure 2.3iii shows the mean fold increase in diaphragm-associated cell numbers at 0, 20 and 100 dpi. At 20 dpi there was a significant, 3.6-fold increase in cells recovered from IL-10 -/- versus WT diaphragm (α=0.05). By 100 dpi the ratio had returned to the pre-infection value.

Figure 2.3 Attenuation of inflammation at the surface of the diaphragm by IL-10. Sections of diaphragm from 20 dpi were stained with H & E (i) or with monoclonal antibodies specific for B220 and CD4 (ii). Alternatively, cells were recovered from diaphragms by digestion, cell suspensions from 3 mice of each strain were pooled, counted and expressed as a ratio (iii). Panel i; (A, B) Normal mesothelia (arrow) cover diaphragms from uninfected WT and IL-10-/- mice. (C) Mild cellular infiltration of lymphatic lacuna in diaphragm of WT mouse (arrow). (D) Pronounced cellular infiltrates and large lymphoid aggregates adhere to the diaphragm of an IL-10 -/- mouse. (E) Enlarged lymphoid nodule from an infected WT mouse. (F) Larger nodule from an infected IL-10 -/- mouse. Panel ii: (A) CD4+ T cells and (B) B cells detected in germinal center in nodule from infected WT mouse. Arrow in A indicates diaphragm. Panel iii: At 20 dpi, IL-10 -/- mice had nearly 4 times as many infiltrating cells associated with the diaphragm compared to WT mice ($p < 0.05$). Relative cell numbers returned to the pre-infection level by 100 dpi. Scale bars = 100 μm (i: A-D, ii: A, B) and 200 μm (i: E, F). U = uninfected.


i
WT
IL-10 -/-
A
B
U
C
D
20 dpi
E
F
ii
CD4
B220
A
B
iii
Fold increase in total cells: IL-10 -/- over WT
0
1
2
3
4
5
-10
10
30
50
70
90
110
Days post-infection

Expansion of B and T cell populations in the pleural cavities in IL-10 -/- mice.

To evaluate the body cavity response to muscle infection, cells were enumerated in pleural and peritoneal exudates collected from WT and IL-10 -/- mice at 0 and 20 dpi. WT mice did not significantly expand body cavity cell populations in response to infection. IL-10 deficient mice demonstrated a > 3-fold expansion of pleural exudate cells ($p < 0.01$) in response to infection (Fig 2.4). Uninfected IL-10 -/- mice yielded greater numbers of peritoneal exudate cells compared to WT mice, but this number did not change significantly upon infection ($p = 0.1$)

Flow cytometric analysis of exudates from infected IL-10 -/- mice revealed modest increases in peritoneal B and T cell numbers while pleural B and T cell numbers increased 6-fold and 9-fold, respectively (Fig 2.5A). In addition to conventional B lymphocytes, or B-2 cells, body cavities possess a population of self-renewing B lymphocytes, so-called B-1 cells (38). B-1 cells can be phenotypically distinguished from B-2 cells by their surface expression of CD11b (6). Evaluation of body cavity B220+ populations for expression of CD11b revealed that IL-10 controlled the expansion of B-2 cells (11-fold) and, to a lesser extent, B-1 cells (4-fold) in the pleural cavity (Fig 2.5B). In the peritoneal cavity, IL-10 inhibited expansion of B-2 cells (3-fold) while IL-10 appeared to be required for maintenance of the B-1 cell population.

Antibody response to muscle infection

The antibody response during oral infections with *T. spiralis* is well documented (1, 18, 45). Our method of infection allowed us to evaluate immunity induced by muscle larvae in the absence of antibody responses to earlier stages of infection. In addition, synchronous arrival of larvae in the

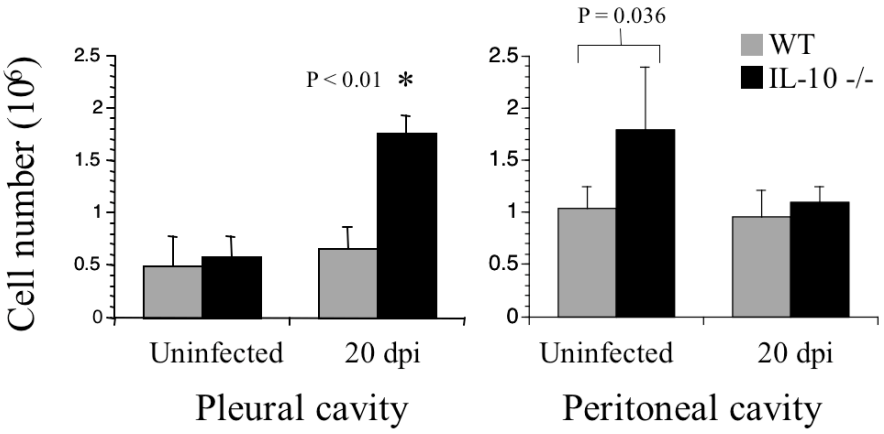
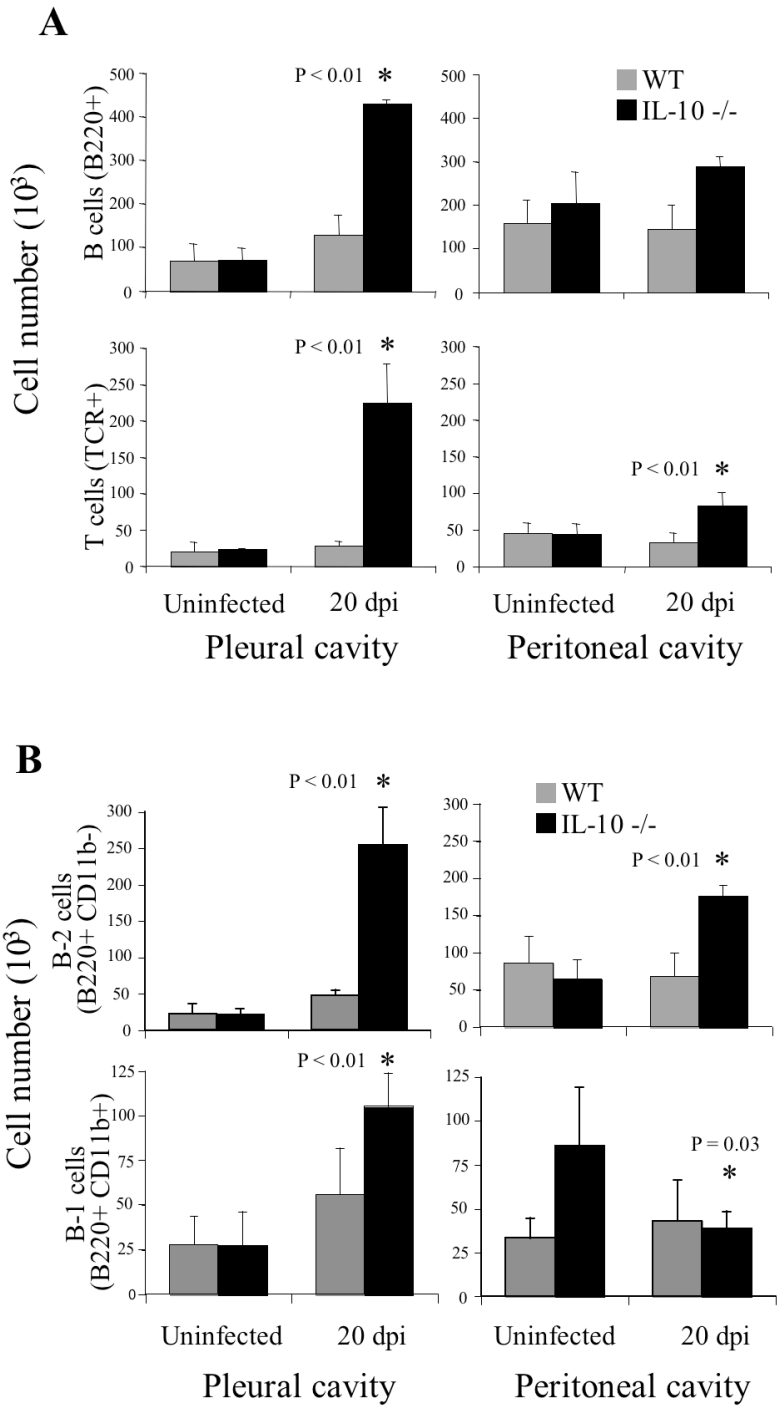


Figure 2.4 Cellular response in pleural and peritoneal exudates following infection. At 0 and 20 dpi, pleural and peritoneal exudates were collected and cells counted (n=4-5 mice per strain). Infection induced a 3-fold increase in pleural exudate cells in IL-10 -/- mice ($p < 0.01$). Uninfected IL-10 -/- mice yielded greater numbers of peritoneal exudate cells compared to WT mice, but this number did not change significantly upon infection ($p = 0.1$).

Figure 2.5 T and B cells recovered from the pleural and peritoneal cavities during muscle infection. Lymphocytes in pleural and peritoneal exudates (n=4-5 mice) were enumerated by flow cytometry following staining with monoclonal antibodies to TCR beta-chain and B220 (panel A), or, in order to identify B cell subsets, CD11b and B220 (panel B). Panel A: Mice lacking IL-10 expanded pleural B and T cell populations 6-fold and 9-fold, respectively. A modest increase in B cells ($p = 0.06$) and a significant increase in T cells ($p < 0.01$) were evident in the peritoneal cavities. Panel B: B-2 cells (11-fold) and B-1 cells (4-fold) were expanded in the pleural cavities of IL-10 -/- mice following infection. In the peritoneal cavity, B-2 cells expanded 3-fold in the absence of IL-10, while the B-1 cell population was diminished 2-fold following infection. Asterisks mark statistically significant changes (student's t test).


A
Cell number (10^3)
B cells (B220+)
T cells (TCR+)
P < 0.01 *
P < 0.01 *
P < 0.01 *
P < 0.01 *
WT
IL-10 -/-
Uninfected
20 dpi
Uninfected
20 dpi
Pleural cavity
Peritoneal cavity
B
Cell number (10^3)
B-2 cells (B220+ CD11b-)
B-1 cells (B220+ CD11b+)
P < 0.01 *
P < 0.01 *
P < 0.01 *
P = 0.03 *
WT
IL-10 -/-
Uninfected
20 dpi
Uninfected
20 dpi
Pleural cavity
Peritoneal cavity

musculature may amplify the host humoral response to muscle stage antigens. To evaluate the host response to synchronized muscle infection, IgG1, IgG2a, IgG2b, IgG3 and IgM specific for somatic antigens or ESA were measured at 0, 5, 10, 20, 24 and 55 dpi by ELISA (Fig 2.6).

Comparison of the responses to somatic antigens with that to ESA revealed two distinct phases of antibody production. The first response, mounted from 10 to 20 dpi, produced antibodies to somatic antigens but not to ESA. The second response, evident at 55 dpi, was directed at ESA. The early response incorporated IgM, IgG2b and IgG3. A weak and variable IgG2a response was detected only in IL-10 -/- mice. The late response incorporated all the isotypes assayed. Although IgG1 was absent from the early response to somatic antigens, it was dominant in the late response.

Antibody responses in WT and IL-10 -/- mice differed only in kinetics, with IL-10 -/- mice initiating IgG3 and IgG2a production more rapidly in the early response, and IgM more rapidly in the late response. Denkers et al. (17) described the murine antibody response to oral infection with *T. spiralis* as biphasic.

The early response is directed against phosphorylcholine that modifies glycans on somatic glycoproteins (40), while the late response is specific for tyvelose-bearing glycoproteins found in ESA. We analyzed mouse sera from our experiments by Western blot in order to determine the specificities of the antibodies produced. We found that sera collected 55 dpi bore antibodies specific for tyvelose-bearing glycoproteins. However, antibodies in sera collected 10 to 20 dpi did not bind phosphorylcholine-bearing somatic glycoproteins (data not shown). The specificity of antibodies produced between 10 and 20 dpi remains unknown.

