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Author
Broadhurst, Mara Jana

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Type-2 Immunity During Therapeutic Helminth Infection

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Mara Jana Broadhurst

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Abstract

TYPE-2 IMMUNITY DURING THERAPEUTIC HELMINTH INFECTION

Mara Jana Broadhurst

Clinical and laboratory studies have demonstrated that helminth infection can ameliorate intestinal inflammation driving inflammatory bowel diseases (IBD). Nonetheless, the cellular and molecular mechanisms mediating the benefits of helminth exposure remain poorly understood. Host immune strategies that have evolved to tolerate chronic helminth infection, including mechanisms for tissue repair and the mitigation of inflammatory pathology, are likely at play. These mechanisms are largely orchestrated by T-helper (T_H)2 cells.

The studies described here investigate the immunologic mechanisms underlying helminthic therapy. First, in an ulcerative colitis patient who achieved clinical remission after infecting himself with *Trichuris trichiura*, we described the induction of a T_H2 response in the colonic mucosa following helminth exposure as well as the expression of IL-22, a cytokine known to promote intestinal barrier functions. The severely impaired mucus production in the inflamed colon was restored, suggesting that disease remission was associated with repair of the mucosal barrier. Similarly, in juvenile rhesus macaques suffering from chronic idiopathic colitis, a putative model for IBD, we found that symptomatic improvement following *T. trichiura* exposure was associated with a mucosal T_H2 response and reduced bacterial attachment to the intestinal epithelium.

We also explored the role of retinoic acid (RA), a vitamin A metabolite that regulates T cell function and is vital for mucosal epithelial maintenance, during helminth infection in mice. RA synthesis was induced during *Schistosoma mansoni* infection and played a critical role in the T_H2 response. Alternatively-activated macrophages were found to be an important source of RA synthesis in this context.

The findings presented here support the broad conclusion that T_H cell responses in the intestinal mucosa elicited by helminth infection, including the canonical T_H2 cytokines as well as IL-22, support mucosal barrier functions that avert pathogenic inflammatory responses to commensal bacteria. The innate immune response elicited by helminth infection drives RA synthesis, which is critical for the development of T_H2 immunity. These studies provide insights into the mechanisms underlying helminthic therapy, and support the development of IBD therapies that target mucosal repair.

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CHAPTER I

BEYOND THE "HYGEINE HYPOTHESIS": EXPLORING THE IMMUNOLOGY OF HELMINTHIC THERAPY

Helminth exposure can ameliorate inflammatory pathology related to autoimmunity and inflammatory disorders.

Helminths are adapted to maintain chronic infections in mammalian hosts

Helminths comprise a diverse group of parasitic nematodes, cestodes and trematodes that infect more than one in four people in the world (Hotez et al, 2010). These parasites have adapted to mammalian hosts for millennia, allowing the evolution of potent mechanisms for modulating mammalian immune responses (Allen and Maizels, 2011). Thus, despite the presence of protective immune responses aimed at killing and expelling helminths, these parasites are able to maintain persistent infections (commonly lasting years, and in some cases decades) with minimal inflammatory pathology in the host (Maizels, 2003).

Helminth infection subverts pathologic inflammation driving autoimmunity and atopy

It was first noted in 1968 that autoimmune diseases are less common in regions of endemic helminth infection (Greenwood, 1968). An inverse relationship between helminth infection and inflammatory disorders such as allergies and asthma has also been recognized (Fallon, 2007). These observations gave rise to the idea that immuno-regulatory mechanisms activated by helminths may protect against autoimmune and inflammatory diseases that became more prevalent in developed countries during the 20th century (Wilson, 2004; Zaccone, 2006). This idea extends the prevailing "hygiene hypothesis," e.g., the notion that a relative reduction in the microbial load (both pathologic and commensal) due to the indiscriminate use of antibiotics, the overuse of pesticides and insecticides, and an emphasis on environmental sterility has precipitated

the increased frequency of autoimmune and allergic diseases in developed nations. The direct relationship of helminth infection to the development of inflammatory disease was first tested in a cohort of Gabonese children harboring intestinal helminth infections (van der Biggelaar et al., 2004). Long-term anti-helminthic treatment in these children increased their risk of developing an allergic response to dust mites.