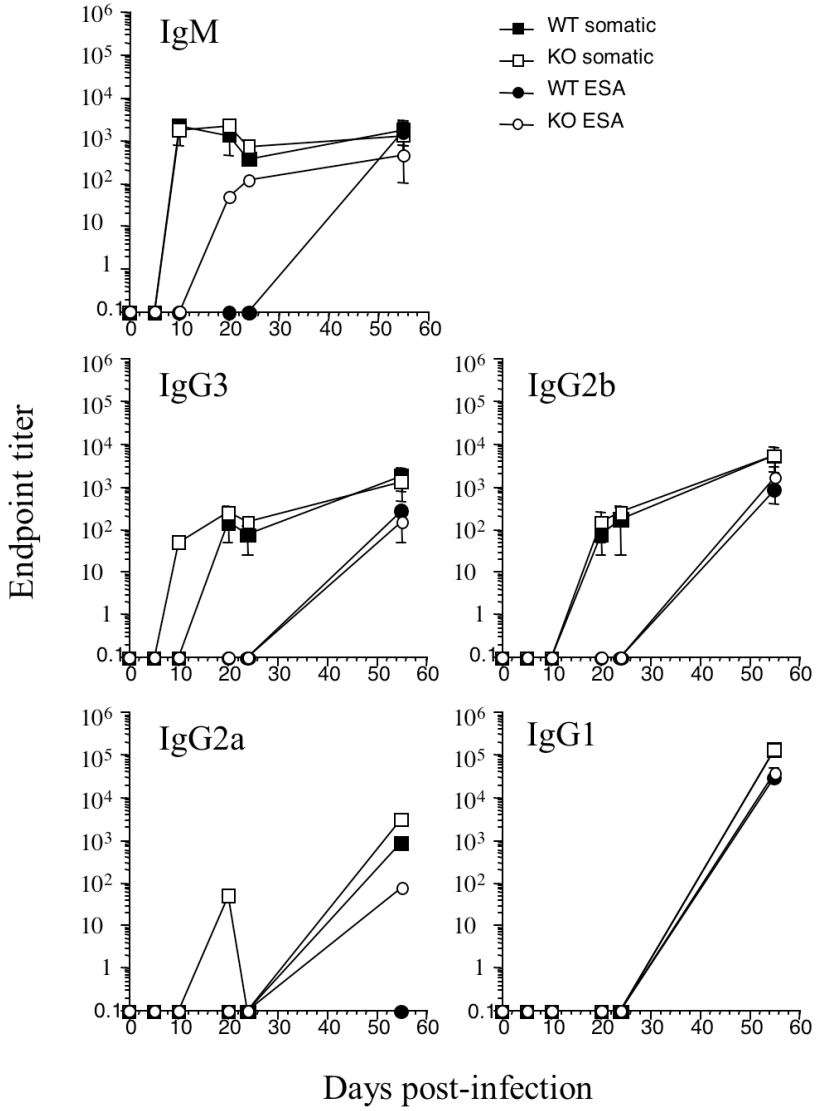


Figure 2.6 The antibody response to synchronized muscle infection. Serum titers of IgG1, IgG2a, IgG2b, IgG3 and IgM specific for somatic antigen (squares) or ESA (circles) in WT (filled) and IL-10 -/- (open) mice. Endpoint titers were specified as the reciprocal of the last dilution before absorbance fell below 0.1 in ELISA. Geometric means and standard deviations for endpoint titers from groups of 4 mice are shown.

Discussion

To address the role of IL-10 in the immune response to *T. spiralis* in muscle, we synchronously infected WT and IL-10 -/- mice by intravenous injection of NBL. We chose this method in order to avoid confounding influences of IL-10 on the intestinal stage of infection, influences that might alter the dose of NBL delivered to the muscle. Concurrent investigations in our lab showed no effect of IL-10 on survival of intestinal *T. spiralis*; however NBL were found to induce large areas of focal necrosis in the livers of IL-10 -/- but not WT mice (7). Contradictory results have been reported by Helmby and Grencis (31) who described delayed worm expulsion together with reduced muscle burdens in orally infected IL-10 -/- mice. The different outcomes may be due to differences in dose (300 versus 600 larvae given orally) or parasite isolate used in the two studies. Helmby and Grencis concluded that reduced muscle burdens result from an IFN-γ-dependent immune response directed against NBL, although it was less clear when or where this influence is effected. The results of our synchronous infections indicate that IL-10 neither promotes nor compromises survival of larvae in the muscle. This outcome is important to the interpretation of our results, as it demonstrates that immune deficient and WT mice acquire comparable antigenic loads, eliminating this important variable as an influence on the immune response. Survival of larvae in the presence of the intense inflammation observed in IL-10 -/- mice suggests that *T. spiralis* is a highly adapted parasitic organism.

Muscle infection with *T. spiralis* elicited a focal cellular immune response. Nurse cells were surrounded by limited infiltrates in which macrophages were the dominant cell type. Remarkably, parasites survived in close association with macrophages, CD8+ and CD4+ T lymphocytes and B

lymphocytes. It will be important to characterize the functional properties of infiltrating cells in order to understand the basis for parasite survival in what appears to be a hostile environment. Shortly after nurse cell formation was complete, the infiltrates decreased in intensity. We found that IL-10 limited the initial inflammatory response to muscle infection, but was not necessary for the down-modulation observed around mature nurse cells. In a similar manner, IL-10 has been shown to control early, but not chronic, granulomatous responses to *S. mansoni* eggs (58).

The shift from IL-10-dependent to IL-10-independent control of inflammation was coincident with completion of parasite development in the nurse cell, together with the induction of a strong IgG1 response to tyvelose-bearing glycoproteins that are synthesized only by mature first-stage larvae. Class-switching from IgM to IgG1 is controlled by IL-4 (50), the preeminent Th2 cytokine. The induction of an IgG1 response suggests a role for Th2 cytokines in controlling inflammation during chronic infection. A mechanism for targeted activation of Th2 cells by tyvelose-bearing glycoproteins has not been elucidated, although a recent report describes Th2 bias in responses to nematode glycans (53). One outcome of an intense and prolonged IgG1 response to ESA may be modulation of macrophage function. Mosser et al. (52) have shown that ligation of the Fc-gamma receptor I, by IgG1 complexed to antigen, inhibits pro-inflammatory activities of macrophages. Furthermore, the Th2 cytokines, IL-4 and IL-13, are key signals in the generation of alternatively activated macrophages, a cell type implicated in modulation of chronic inflammation in other mouse models of helminth disease (25, 32). There is evidence that *T. spiralis* infection promotes alternative activation of macrophages. Macrophages recovered from the peritoneal cavities of mice

infected orally with *T. spiralis* produce the protein Ym1 (12) and are impaired in their ability control growth of intracellular *Toxoplasma gondii* (57). These features are consistent with an alternatively activated phenotype. Muscle stage infection with *T. spiralis* may polarize infiltrating macrophages toward an alternative phenotype, suppressing their destructive properties. In addition, IL-13 inhibits inflammation by enhancing the production of IL-1 receptor antagonist (36, 56) and suppressing the production of pro-inflammatory cytokines (16, 22). In the context of anti-inflammatory cytokines, consideration must be given to TGF-β, a potent deactivator of macrophage activity *in vitro* (55) that has been shown to cooperate with IL-10 in the protection of mice against experimentally induced colitis (28). Our future work will investigate the function of macrophages in chronic *T. spiralis* infection, specifically addressing the interplay among Th2 cells, macrophages, tyvelose-bearing glycoproteins, and specific antibodies.

In our model, the effects of IL-10 are likely to be exerted at the time of immune response activation. Although it is conceivable that IL-10 deficiency causes developmental defects of the immune system, in our experiments, IL-10 did not influence the cell types recruited to or retained at the site of infection, but instead limited the intensity of the response. These observations are consistent with the known effects of IL-10 in suppressing the activity of antigen presenting cells (9, 15, 23, 27, 41). Our results suggest that IL-10 mediates its anti-inflammatory effect in mice infected with *T. spiralis* by influencing the activities of large numbers of macrophages that surround infected muscle cells.

In addition to moderating inflammation surrounding the nurse cell, we found that IL-10 also influenced regional inflammation during muscle infection.

The unique physiology of the diaphragm (49) prompted us to examine regional responses during *T. spiralis* infection. We observed cellular infiltrates at the surface of the diaphragm and in the body cavities following infection. Our data indicated that aggregates and nodules observed on the surface of the diaphragm were on the pleural aspect of the muscle. We were able to recover these aggregates by enzymatic digestion and found them to be rich in B and T lymphocytes. When we recovered cavity lymphocytes by lavage, we found that IL-10 limited the expansion of B-2, B-1 and T lymphocytes in the pleural cavity. The magnitude of the response in the pleural cavity most likely was induced by the migration of NBL through the lungs (10). IL-10 controlled expansion of pleural B cell populations but did not yield dramatic differences in parasite-specific, serum immunoglobulins, although production of some isotypes was accelerated in IL-10 -/- mice. Currently, we are evaluating the contribution of B lymphocytes to immune modulation in synchronously infected, B cell-deficient mice.

In summary, we have found that IL-10 limits the initial inflammatory response to muscle infection by *T. spiralis*. This inhibition is evident at the site of infection and in the pleural cavity. Although the source(s) of IL-10 was not identified, candidates include macrophages, CD4+ T cells and B lymphocytes. Inhibition of inflammation during chronic muscle infection was shown to be IL-10-independent. The transition from IL-10-dependent to IL-10-independent control of inflammation was abrupt, coinciding with parasite maturation and the induction of a dramatic IgG1 response directed at tyvelose-bearing glycoproteins. Thus, muscle infection by *T. spiralis* serves as a dramatic example of a chronic infection wherein the immune response appears to be controlled in order to promote the survival of both parasite and host.

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CHAPTER THREE

Synthesis of syndecan-1 by skeletal muscle cells is an early response to infection with *Trichinella spiralis* but is not essential for nurse cell development[*].

[*] Daniel P. Beiting, Pyong Woo Park, Judith A. Appleton. Synthesis of syndecan-1 by skeletal muscle cells is an early response to infection with *Trichinella spiralis* but is not essential for nurse cell development. Infection and Immunity 2006; 74(3): 1941-1943

Summary

Trichinella spiralis creates a unique, intracellular habitat in striated muscle. We report that a proteoglycan, syndecan-1, is induced early in infection yet is not essential for habitat development and exerts a modest influence on the immune response. This report is the first to address the requirement for a specific muscle protein in Trichinellosis using mice deficient in the relevant gene.

Body of paper

Muscle infection by *Trichinella spiralis* is marked by a series of dramatic morphological and biochemical changes in the host cell, producing a structure referred to as a nurse cell (12). The molecular mechanisms that regulate these complex cellular changes are largely unknown, and protein markers for induction of the infected cell phenotype are limited (8). We have found that syndecan-1, a heparan sulfate (HS)-bearing proteoglycan (HSPG), is produced early in infection and is retained in the cytoplasm rather than transported to the surface of the nurse cell. Syndecan-1 binds to fibroblast growth factors (6), inhibits myoblast differentiation (10), and anchors cells to the extracellular matrix (15). In addition, syndecan-1 modulates immune responses, as demonstrated in a model of lung allergy induced by *Aspergillus* antigen (16). Similarly, HSPGs have been implicated in the control of myeloid cell proliferation in granulomas induced by *Schistosoma mansoni* (1, 7).

Our observation that infected cells produce syndecan-1, together with the knowledge that syndecan-1 binds to collagen types IV and VI (14) (components of the nurse cell capsule (11)), prompted us to hypothesize that syndecan-1 would be influential in parasite development or host cell

transformation. To test this hypothesis, eight-week old C57BL/6J (WT) (Jackson Laboratory, Bar Harbor, ME) and syndecan-1 deficient ($sdc1^{-/-}$) mice (Baylor College of Medicine, Houston, TX) were infected intravenously with 25,000 NBL. Parasite preparation and synchronous infections were carried out as described previously (3). At 5, 10, 22 and 51 days post-infection (dpi), mice were killed and histologic sections of tongue were prepared and stained with rat monoclonal antibody to syndecan-1 (clone 281-2; BD Pharmingen, San Diego, CA) using the ABC Vectastain Elite kit (Vector labs, Burlingame, CA) (3). Syndecan-1 was evident as early as 5 dpi (Figure 3.1A), remained detectable into chronic infection (Figure 3.1D), and localized to the cytoplasm of infected cells. Infected tongue from $sdc1^{-/-}$ mice did not stain with antibody to syndecan-1 (Figure 3.1E), nor did uninfected WT muscle cells. Despite prolonged production of syndecan-1 by infected cells, we found that *T. spiralis* infection progressed normally in mice lacking the proteoglycan. Larvae invaded cells and developed normally in $sdc1^{-/-}$ mice as evidenced by larval morphology and collagen capsule formation (Figure 3.1E, arrow). Similar numbers of mature L1 were recovered from WT and $sdc1^{-/-}$ mice and were comparable in their ability to infect, mature and reproduce when passaged into C57BL/6 mice (data not shown). Although we observed no requirement for syndecan-1 up to 50 dpi in our experiments, *T. spiralis* survives in the muscle for months to years and our data cannot rule out a role for syndecan-1 in the longer term.

Cytoplasmic localization of syndecan-1 in nurse cells contrasts with the cell surface location of the proteoglycan in epithelial cells and plasma cells, where the molecule facilitates adhesion and proliferation (4). Modification of syndecan-1 by HS is critical for biological activity (9), localization (17) and

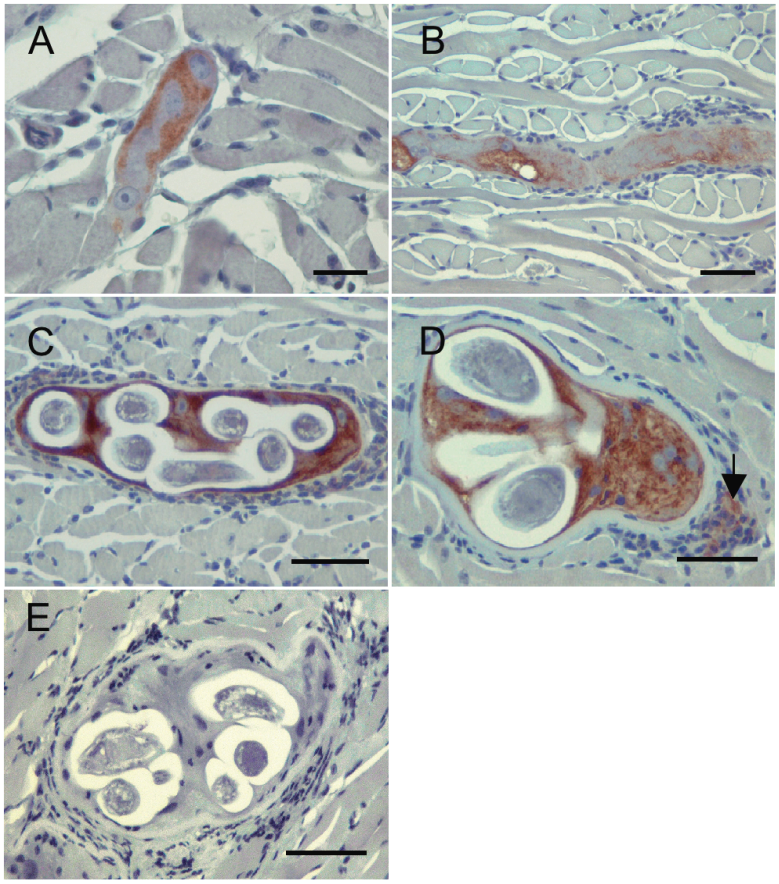

Figure 3.1 Syndecan-1 is produced by muscle cells infected with *T. spiralis,* but is not required for nurse cell development. Sections of infected tongue from WT (A-D) or sdc1$^{-/-}$ (E) stained with antibody to syndecan-1. All sections were counterstained with hematoxylin. (A) 5 dpi; syndecan-1 in cytoplasm of infected cell. (B) 10 dpi; syndecan-1 is diffusely distributed within infected cell. (C) 20 dpi and (D) 50 dpi; cytoplasm of mature nurse cell stains intensely with anti-syndecan-1. Diffuse, extracellular syndecan-1 is detected in the infiltrate surrounding nurse cells (D, solid arrow). (E) 20 dpi, nurse cells of sdc1$^{-/-}$ mice do not contain syndecan-1. Scale bars = 25 μm (A) and 50 μm (B-E).

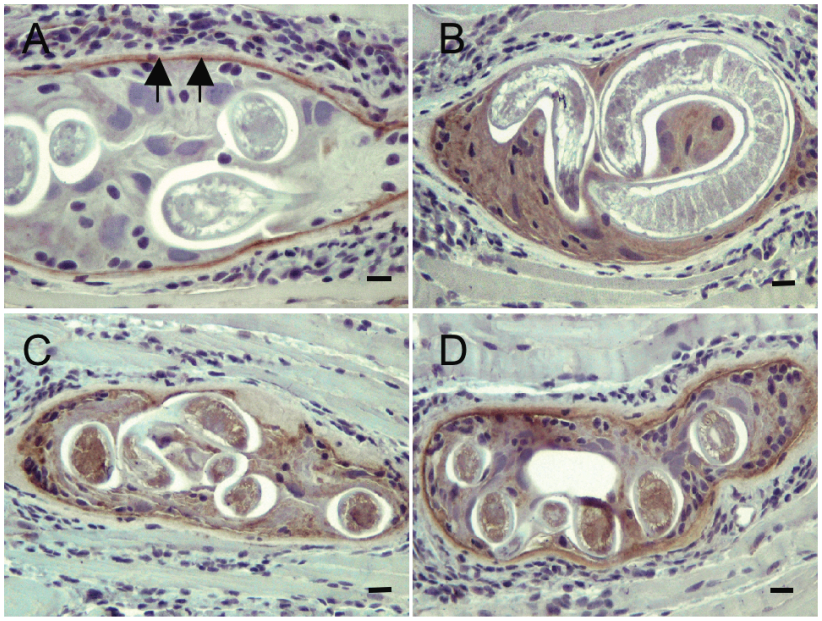

Figure 3.2 Investigation of nurse cell syndecan-1 structure. Infected diaphragm from WT (A-C) or sdc1$^{-/-}$ (D) mice at 22 dpi was stained with (A) antibody to perlecan, (B) polyclonal antibody to carboxy-terminus of syndecan-1 or (C, D) antibody to heparan sulfate. Scale bars are 10 μm (A) and 20 μm (B-D). Arrows indicate perlecan in the nurse cell basement membrane (A).

immuno-regulatory capacity (16). We explored potential causes for retention of syndecan-1 in the nurse cell cytoplasm. In order to detect a general defect in proteoglycan synthesis or transport, we stained infected muscle with antibodies specific for HS (clone F58-10E4; Seikagaku Corporation, Tokyo, Japan) or for perlecan (clone A7L6; NeoMarkers, Fremont, CA), a large (>500 kDa) multidomain HSPG that is a common constituent of basement membrane. We detected HS in the cytoplasm and cell margins of both WT (Figure 3.2C) and sdc1$^{-/-}$ mice (Figure 3.2D), indicating that infected muscle cells synthesize HS irrespective of syndecan-1 production.

The HS content of nurse cells from sdc1$^{-/-}$ mice may be associated with other HSPGs. Perlecan was detected at the margins of uninfected and infected myotubes, indicating that infected cells can synthesize large, complex HSPGs and transport them appropriately (Figure 3.2A). If syndecan-1 mRNA were alternatively spliced in muscle cells, as has been reported to occur in mouse embryos (13), a truncated core protein may exhibit altered activity or localization. We did not detect alternatively spliced transcripts with PCR (data not shown). Staining with antibodies specific for the amino-terminus of the protein (Figure 3.1; clone 281-2) or the carboxy-terminus (16) yielded similar patterns (Figure 3.2B), suggesting that the protein was intact in the infected cell. It is possible, however, that nurse cell syndecan-1 is heterogeneous including intact as well as truncated molecules.

Based on recent findings that syndecan-1 limits TH2-driven inflammation by an HS-dependent mechanism (16), we hypothesized that sdc1$^{-/-}$ mice would generate larger inflammatory reactions around nurse cells. To test this hypothesis we measured cytokine production by cervical lymph node cells restimulated *in vitro* with plate-bound anti-CD3 (5). Cells cultured

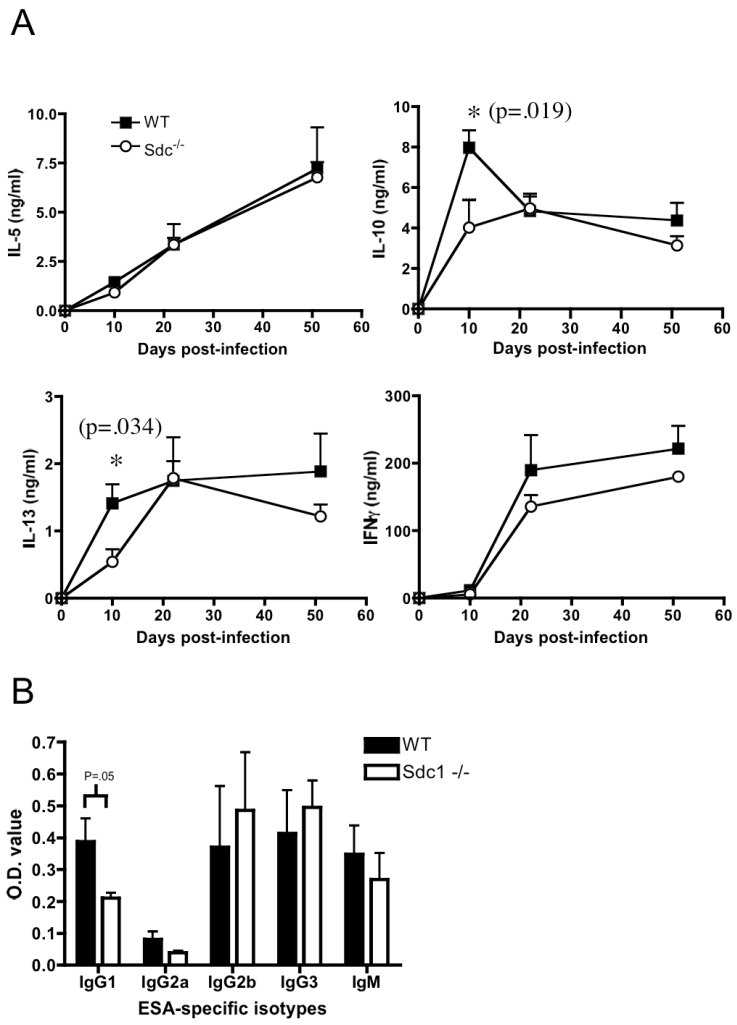

Figure 3.3 Sdc1$^{-/-}$ mice produce less IL-10, IL-13 and parasite-specific IgG1 compared to WT mice. (A) Cytokine responses of CLN cells from WT and sdc1$^{-/-}$ mice infected with *T. spiralis.* Leukocytes were collected at indicated times post-infection, cultured and restimulated with anti-CD3. IL-5, 10, 13 and IFNγ were measured in supernatants collected after 72 hours. (B) Sera collected at 50 dpi were diluted 5000-fold (IgG1) or 100-fold (IgG2a, IgG2b, IgG3 and IgM) and evaluated by ELISA for ESA-specific antibodies. Means and standard deviations are shown for groups of 4-5 mice. Asterisks indicate statistical significance as determined using Student's t test.

from sdc1$^{-/-}$ mice at 10 dpi yielded significantly reduced concentrations of IL-10 and IL-13 compared with WT cells (Figure 3.3A). IL-4 is rarely detected in our cultures (WT or sdc1$^{-/-}$, data not shown), for reasons that have not been elucidated.

To test whether the reduced levels of IL-13 early in infection influenced the B cell response, we measured serum antibodies to parasite excretory-secretory antigens (ESA; prepared as described in (2)) by ELISA (3) Deficiency in IL-13 production correlated with a reduced TH2-driven, parasite specific IgG1 response in sdc1$^{-/-}$ mice (Figure 3.3B). Despite these differences, the volume (Figure 3.1C and D) and composition (CD4, CD8, B220 and MHCII positive cells; data not shown) of local cellular infiltrates were similar in both groups of mice. We did not detect syndecan-2 or -4 in infected cells from either sdc1$^{-/-}$ or WT mice (data not shown). Thus, these members of the syndecan family did not compensate for the absence of syndecan-1 in nurse cells of sdc1$^{-/-}$ mice.

In summary, we have shown that syndecan-1 is produced by muscle cells following infection by *T. spiralis*. Mice deficient in syndecan-1 support normal larval development but display modestly reduced TH2 responses during infection. The lack of a more dramatic phenotype in these mice during infection might be explained by compensatory activities of HSPGs in the *T. spiralis* nurse cell.

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CHAPTER FOUR

Complementary roles for interleukin-10 and naturally-occurring regulatory T cells in suppressing immunity to muscle stage *Trichinella spiralis*[*]

[*] Daniel P. Beiting, Matthias Hesse, Lucille F. Gagliardo, Susan K. Bliss and Judith A. Appleton. Complementary roles for IL-10 and naturally-occurring regulatory T cells in suppressing immunity to muscle stage *Trichinella spiralis*. Manuscript in preparation.

Summary

We previously demonstrated that IL-10 is critical in the control of acute inflammation during *T. spiralis* development in the muscle. Here we use gene-targeted knockout mice, adoptive transfer of specific T cell populations, and *in vivo* antibody treatments to determine the mechanisms by which inflammation is controlled and effector T cell responses are moderated during muscle infection. We report that $CD4^+CD25^-$ effector T cells, rather than $CD4^+CD25^+$ regulatory T (Treg) cells, suppress inflammation by an IL-10-dependent mechanism that limits IFN-γ production and local iNOS induction. Conversely, we show that depletion of Treg cells during infection results in exaggerated TH2 responses. Finally, we provide evidence that, in the absence of IL-10, TGF-β is required to control local inflammation and promote parasite survival during muscle infection.

Introduction

Helminth infections are associated with increased production of interleukin-10 (IL-10) and suppression of host T cell responses (36, 43, 44, 52). IL-10 can be synthesized by a wide range of cells, including T helper type 2 (TH2) cells (19), specialized suppressor T cell subsets (25), B lymphocytes (48), granulocytes (49) and macrophages (17, 34), yet it is unclear which of the cell types contribute to immune suppression during helminth infection. IL-10 has been extensively studied in mice infected with the human parasite *Schistosoma mansoni*. In this system, IL-10 limits IFN-γ production, promotes TH2-driven granuloma formation and prolongs host survival (27, 65). Both $CD4^+CD25^+$ regulatory T cells (Treg) and $CD4^+CD25^-$ effector T cells (Teff) isolated from liver granulomas produce IL-10 in response to egg antigens (26,

46), and suppress egg-specific IFN-γ production after adoptive transfer into IL-$10^{-/-}$ mice (46). In addition, cells of the innate system are also an important source of IL-10 during *S. mansoni* infection (26). Taken together, these studies suggest that IL-10, produced by multiple cell types, may be required for complete suppression of helminth-induced disease.

In addition to modulating TH1 responses during *S. mansoni* infection, IL-10 has also been implicated in suppression of IFN-γ production by peripheral blood mononuclear cells (PBMC) from humans infected with filarial nematodes (36, 44, 52). Based on reports that $CD4^{+}CD25^{+}$ Treg limit TH1-driven intestinal inflammation by IL-10 dependent mechanisms (6, 45), it might be expected that depletion of this cell type during helminth infection would result in enhanced TH1 responses. To the contrary, when Treg cells are depleted in mice infected with the filarial nematodes *Litomosoides sigmondontis* or *Brugia pahangi,* production of IL-4, IL-5 and IL-10 increases (23, 59). A recent and detailed study of Treg cells derived from mice infected with the intestinal nematode *Heligmosomoidies polygyrus* showed that helminth-induced Tregs successfully suppressed allergic TH2 responses and did not require IL-10 to achieve this effect (64). We show here that Treg cells are, in fact, potent mediators of TH2 responses during nematode infection but do not influence inflammation. In contrast, we report that effector T cell-derived IL-10 is critical in suppressing IFN-γ production and limiting myositis.

Trichinella spiralis is a natural pathogen of rodents that establishes chronic infection in skeletal muscle and is well suited for the study of helminth-induced immune suppression. Following a relatively brief intestinal phase, adult female worms release newborn larvae (NBL) that rapidly enter mesenteric venules (11), disseminate throughout the host, and eventually

enter skeletal muscle. The diaphragm, tongue and masseter muscles are all preferred sites of infection (58). Each NBL invades a single, terminally differentiated muscle cell (myotube) and over a period of 20 days (18) both the parasite and the host cell undergo a process of coordinated growth and development, marked by dramatic host cell remodeling (18, 30, 31, 54). If larvae survive this developmental period, they establish a chronic infection that is essential for transmission. To investigate the mechanisms that control T cell responses specifically to muscle stage *T. spiralis*, we synchronously infected mice with NBL delivered intravenously. Using this route of infection, we can evaluate the immune response to muscle larvae without the confounding influence of intestinal worm survival or fecundity. Here we use gene-targeted knockout mice, adoptive transfer of specific T cell populations, and *in vivo* antibody treatments to determine the mechanisms by which inflammation is controlled and effector T cell responses are moderated during muscle infection.

Materials and Methods

Rats and mice

Adult Albino Oxford (AO) strain rats were produced and maintained in the James A. Baker Institute vivarium. C57BL/10$^{SgSnAi\text{-}[ko]IL10}$ (IL-10$^{-/-}$) and C57BL/10$^{SgSnAi\text{-}[ko]IL10\text{-}[ko]RAG2}$ (RAG2$^{-/-}$/IL-10$^{-/-}$) were bred at the Cornell Transgenic Mouse Core Facility and maintained at the James A. Baker Institute. Six-week old C.129S2-STAT6^{tm1Gru}/J (STAT6$^{-/-}$) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Age and gender-matched C57BL/10^{SgSnAiTac} (WT) and BALB/c^{AnNTac} mice were obtained from Taconic (Hudson, NY). RAG2$^{-/-}$/IL-10$^{-/-}$ mice were maintained in a Bioclean isolation

units (Lab Products Inc, Seaford, DE). All animals were cared for in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care. Mice and rats were fed autoclaved, pelleted ration (5K67; Jackson Laboratories, Bar Harbor, ME) and provided acidified water (pH 3).

Parasite

T. spiralis (pig strain) infectious larvae (L1) were recovered from muscles of irradiated AO rats by digestion with 1% pepsin in acidified water, as described by Crum et al. (15). Newborn larvae (NBL) were recovered from adult worm cultures prepared as described previously (7). Mice were administered a single intravenous (I.V.) injection (lateral tail vein) of 25,000 NBL suspended in 0.25 ml serum-free Dulbecco's Modified Eagle's Medium (DMEM; Mediatech Incorporated, Herndon, VA). Infection by this route bypasses the intestinal phase of infection, eliminating the intestinal immune response as a confounding variable. In addition, I.V. injection of NBL results in synchronous development of nurse cells and the host response to muscle infection. Typically, we find that approximately half of the NBL injected establish chronic infection. In other experiments, mice were infected with 5,000 NBL I.V., rested for three months and then challenged with 20,000 NBL I.V. Mice were killed by CO_2 inhalation at the times indicated in each experiment. Muscle larvae burdens were determined as described previously (7). To evaluate infectivity, muscle larvae recovered from individual mice were pooled and used to orally infect C57BL/10 mice with 250 L1. At approximately 30 days post-oral infection, muscle burdens were determined to confirm that larvae from different treatment groups were equally infective. Somatic

antigens from muscle larvae were prepared from whole larval homogenate as described previously (4).

Antibodies

Rat monoclonal antibody (mAb) to the IL-2 receptor alpha-chain (CD25) (IgG1, clone PC61 (42)), mouse mAb to TGF-β (IgG1, clone 1D11 (16); The American Type Culture Collection, Manassas, VA) and mouse mAb to equine influenza virus (IgG1, clone 5 (3)) were affinity-purified from culture supernatant using protein G and fast performance liquid chromotography (ÄKTA FPLC; Amersham Biosciences, Uppsala, Sweden). The column was equilibrated with 0.02 M sodium phosphate (pH 7.0), and bound protein was eluted with 0.1 M glycine-HCl (pH 2.7). Fractions were neutralized with 1 M Tris-HCl (pH 9.0). Purified antibody was dialyzed against phosphate buffered saline (PBS). Total rat immunoglobulin (Ig) was precipitated from normal sera with 40% ammonium sulfate (Sigma, St. Louis, MO) and dialyzed as described above. After endotoxin removal over polymyxin B columns (Detoxi-gel, Pierce, Rockford, IL), antibody preparations contained less than 1.0 unit endotoxin/mg as determined using the Limulus Amebocyte Lysate Pyrochrome assay (Associates of Cape Cod Inc., Falmouth, MA).

For flow cytometry experiments, fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (clone GK1.5, 1 μg/ml, eBioscience, San Diego, CA), allophycocyanin (APC)-conjugated anti-Foxp3 (clone FJK-16S, 5 μg/ml, eBioscience), biotinylated anti-CD25 (clone 7D4; recognizes an epitope distinct from that bound by clone PC61, 1 μg/ml, BD Pharmingen, San Diego, CA), and streptavidin-PE (1 μg/ml, eBioscience) were used. Rabbit polyclonal antibody raised against the C-terminus of mouse inducible nitric oxide

synthase (iNOS, 0.34 μg/ml) was from Lab Vision Corporation (Fremont, CA). Negative control sections were treated with normal rabbit serum (Pel-Freez, Rogers, AR).

Histology and Immunohistochemistry

Tissues for microscopic examination were prepared and stained with hematoxylin and eosin (H & E) as described previously (7). For immunohistochemistry, formalin-fixed, paraffin-embedded sections were mounted on poly-L-lysine-coated glass slides, deparaffinized for 10 min in xylene and rehydrated through graded alcohol baths. Antigen retrieval was carried out by steaming tissue sections for 40 min in 10 mM citric acid (pH 6.0) using a rice steamer (Black & Decker, Hampstead, MD). Sections were allowed to cool for 20 min at room temperature. Staining was carried out as described previously (7). Slides were dehydrated through graded alcohol baths, cleared in xylene and mounted in Permount (Fisher Scientific, Pittsburgh, PA).

Recovery and culture of lymphocytes from cervical lymph nodes.

Cervical lymph nodes (CLN) drain the tongue and masseter muscles in the mouse and were assayed to monitor the immune response to muscle infection. CLN were excised from infected mice and kept on ice in DMEM supplemented with 10% FCS (Atlanta Biologicals, Lawrenceville, GA), 0.1 M non-essential amino acids (Gibco, Grand Island, NY), 30 mM HEPES (Gibco), and 50 μM 2-mercaptoethanol (Sigma). CLN from individual mice were manually dispersed, under aseptic conditions, in petri dishes using a 12cc syringe pestle. Cells were passed through sterile 100 μm screens, washed

with medium, and cell number was determined using a Coulter Counter (Model Z2, Beckman Coulter, Fullerton, CA). Cells were plated in triplicate at 1 x 10^6 cells/well in 200 μl on 96-well tissue culture plates (Costar, Corning Inc., Corning, NY) and restimulated with 10 μg/well of somatic larval antigens to elicit cytokine secretion from parasite-specific T cells. Cells were incubated at 5% CO_2 and supernatants were collected after 72 hours and stored at -20ºC.

Cytokine-specific enzyme-linked immunosorbant assays (ELISA)

Cytokine ELISA to measure concentrations of IL-4, IL-5, IL-10 and IFN-γ were carried out as described previously (12). IL-13 ELISA used capture (clone 38321; 2 μg/ml) and detecting antibodies (goat polyclonal; 0.1 μg/ml) from R & D Systems (Minneapolis, MN).

Flow Cytometry

Cells were recovered from individual diaphragms by digestion in collagenase I and stained for flow cytometric analysis as described previously (7).

Adoptive transfer experiments

Single cell suspensions were prepared from CLN recovered from WT (n=8) and IL-$10^{-/-}$ (n=8) donor mice at 20 dpi as described above. $CD4^+CD25^+$ cells (Tregs) and $CD4^+CD25^-$ cells (Teff) were purified from CLN using the Mouse Regulatory T cell Isolation Kit and an AutoMACS magnetic cell separator (Miltenyi Biotec, Auburn, CA). CLN cells were subjected to two rounds of negative selection for $CD4^+$ T cells and were 97% pure by flow cytometric analysis. Treg and Teff populations were then enriched by positive

and negative selection and were 83% and 91% enriched, respectively. One day prior to infection, RAG2$^{-/-}$/IL-10$^{-/-}$ mice received 1x10^4 Treg cells and 1x10^5 Teff cells, intraperitoneally. Mice were intravenously infected with 25,000 NBL the following day. At 13 dpi, CLN cells and tongue were collected for cytokine assays and histopathology/immunohistochemistry, respectively.

In vivo antibody treatments

For depletion of CD25$^+$ cells, mice were injected once, intraperitoneally, with 750 μg of anti-CD25 or rat Ig (control). The following day, mice were injected with 25,000 NBL. At 20 dpi, cells were recovered from diaphragm and stained for flow cytometric analysis to confirm extent of Treg depletion. In separate experiments, mice were administered 1 mg of antibody to TGF-β or mouse influenza virus (control) each week beginning at day –1. Mice were infected at day 0 and sera collected at 20 dpi were analyzed for active TGF-β using a luminescence-based bioassay (see below). At times indicated, tongues, diaphragms, CLN and carcasses were recovered for histopathology/immunohistochemistry, flow cytometry, cytokine assays and muscle burdens, respectively.

Luminescence-based TGF-β bioassay

Serum levels of active TGF-β were measured as described previously (59) using mink lung epithelial cells (clone 32, provided by Dr. Daniel Rifkin; New York University Medical Center, New York, NY) cultured in X-VIVO-15 serum-free medium (Cambrex Bio Science, Walkersville, MD). Luminescence was measured using the Luminescent Substrate System (Promega, Madison, WI) and a Veritas Microplate Luminometer equipped with reagent injectors

(Turner Biosystems, Sunnyvale, CA). Concentrations of TGF-β in sera were estimated using a standard curve prepared with recombinant human TGF-β1 (Roche Diagnostics GmbH, Mannheim, Germany).

Statistical Analysis

Means and standard deviations were calculated from data (diaphragms, CLN, sera, etc.) collected from individual mice. Significance was determined using Student's t test or one-way ANOVA with Tukey's posthoc analysis. Statistical analysis was performed with GraphPad Prism 4 software.

Results

Influence of IL-10 on the T cell response to muscle infection.

We previously demonstrated that IL-10 limits inflammation during parasite development in the muscle, but not during chronic infection (7). To determine the influence of IL-10 on the T cell response to muscle infection, we examined the cytokine profile of CLN cells recovered from WT and IL-10$^{-/-}$ mice during parasite development (8 dpi; acute muscle infection) and at the completion of parasite development (21 dpi; transition to chronic infection). After restimulation with somatic larval antigens, WT CLN cells produced IL-10 and IFN-γ at 8 dpi (Figure 4.1A). By 21 dpi, IL-10 levels were increased, IL-5 and IL-13 production was detectable and IFN-γ production was unchanged (Figure 4.1B). In the absence of IL-10, mice produced nearly 8-times more IFN-γ at 8 dpi (Figure 4.1C) and 21 dpi (Figure 4.1D), compared to WT counterparts. IFN-γ is a potent activator of macrophage activity, resulting in increased production of inducible nitric oxide synthase (iNOS) and enhanced generation of reactive nitrogen intermediates. We have previously shown

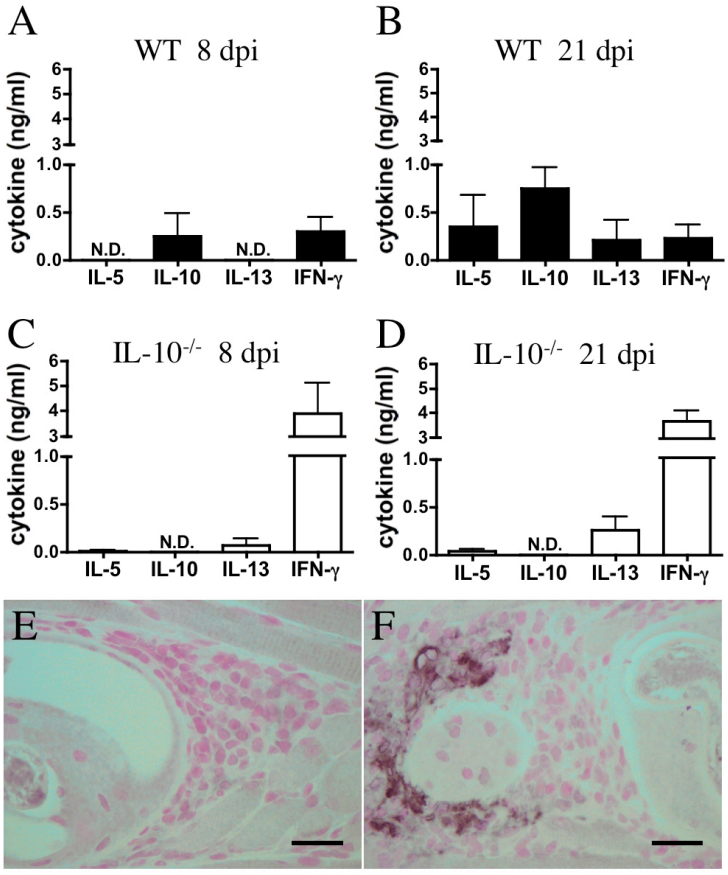


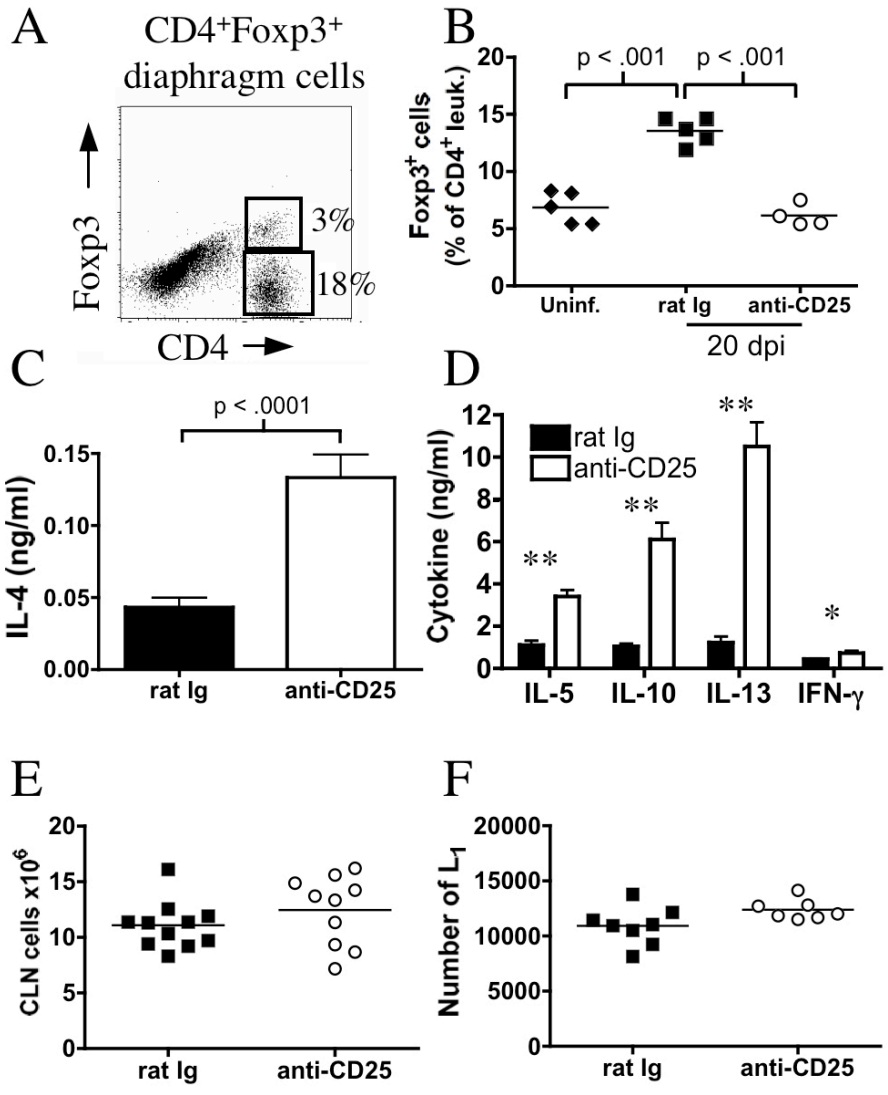
Figure 4.1 The influence of IL-10 on development of the T cell response to muscle infection. (A-D) Cytokines produced by CLN from C57BL/10 (WT) mice and IL-10$^{-/-}$ mice at 8 and 21 dpi after restimulation with somatic larval antigen. Means and standard deviations are shown from groups of 3-5 mice. (E and F) Detection of inducible nitric oxide synthase (iNOS) at 21 dpi; brown indicates iNOS-producing cells. Scale bars = 25 μm.

that the cellular infiltrate recruited to infected muscle is rich in macrophages. Sections of infected tongue were stained with antibody to iNOS to determine whether the increase in IFN-γ measured in the cytokine assays would correlate with altered macrophage phenotype *in vivo*. Nurse cells in WT mice rarely had $iNOS^+$ cells at 21 dpi (Figure 4.1E). In contrast, $IL\text{-}10^{-/-}$ mice had abundant numbers of $iNOS^+$ cells surrounding infected myotubes (Figure 4.1F). Taken together, these data show that IL-10-mediated suppression of inflammation is associated with reduced IFN-γ production and local iNOS synthesis, but does not prevent the development of a TH2 response later in infection.

Depletion of naturally-occurring regulatory T cells during muscle infection.

IL-10 is a product of $CD4^+CD25^+$ Treg cells that suppress TH1-driven inflammation (6, 45). To evaluate whether Treg cells were required for the IL-10-dependent suppression of IFN-γ during muscle infection, WT mice were injected with antibody to CD25 (clone PC61, n = 10) or rat Ig (n = 11). This treatment has been reported to deplete Treg cells; however, recent evidence suggests that anti-CD25 functionally inactivates these cells by inducing down-regulation of the IL-2 receptor (39). To determine the extent of Treg depletion/inactivation, we recovered cells that infiltrate the diaphragms of Treg-depleted or control mice at 20 dpi. Cells were stained with antibodies to CD4 and CD25 and analyzed by flow cytometry. We found that 8.4% ± 1.5% of diaphragm leukocytes expressed CD25 at 20 dpi. Treatment with anti-CD25 reduced this percentage to 4.6% ± 1.0%. In addition to being expressed on the surface of Tregs, CD25 is also transiently up-regulated on the surface of effector T cells after activation (40, 41). To confirm that anti-CD25

Figure 4.2 Depletion of naturally-occurring Treg cells during muscle infection. (A) Representative dot plot of diaphragm cells showing $CD4^+$ and $CD4^+Foxp3^+$ cells as a percentage of gated lymphocytes at 20 dpi. (B) $Foxp3^+$ cells as a percentage of $CD4^+$ cells from uninfected and 20 dpi diaphragm from mice treated with anti-rat Ig or anti-CD25. (C-D) IL-4, IL-5, IL-10, IL-13 and IFN-γ concentrations in antigen restimulated cultures of CLN cells from mice treated with anti-CD25 (n=10) or rat Ig (n=11). Significance was determined by (B) one-way ANOVA with Tukey's posthoc analysis or (C-F) Student's t test; $p < 0.001$ (**) and $p = 0.016$ (*). (E) Cellularity of CLN and (F) larval burdens. Symbols in B, E and F represent individual mice.


A
CD4+Foxp3+
diaphragm cells
Foxp3
CD4
3%
18%
B
p < .001
p < .001
Foxp3+ cells
(% of CD4+ leuk.)
Uninf.
rat Ig
anti-CD25
20 dpi
C
p < .0001
IL-4 (ng/ml)
rat Ig
anti-CD25
D
rat Ig
anti-CD25
Cytokine (ng/ml)
IL-5
IL-10
IL-13
IFN-γ
E
CLN cells x10^6
rat Ig
anti-CD25
F
Number of L1
rat Ig
anti-CD25

treatment had an effect on the Treg population specifically, we analyzed the proportion of diaphragm $CD4^+$ T cells that expressed Foxp3 at 20 dpi. Foxp3 is a definitive Treg marker (20, 29) that is required for suppressor cell development (21). Staining of diaphragm cells with anti-CD4 and anti-Foxp3 revealed a discrete $CD4^+Foxp3^+$ cell population (Figure 4.2A). In diaphragms of uninfected mice, approximately 7% of the $CD4^+$ cells expressed Foxp3 (Figure 4.2B). This percentage increased to approximately 14% at 20 dpi, and was reduced by half after *in vivo* treatment with anti-CD25 (Figure 4.2B).

Reduction of $Foxp3^+$ cells correlated with dramatically elevated production of IL-4, IL-5, IL-10 and IL-13 by CLN cells restimulated with parasite antigen (Figure 4.2C and 4.2D). A modest but significant increase in IFN-γ was also detected. Despite exaggerated TH2 responses, cellularity in CLN (Figure 4.2E) and larval burdens (Figure 4.2F) were unaffected. Larvae recovered from Treg depleted and control mice at 20 dpi were equally infective when passaged into WT mice, producing muscle burdens of 40,250 ± 8826 L1 (n = 4) and 38,333 ± 6691 L1 (n = 3), respectively.

These results show that naturally-occurring Treg cells accumulate in infected muscle. Reducing this population of cells by *in vivo* antibody treatment led to a dramatic increase in TH2, but not TH1, cytokine production. Increased TH2 responses did not affect local inflammation or larval burdens. Moreover, since Treg depletion caused only a modest change in IFN-γ production, it is unlikely that these cells are a critical source of IL-10 during infection.

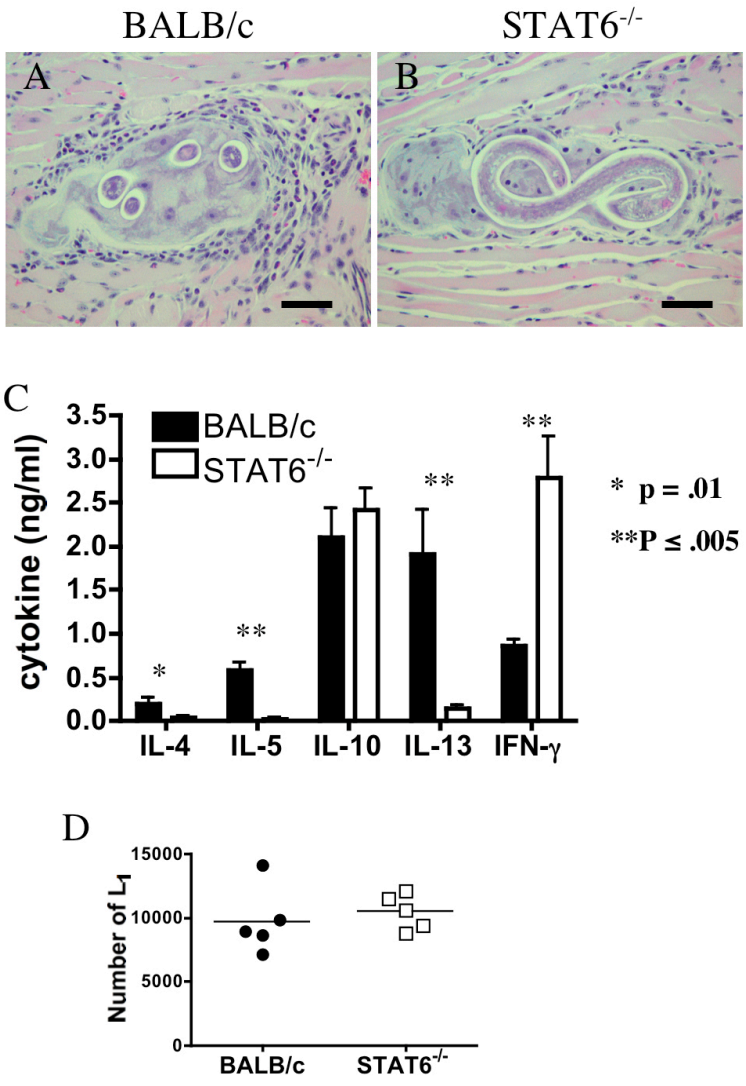


Figure 4.3 The influence of STAT6 signalling on the inflammatory response to muscle infection. (A and B) H & E stained tissue sections of tongues from BALB/c and STAT6$^{-/-}$ mice at 18 dpi. (C) Cytokine concentrations in antigen restimulated cultures of CLN cells from STAT6$^{-/-}$ (n=6) and BALB/c mice (n=5). (D) Larval burdens at 18 dpi. Symbols in D represent individual mice. Scale bars = 50 μm. Significance was determined by Student's t test.

The influence of TH2 cytokines on the inflammatory response to muscle infection.

To further investigate the importance of TH2 cytokines on the outcome of muscle infection, we infected mice that were deficient in signal transducer and activator of transcription-6 (STAT6). These mice are on a BALB/c background and would be expected to mount a stronger TH2 response than C57BL/10 mice used in previous experiments. STAT6 is required to transduce signals from IL-4 and IL-13 after binding to their surface receptors (33). $STAT6^{-/-}$ (n = 6) or BALB/c (n = 5) control mice were synchronously infected. H & E stained sections of tongue recovered from BALB/c mice at 18 dpi show numerous mature larvae and fully developed nurse cells surrounded by focal infiltrates (Figure 4.3A). In contrast, infiltrates around nurse cells of $STAT6^{-/-}$ mice were sparse (Figure 4.3B). When cultured in the presence of somatic larval antigens, CLN cells from BALB/c mice produced significantly more IL-4, IL-5 and IL-13, but less IFN-γ, compared to $STAT6^{-/-}$ counterparts at 18 dpi (Figure 4.3C). IL-10 levels were comparable, suggesting that, in the presence of IL-10, inflammation is normally driven by TH2 cytokines. Finally, despite impairment of TH2 responses and inflammation in $STAT6^{-/-}$, larval burdens were similar at 18 dpi (Figure 4.3D). Larvae recovered from BALB/c and $STAT6^{-/-}$ mice at 18 dpi were equally infective when passaged into WT mice, producing muscle burdens of 25,867 ± 6275 L1 (n = 3) and 17,133 ± 4868 L1 (n = 3), respectively.

Adoptive transfer experiments reveal a critical source of IL-10 during muscle infection.

To determine the T cell subset responsible for IL-10-mediated suppression of IFN-γ production during infection, we established an adoptive transfer system in which we could limit the source of IL-10 to donor-derived $CD4^+CD25^+$ Treg or $CD4^+CD25^-$ Teff cells. Enriched Treg and Teff cell populations were prepared from CLN recovered from WT and IL-$10^{-/-}$ mice at 20 dpi, a time when we observe the strongest influence of IL-10 (7). 1 x 10^4 Treg cells were transferred together with 1 x 10^5 Teff cells, a ratio that is normally found in mice (57). Recipient $RAG2^{-/-}$/IL-$10^{-/-}$ mice are devoid of T and B lymphocytes and have no endogenous source of IL-10. Thus, transferred Treg or Teff cells were the only source of IL-10.

Mice that received WT Teff cells were able to control myositis, regardless of whether these cells were co-transferred with WT (Figure 4.4A) or IL-$10^{-/-}$ Treg cells (Figure 4.4B). In contrast, mice receiving IL-$10^{-/-}$ Teff cells, whether in combination with WT (Figure 4.4C) or IL-$10^{-/-}$ Treg cells (Figure 4.4D), had more extensive myositis. Antigen-specific IFN-γ production was observed only in mice that received Teff cells from IL-$10^{-/-}$ donors, irrespective of the source of Treg cells (Figure 4.4E). These findings support the conclusion that IL-10, produced by Teff cells but not Treg cells, is necessary and sufficient to suppress IFN-γ and inflammation during muscle infection (Figure 4.4E).

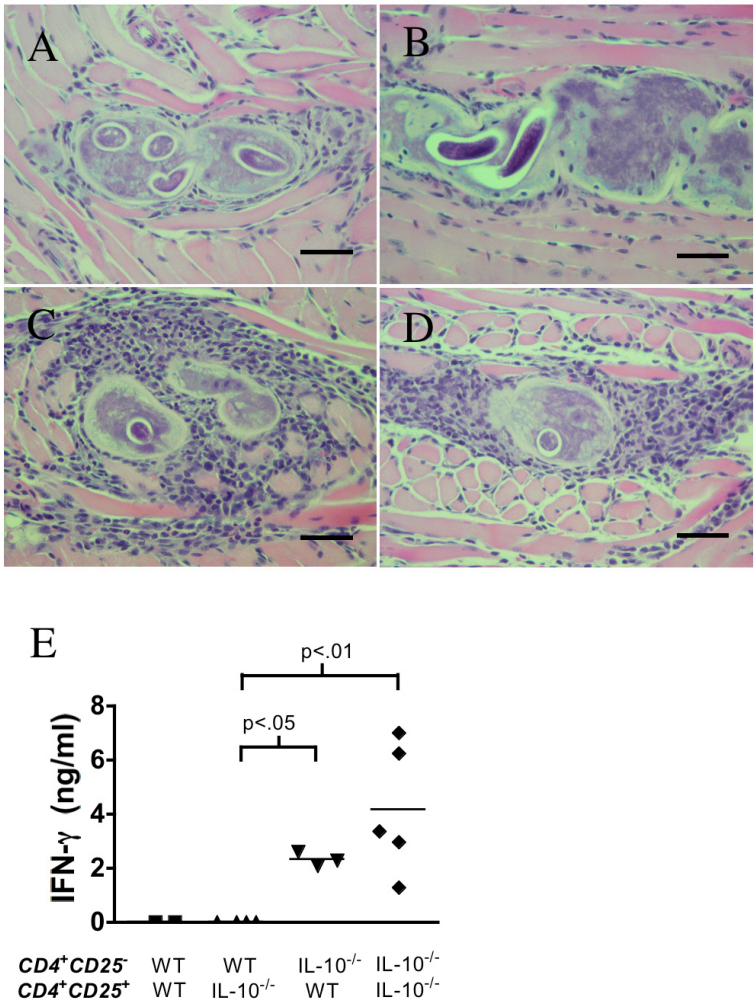


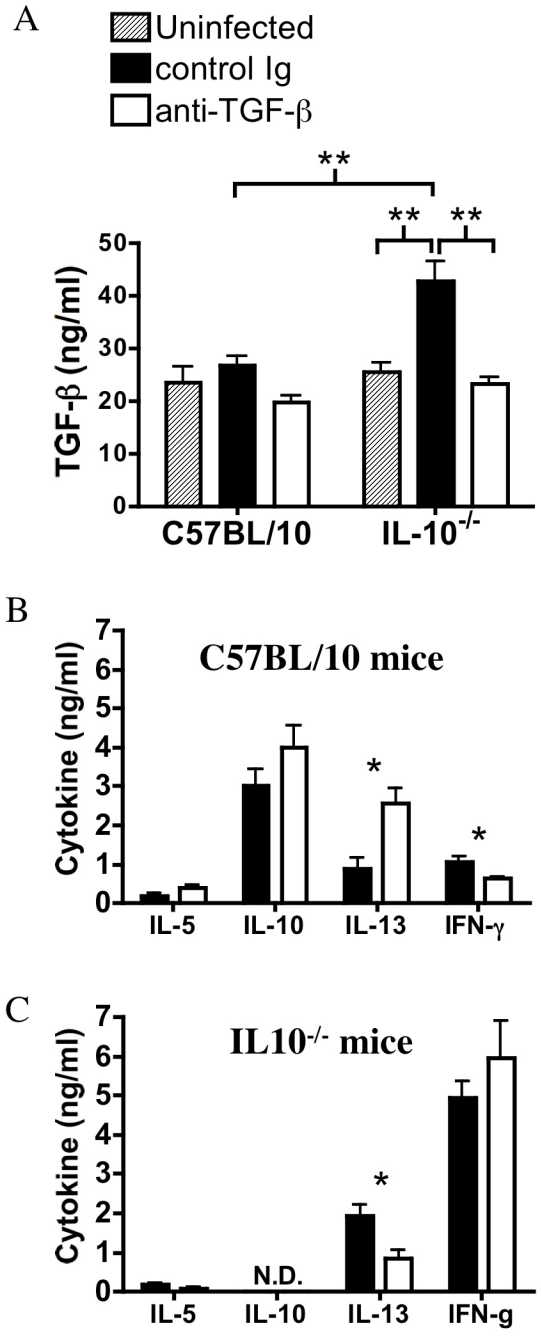
Figure 4.4 Relative contributions of $CD4^+CD25^-$ and $CD4^+CD25^+$ cell-derived IL-10 production to suppression of TH1-driven inflammation in infected muscle. (A-D) H & E stained sections of diaphragm from $RAG2^{-/-}$/$IL\text{-}10^{-/-}$ mice (13 dpi) that received $CD4^+CD25^-$ and $CD4^+CD25^+$ cells from either WT or $IL\text{-}10^{-/-}$ donors. (E) IFN-γ concentrations in antigen restimulated cultures of CLN cells from recipient mice. Symbols represent individual mice. Significance was determined by one-way ANOVA with Tukey's posthoc analysis. Scale bars = 50 μm.

Effect of TGF-β neutralization on the cytokine and inflammatory response to muscle infection.

Both surface-bound and secreted TGF-β have been shown to be important mechanisms of Treg-mediated control of effector T cells (37, 50, 51). To evaluate a role for TGF-β in control of T cell responses, we neutralized this cytokine *in vivo* by weekly administration of antibody that binds both TGF-β1 and -β2 isoforms (16). We determined the extent of neutralization using a bioassay that measures the active form of TGF-β (1). Muscle infection with *T. spiralis* induced a significant elevation of active TGF-β in sera of IL-10$^{-/-}$ but not WT mice (Figure 4.5A). Treatment with TGF-β neutralizing antibody reduced serum concentrations of active cytokine to pre-infection levels (Figure 4.5A). CLN from WT mice treated with anti-TGF-β produced significantly more IL-13 and less IFN-γ compared to control Ig-treated WT mice (Figure 4.5B). In contrast, neutralization of TGF-β in IL-10$^{-/-}$ mice caused a reduction of IL-13 (Figure 4.5C).

As expected, WT mice treated with control Ig had limited myositis at 20 dpi (Figure 4.6A), while IL-10$^{-/-}$ mice treated with control Ig had more intense cellular infiltrates surrounding nurse cells (Figure 4.6C). Compared to control Ig treated WT mice, WT mice treated with anti-TGF-β had more pronounced myositis (Figure 4.6B). Mice deficient in IL-10 and treated with anti-TGF-β had the most extensive cellular infiltration of muscle (Figure 4.6D) and the greatest cellularity in CLN (Figure 4.6E). Although IL-10 or TGF-β alone did not significantly protect muscle larvae from destruction, the combined deficiency in both cytokines resulted in a significant reduction in muscle larvae compared to WT mice treated with control Ig (Figure 4.6F). Viable larvae recovered from

Figure 4.5 Influence of TGF-β on T cell responses in WT and IL-10$^{-/-}$ mice. (A) Active TGF-β in sera of uninfected (n=5) and 20 dpi WT and IL10$^{-/-}$ mice. (B) Cytokines concentrations in antigen restimulated cultures of CLN cells from WT mice treated with anti-TGF-β (n=7) or control Ig (n=8), and (C) IL-10$^{-/-}$ mice treated with anti-TGF-β (n=8) or control Ig (n=6). N.D.= not detected. Asterisks mark significance (*) $p < 0.05$, (**) $P < 0.001$ as determined by (A) one-way ANOVA with Tukey's posthoc analysis or (B and C) Student's t test.


A
Uninfected
control Ig
anti-TGF-β
**
**
**
TGF-β (ng/ml)
50
40
30
20
10
0
C57BL/10
IL-10-/-
B
C57BL/10 mice
Cytokine (ng/ml)
7
6
5
4
3
2
1
0
*
*
IL-5
IL-10
IL-13
IFN-γ
C
IL10-/- mice
Cytokine (ng/ml)
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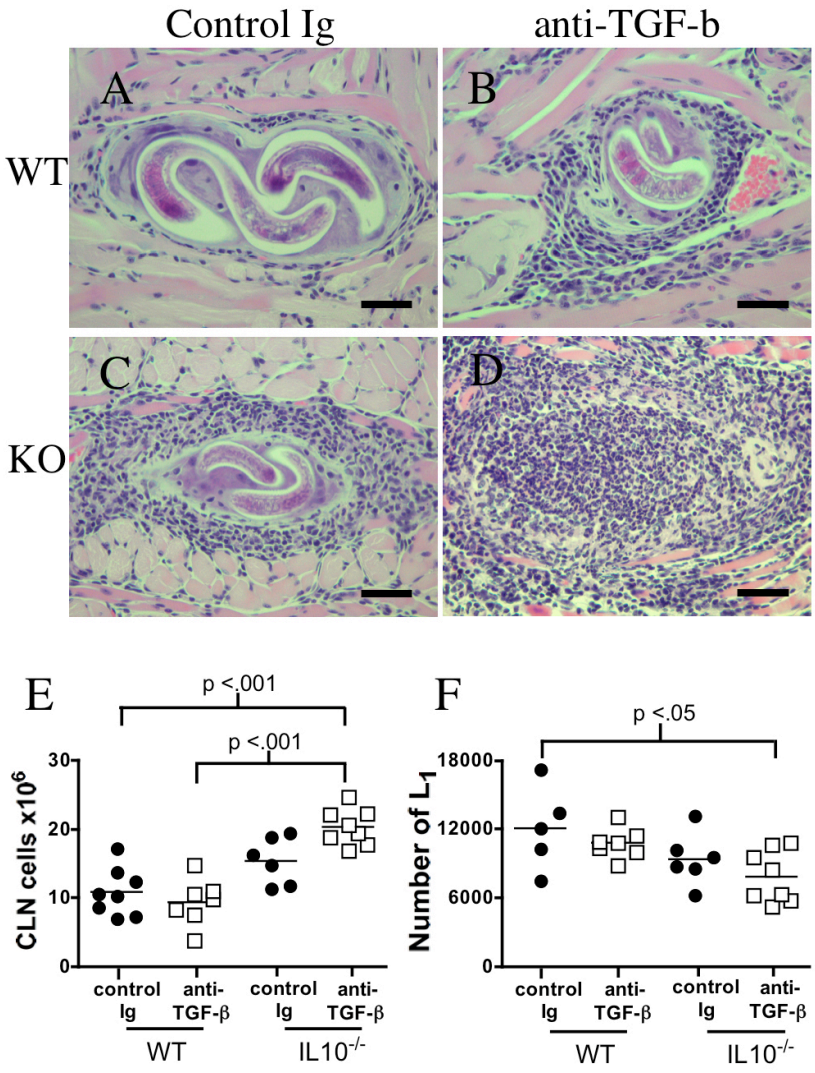

Figure 4.6 TGF-β and control of myositis and parasite survival. (A-D) H & E stained tongue sections from WT and IL-10$^{-/-}$ mice at 20 dpi treated with either control Ig or anti-TGF-β. (E) Cellularity in CLN and (F) larval burdens. Symbols represent individual mice in panels E and F. Significance was determined by one-way ANOVA and Tukey's posthoc analysis.

all 4 groups of mice were equally infective when passaged into WT mice (n = 5 for each group) producing muscle burdens of 15,200 ± 4361 L1 (WT, control Ig); 23,600 ± 4347 L1 (WT, anti-TGF-β); 25,880 ± 11,140 L1 (IL-10$^{-/-}$, control Ig) and 16,240 ± 6121 L1 (IL-10$^{-/-}$, anti-TGF-β).

Effects of TH2 or mixed TH1/TH2 responses on immunity to muscle stage parasites.

To better understand the type of immune response that is required for destruction of muscle larvae, we developed an *in vivo* challenge model in which WT and IL-10$^{-/-}$ mice were first infected with a low dose (5000 NBL), rested for 3 months to allow the larvae to establish a chronic infection, and then challenged with 20,000 NBL to elicit a memory response (1º/2º). For controls, additional mice received either the priming dose only (1º/-), or challenge dose only (-/2º). WT mice receiving both a prime and challenge dose mounted a polarized TH2 response characterized by IL-5, IL-10 and IL-13 production, with low levels of IFN-γ evident by 8 days post-challenge (Figure 4.7A). If larvae survived this TH2-polarized response, we would expect WT 1º/2º mice to harbor the combined burdens of the 1º infection (2221 ± 575 L1) and the 2º infection (9066 ± 2505 L1) to give an expected burden of approximately 11,287 L_1. To the contrary, 1º/2º mice had a mean muscle burden of 8874 L_1 ± 3679 at 20 dpc (Figure 4.7B), demonstrating killing of approximately 21% of the larvae. IL-10$^{-/-}$ 1º/2º mice produced significantly more IFN-γ, but similar levels of IL-5 and IL-13, compared to WT mice (Figure 4.7A). If larvae survived the mixed response seen after challenge of the IL-10$^{-/-}$ mice, we would expect muscle burdens of approximately 9432 L1, rather than the observed mean burden of 4915 ± 2878 L1 (48% killing; Figure 4.7C).

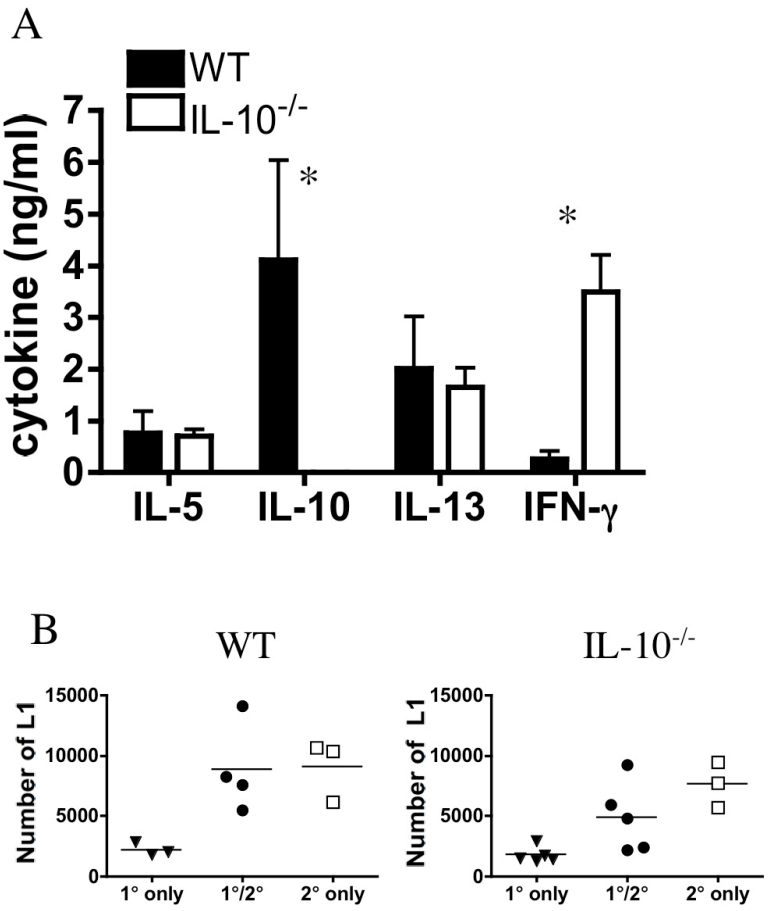

Figure 4.7 TH2-polarized and mixed TH1/TH2 responses in parasite killing. (A) Cytokine concentrations in antigen restimulated cultures of CLN cells from C57BL/10 (WT) and IL-$10^{-/-}$ mice 8 days post-challenge. (B) Larval burdens at 21 days post-challenge. Symbols represent individual mice. Asterisk marks statistical significance ($p < 0.001$) as determined by Student's t test.

Killing of worms was evident in histologic sections of diaphragm as well. WT mice receiving only the secondary infection had no inflammation surrounding developing larvae at 8 dpi (Figure 4.8A). In contrast, WT 1º/2º mice had extensive inflammation in muscle (Figure 4.8C), characterized by a cellular infiltrate rich in eosinophils (Figure 4.8C, inset). Compared to WT mice, IL-10$^{-/-}$ receiving only a secondary infection had more intense inflammatory reactions surrounding developing larvae (Figure 4.8B). The most severe myositis was seen in IL-10$^{-/-}$ 1º/2º mice (Figure 4.8D), in which an infiltrate largely comprised of macrophages and nearly devoid of eosinophils (Figure 4.8D, inset) incarcerated some larvae. Large numbers of iNOS producing cells were detected in diaphragms of IL-10$^{-/-}$ (Figure 4.8F) but not WT mice (Figure 4.8E) that had been primed and challenged.

Discussion

Our previous work demonstrated a role for IL-10 in limiting inflammation during *T. spiralis* development in the muscle, yet the cell types responsible for this modulation, as well as the mechanism by which IL-10 mediated suppression, were not identified. The data presented here show that IL-10 limits acute inflammation, suppresses IFN-γ levels, and prevents iNOS production by inflammatory cells recruited to the site of infection. In our system, IL-10 deficiency did not influence the production of IL-4, IL-5 or IL-13, nor did it reduce titers of parasite-specific IgG1 (7), suggesting that IL-10 producing cells specifically suppress TH1 responses during muscle infection.

The influence of IL-10 on the inflammatory response in muscle is only evident during parasite development, a time that is coincident with significant damage to host muscle cells caused by parasite migration and growth. It has

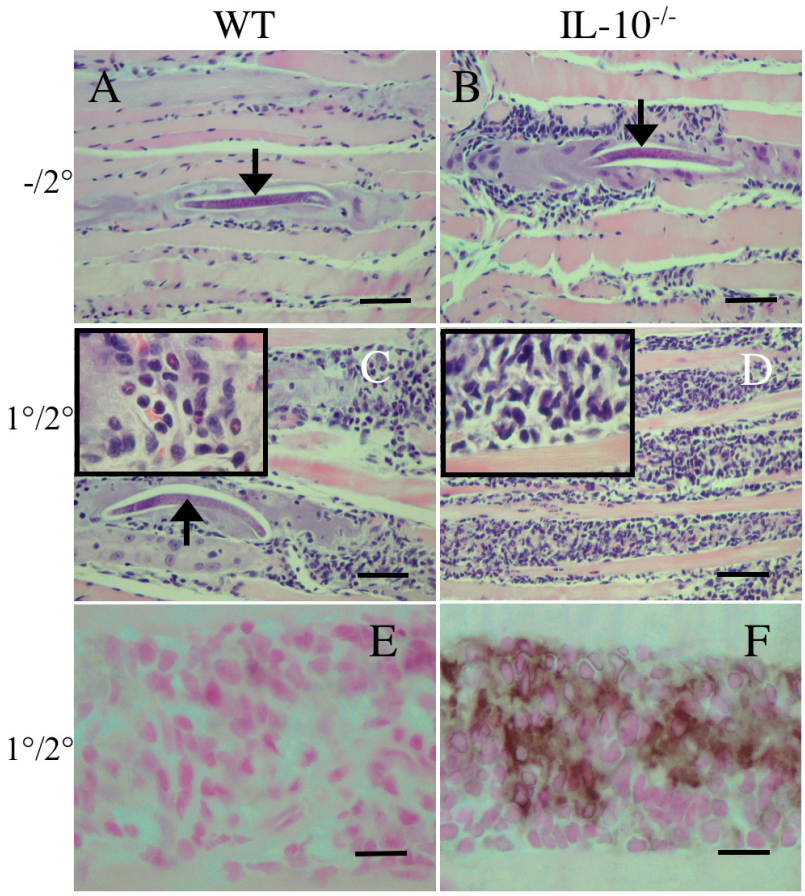

Figure 4.8 Histological evidence of worm destruction during NBL challenge. (A-D) H & E stained sections of tongue from C57BL/10 (WT) and IL-$10^{-/-}$ mice receiving prime and challenge (1°/2°) or only the secondary infection (-/2°). (E and F) Immunohistochemical detection of iNOS in sections of diaphragm from WT and IL-$10^{-/-}$ mice. Arrows indicate immature larvae. Scale bars = 50 μm (A-D) or 30 μm (E and F).

been reported that tissue damage releases extracellular matrix (ECM) components that can trigger activation of dendritic cells and macrophages. For example, heparan sulfate, a major constituent of ECM and cell surface proteoglycans, stimulates Toll-like receptor-4 (TLR4)-dependent maturation of dendritic cells resulting in production of the proinflammatory cytokines TNF-α, IL-6, and IL-1β (32, 38). Coincidently, we have recently reported that the *T. spiralis* nurse cell is rich in cytoplasmic heparan sulfate (8). It is possible that early IL-10 production stems directly from the need to control pathogenic responses to tissue damage, but specific experiments need to be designed to test this hypothesis. While increased IFN-γ levels may be responsible for exaggerated inflammation seen in *T. spiralis* infected IL-$10^{-/-}$ mice, we cannot rule out the possibility that IL-10 deficiency may influence other pro-inflammatory mediators that were not examined in this study.

It has been demonstrated that Treg cells require IL-10 to mediate protection from TH1-driven intestinal inflammation (6, 45). We investigated whether this cell population was important in control of TH1 responses and myositis during *T. spiralis* infection. We determined the frequency $CD4^{+}Foxp^{+}$ Treg cells in infected diaphragm by flow cytometry and found that infection induced an enrichment of this population in muscle. Infection with the protozoan parasite *Leishmania major* also causes enrichment of Treg cells at the site of infection in the skin (10). In this setting, IL-10 produced by Treg cells is critical in limiting TH1-driven parasite clearance (9) and establishing host protective immunity (47). In contrast, we found that a reduction in the Treg population in infected diaphragm by anti-CD25 treatment was associated with increased TH2 cytokine production. Similarly, treatment with anti-CD25 to

deplete Treg cells during infection with the filarial nematodes *B. pahangi* (23) and *L. sigmondontis* (59) elevated parasite-specific TH2 responses.

Despite the exaggerated TH2 response seen after Treg depletion during muscle infection, we did not observe increased inflammation or parasite killing. To further evaluate the impact of TH2 cytokines on myositis we infected BALB/c and STAT6$^{-/-}$ mice. As expected, based on their genetic background, BALB/c mice (Figure 4.3C) mounted more intense TH2 response than C57BL/10 mice (Figure 4.1B). In the absence of STAT6, IL-10 production was unaffected but TH2 responses were significantly impaired, and mice had reduced focal infiltrates in muscle. These studies suggest that in the presence of IL-10, TH2 cytokine signaling promotes development of the inflammatory response in muscle. Taken together with our Treg depletion data, we hypothesize that Treg cells induced during infection suppress TH2-responses that have the potential to promote inflammation.

We did not phenotype IL-10 producing cells during muscle infection. Instead, we took a mechanistic approach to identifying cells that mediate IL-10-dependent suppression of inflammation *in vivo*. Focusing on CD4 T cell populations, we conducted adoptive transfer experiments with CD4^{+}CD25^{-} Teff and CD4^{+}CD25^{+} Treg cells derived from infected WT and IL-10$^{-/-}$ donors. Since recipient mice were RAG2$^{-/-}$/IL-10$^{-/-}$ the source of IL-10 would be restricted to transferred Treg and/or Teff cells. There are potential drawbacks to this system. First, transferred cells would be expected to undergo expansion after transfer, possibly resulting in cell numbers or distributions not normally found in WT mice. To address this concern, we transferred small numbers of cells, infected mice the following day, and terminated the experiment 13 days later. Regardless, we found that IL-10 produced by

$CD4^+CD25^-$, but not $CD4^+CD25^+$ cells, was required to control myositis and IFN-γ production (Figure 4.4). IL-10 production has been described for suppressive CD4 T cell populations that are distinguished based on their ability to produce IL-4 (TH2 cells), IFN-γ (TH1 cells), both IL-4 and IFN-γ (Tr1 cells), or TGF-β (TH3 cells) (Reviewed in (25)). Since none of these cells typically express CD25, it is possible that one or more of the subsets may be partially or entirely responsible for the suppressive effect of the $CD4^+CD25^-$ cells in our adoptive transfers.

TGF-β and IL-10 have been shown to have an additive effect on suppression of T cell activity *in vitro* (66). We observed a similar relationship between these two cytokines during *T. spiralis* infection. Neutralization of TGF-β in IL-$10^{-/-}$ mice resulted in myositis that was more severe than that seen in mice deficient in IL-10 or TGF-β alone. These observations contrast with results in *S. mansoni* studies where neutralization of TGF-β, administration of soluble TGF-β receptor, or deficiency in Smad3 signaling did not significantly alter granuloma formation (35). Our data has also shown that, although IL-10 and TGF-β both limited inflammation, they had very different effects on the T cell response to infection. Unlike IL-10, TGF-β did not limit IFN-γ or iNOS production (data not shown). It is possible that IL-10 and TGF-β may target different cell types to achieve suppression, but this remains to be examined.

Only the combined deficiency in IL-10 and TGF-β resulted in significant parasite destruction. Arasu and collegues demonstrated that TGF-β is sufficient to induce reactivation of the canine hookworm *Ancylostoma caninum in vitro* (5). Moreover, orthologues of mammalian TGF-β regulate the development of free-living nematodes (53) and have been identified in a number of parasitic nematode species (14, 22, 24). Since we do not observe

significant parasite destruction after TGF-β neutralization in WT mice, it is likely that killing observed in IL-10$^{-/-}$ mice is immune-mediated rather than a result of direct influences of TGF-β on larval development.

To better understand the cytokine profile required for parasite killing, we primed and challenged WT and IL-10$^{-/-}$ mice with NBL. We reasoned that destructive responses would be relevant targets for immune suppression. Our results show that in WT mice, the memory response to infection is TH2 polarized and has limited ability to kill larvae; however, in the absence of IL-10 a mixed response dominates and results in enhanced parasite killing. A similar observation has been made after vaccination of mice with *S. mansoni* cercariae, where it was found that both TH1- and TH2-polarization protect from a challenge infection (2, 13, 28), while a strong but mixed response to vaccination affords the highest protection (28). The mechanisms of larval killing activated by different T cell environments are not well understood, but there is evidence that TH1 responses may be effective in killing larval stages of parasites (55, 63). When eggs of the tapeworm *Taenia solium* are ingested, oncospheres hatch in the intestine and migrate to muscle where they establish chronic infection as cysticerci. Studies in a murine model of cysticercosis showed that elevated TH2 responses during chronic infection correlate with increased parasite load in susceptible BALB/c mice (60, 62). Conversely, resistant C57BL/6 mice, mount a protective TH1 response that can be reversed by administration of neutralizing antibodies to IFN-γ (61) or gene deficiency in STAT4 (56).

Collectively, our data support a model of T cell suppression during muscle infection in which effector T cell-derived IL-10 limits acute myositis, IFN-γ production and synthesis of iNOS by infiltrating cells. This inhibition is

important to the success of the parasite, as TH1 responses can kill developing *T. spiralis* larvae. In the presence of IL-10, TH2 cytokines are produced as infection progresses, promoting the development of an inflammatory response in muscle. Uncontrolled TH2 cytokine production is potently suppressed by $CD4^{+}CD25^{+}$ Treg cells. TGF-β facilitates control of both inflammation and T cell responses during infection. The combined effects of these suppressive mechanisms protect parasites in the muscle and limit disease, setting the stage for chronic infection of the skeletal muscle and maximizing the potential for transmission.

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CHAPTER FIVE

Conclusions and Future Directions

The work described in this dissertation identifies several regulators of the inflammatory response to muscle-stage *Trichinella spiralis*. Prior to these studies only limited, descriptive information relating to the histologic nature of focal infiltrates in infected muscle was published (28, 29). All of our experiments utilized synchronous (intravenous) infections, thereby bypassing the intestine and ensuring that the mechanisms of immune suppression that we identified were activated in response to infection of the muscle.

Collectively, the data presented here support a model (Figure 5.1) in which acute muscle infection triggers a TH1 response characterized by parasite-specific IFN-γ production, increased inflammation and local induction of inducible nitric oxide synthase (iNOS). In order to establish a direct connection between IFN-γ and myositis, experiments need to be conducted in which IFN-γ is neutralized in IL-$10^{-/-}$ mice during infection. Although IL-10 can be produced by many different cell types (4, 5, 10, 16, 23, 25), our adoptive transfer studies revealed that effector T cells (Teff) were the critical source of this cytokine and were sufficient to limit IFN-γ and iNOS production. We defined Teff cells as CD4+CD25-. In reality, this population of cells may include several distinct IL-10-producing subsets separable based on co-secretion of other cytokines (reviewed in (10)). It will be important to identify whether IL-10-mediated suppression is restricted to cells that produce TH2 cytokines, or is a quality of distinct CD4+CD25- suppressor cells. To address this question, mice that report IL-4 production with green fluorescent protein (GFP) could be back-crossed to an IL-10 deficient background and infected with *T. spiralis* (24).

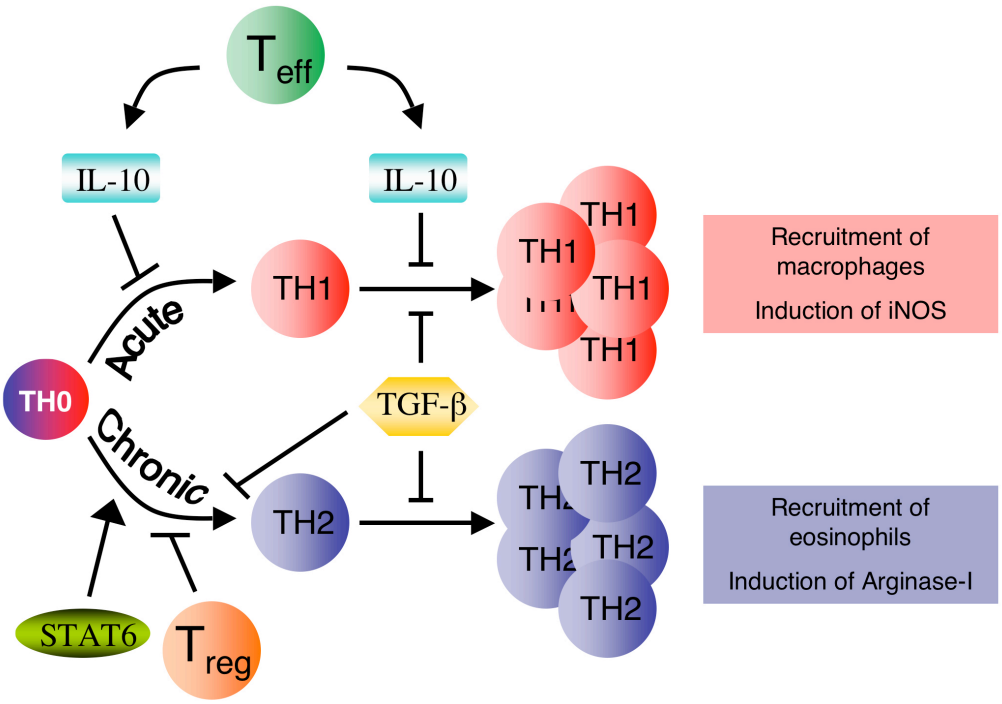

Figure 5.1 Model of mechanisms of immune modulation during T. spiralis muscle infection. Cellular sources of TGF-β have not been determined.

GFP+ and GFP- Teff cells could be purified from infected WT and IL-10$^{-/-}$ mice by fluorescence activated cell sorting and transferred to RAG2$^{-/-}$/IL-10$^{-/-}$ to evaluate their ability to limit IFN-γ production following infection. If GFP+ cells from WT mice, but not IL-10$^{-/-}$, were suppressive, this would indicate that TH2 effectors are controlling TH1-driven inflammation specifically through the production IL-10. In contrast, if GFP- cells were suppressive, it would implicate "induced" Treg cells, also know as Tr1 cells (6), in suppression. *In vitro,* Tr1 cells can be generated by the addition of dexamethasone and vitamin D3 to cultures (1), or with T cell receptor stimulation and ligation of the complement inhibitor CD46 (18). The mechanisms by which they are expanded *in vivo* are poorly characterized. In general, the phenotypic and functional attributes of IL-10-producing regulatory T cells are not well described (10), and *T. spiralis* muscle infection presents a unique opportunity to study these cells in more detail.

It is unclear which signals are required for induction of IL-10 during helminth infection. We can ascertain, however, that excreted/secreted products derived from larval stichocytes are not involved, since the influence of IL-10 preceeds the development of the parasite's secretory organ. It is possible that NBL antigens may drive IL-10 production; however, we favor the hypothesis that IL-10 production develops as a response to tissue damage caused by NBL migration and disruption of skeletal muscle cell integrity. Certain components of extracellular matrix have been shown to stimulate dendritic cells and macrophages in a TLR4 dependent manner (15, 26). The immune response to muscle infection has never been examined in TLR4 or MyD88 deficient mice, but such experiments would provide novel information about the importance of pattern recognition molecules in induction of IL-10 and

protection from myositis during *Trichinella* infection. Although the notion that TLR ligation could result in anti-inflammatory responses seems contradictory the general role for TLRs in bacterial clearance, a recent study showed that engagement of TLR4 on T lymphocytes in the intestinal lamina propria during helminth infection resulted in production of the anti-inflammatory cytokine TGF-β (14). Many parasitic helminths migrate extensively through tissues, likely inducing significant cellular destruction. Understanding how this process influences the development of antigen-specific immune responses would be highly informative in the broad context of infectious diseases.

As *T. spiralis* matures in the muscle there is an induction of TH2 cytokines, generation of high titers of parasite-specific IgG1 and IL-10 independent control of inflammation. We have shown here that naturally-occurring regulatory T cells are potent suppressors of this TH2 response, and likely do not require IL-10 to have this effect. There is increasing evidence supporting the observation that Treg cells act independently of IL-10. For example, recent work by Baumgart et al. show that the majority of Foxp3+ cells recovered from the liver of *S. mansoni* infected mice do not produce IL-10. In contrast, IL-10 production is almost exclusively observed in cells that also make IL-4 (Baumgart, J. Immunol., May 2006). Similarly, Treg cells generated during intestinal nematode infection were found to be potent suppressors of allergic TH2 responses regardless of whether they were derived from WT or IL-$10^{-/-}$ mice (30).

If IL-10 is not required for Treg-mediated suppression, then what is? Addressing this question *in vivo* requires complex manipulation of specific cell populations and effector molecules using adoptive transfer strategies and cells derived from transgenic donor mice. A good starting point would be the

generation of reporter mice in which IL-10 production is marked by green or red fluorescent protein. These mice would be invaluable in identifying, isolating and tracking IL-10-producing cells without the need for restimulation. Another useful tool would be cell-specific IL-10 knockout mice. Roers et al. used the Cre/Loxp system to target disruption of the IL-10 gene in CD4 T cells (27). These mice would be a more "natural" alternative to $RAG2^{-/-}/IL\text{-}10^{-/-}$ in determining the importance of innate sources of IL-10 in our system.

Although Treg depletion resulted in exaggerated TH2 cytokine production during *T. spiralis* infeciton, this did not translate to an observable increase in inflammation or parasite killing. Because these experiments required large amounts of antibody, we did not extend our studies of Treg suppression past 20 days post-infection. It is possible that a prolonged inability to limit TH2 responses during chronic infection may be detrimental to either the parasite or the host. In addition to elevated TH2 cytokines, we also observed increased production of IL-10 in Treg-depleted mice. This raises the possibility that, although it does not influence TH2 driven inflammation in normal mice, IL-10 may be important in limiting myositis under conditions of excessive, or pathogenic, TH2 polarization. We would need to neutralize IL-10 in Treg-depleted mice in order to address this hypothesis. The assertion that IL-10 may limit pathogenic TH1, as well as TH2, responses is consistent with the role for this cytokine in balancing immune-mediated pathogenesis during *S. mansoni* infection (12). Finally, our observation that TGF-β is potent suppressor of inflammation, independent of IL-10, and has a modest influence of TH2 cytokine production led us to hypothesize that this molecule may be a component of Treg-mediated suppression, but is likely produced by other cells as well.

Now that specific mechanisms of immune suppression have been identified during muscle infection, it would be informative to test whether these operate under the conditions of a natural infection. Neutralizing antibodies to IL-10, Treg cells and TGF-β could be administered at times that might minimize effects on intestinal worms. It is possible that the potent TH2 response observed during oral infection may reveal a more critical role for elements that inhibit this arm of the immune response, such as Treg cells and TGF-β. Our synchronous model of infection will continue to be critical in the study of immunity to muscle larvae, by allowing us to evaluate responses in transgenic mice that would otherwise be susceptible to serious intestinal disease during a natural infection.

Although our cytokine assays were a quantitative measure of the immune response during muscle infection, they did not provide direct information about what constituted muscle disease. Instead, the studies described in this dissertation relied on interpretation of histologic data to evaluate muscle damage. Future work on immune suppression during *T. spiralis* infection should incorporate rigorously quantitative assays that would reflect host muscle damage. Serum creatine kinase (CK) activity has been widely used as an indicator of cardiac and skeletal muscle trauma (2). We have generated preliminary data that showing that CK levels are elevated early in infection (data not shown). To distinguish muscle damage elicited by invasion of muscle cells from damage caused directly by cellular infiltration, sera from synchronously infected IL-10$^{-/-}$ and RAG2$^{-/-}$ mice (no inflammation) could be evaluated for CK activity. If cells recruited to infected muscle damage host cells, we would expect to see an elevation of CK in IL-10$^{-/-}$ compared to RAG2$^{-/-}$ mice. CK activity has a short half-life and is susceptible

to interference (20). As an alternative, it would also be useful measure the production of nitric oxide (NO) in muscle tissue as a reflection of disregulated TH1 responses, increased inflammation and tissue damage. NO can be indirectly measured by a sensitive fluorometric assay that determines total levels of NO breakdown products (nitrates and nitrites) (22). Since we have yet to observe mortality in any of our experiments, CK and NO measurements would be a useful read-out that would allow us to extend our conclusions beyond control of inflammation and into the area of protection and preservation of host tissue.

Experiments conducted in challenged WT and IL-10$^{-/-}$ mice suggested that effector T cell responses successfully killed parasites. The mechanisms of parasite destruction are entirely unresolved. Reports of eosinophils mediating destruction of *T. spiralis* larvae are conflicting (7-9, 11, 13). The susceptibility of NBL to damage from oxidative stress (17), combined with the dependence on IL-10 to limit TH1 responses and iNOS production during parasite development, led us to hypothesize that production of NO at the site of infection could kill immature parasites. The iNOS-specific inhibitor aminoguanidine sulfate could be administered to challenged IL-10$^{-/-}$ mice to directly test this hypothesis (3, 21), or iNOS$^{-/-}$ mice (19) could be infected. If correct, it would be intriguing to determine which parasite and/or host cell proteins are targets of s-nitrosylation, thereby identifying molecules that are critical in parasite survival in the muscle.

In summary, the data presented here argue that IL-10 and Treg cells operate independently to control inflammation and promote parasite survival. These suppressive mechanisms complement each other by allowing control of both acute (TH1) and chronic (TH2) responses. Such long lasting modulation

of the host response would facilitate the persistence of larvae in skeletal muscle, prevent overwhelming myositis and morbidity, and promote transmission of the parasite. The ability of *Trichinella* to induce multiple and distinct immune suppressive pathways may have been an essential adaptation that has allowed this organism to infect virtually any animal species in every region of the planet.

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