It has since been widely demonstrated that chronic helminth infections subvert both parasite-specific and bystander immune responses. Studies in animal models have substantiated the ability of a remarkable diversity of helminth species to prevent or ameliorate inflammatory pathology. For example, infection with the trematode *Schistosoma mansoni* attenuates chemically-induced colitis (Moreels et al, 2004; Smith et al., 2007), autoimmune diabetes (Cooke et al, 1999), experimental autoimmune encephalomyelitis (a model of multiple sclerosis; Sewell et al., 2002), and allergic airway inflammation (Smits et al., 2007) in mice. Similar findings have been reported for intestinal nematodes (roundworms, e.g. *Heligmosomoides polygyrus*; Maizels et al., 2011), filarial nematodes (e.g. *Litomosoides sigmodontis*; Hubner et al., 2009), and cestodes (tapeworms, e.g. *Hymenolepsis diminuta*; McKay, 2010).

Whipworm as a pioneering agent of "helminthic therapy"

The therapeutic potential of helminth exposure in humans has been most convincingly demonstrated in the context of inflammatory bowel disease (IBD). Initial clinical trials (including one randomized, double-blind, placebo-controlled trial) have shown the safety and efficacy of *Trichuris suis* (porcine whipworm) exposure in treating Crohn's disease (Summers et al., 2005) and ulcerative colitis (Weinstock et al., 2005), the

two major forms of IBD. The related human parasite, *T. trichiura*, infects more than 1 billion people globally (Bradley and Jackson, 2004). While a heavy worm burden can lead to dystentery and rectal prolapse, especially in children, the vast majority of *T. trichiura* infections are asymptomatic. *Trichuris* species are strictly gastrointestinal parasites, unlike many other helminth parasites that can cause significant organ damage as they migrate through multiple tissues before taking up residence in the intestine. After ingested eggs hatch in the small intestine, larvae mature in the colon and live for 2-3 years, depositing eggs that are passed in the stool. *T. suis* does not establish patent infection in human hosts, necessitating regular egg ingestion to maintain a therapeutic effect. *T. suis* eggs are now marketed globally, and a growing number of IBD patients are pursuing this experimental therapy.

Helminthic therapy has thus gained a controversial foothold in the treatment of IBD patients, particularly for patients who no longer respond to, or who cannot tolerate standard medical therapy. However, no clinical studies have described the mechanism by which helminth exposure modulates mucosal inflammation in IBD patients, and it remains unclear which patients may benefit, and conversely which may be harmed, by such exposure. Importantly, studies in both humans and mice have also revealed helminth-mediated suppression of beneficial immune responses in the context of vaccination (Elias et al., 2006; Urban et al., 2007) and co-infection (Hartgers and Yazdanbakhsh, 2006; Resende et al., 2007). Given these wide-ranging implications, a more complete mechanistic description of immune regulation associated with specific helminths is imperative. We will begin the exploration of these mechanisms within the context of the immune response elicited by helminth infection: the type-2 response.

Chronic helminth infection activates a modified type-2 immune response

Helminths are large, metazoan organisms (ranging in size from a few millimeters to several meters) with the potential to cause significant tissue injury as they mature and migrate through the host. Furthermore, due to effective immune evasion strategies, these parasites can persist in the host for many years. The immune response that optimizes host fitness must therefore address each of these challenges with mechanisms for 1) killing and expelling large, multicellular pathogens, 2) wound healing and tissue repair, and 3) mitigating inflammatory pathology associated with chronic infection. These mechanisms are encompassed within the type-2 immune response elicited by helminth infection. The most common helminth parasites reside in the intestine. The type-2 response to these infections is tailored to the intestinal environment, and a strong cross-talk between infiltrating immune cells and resident intestinal tissue cells (e.g. epithelial cells) plays a fundamental role in host protection.

Initiation of the type-2 response

Helminth antigens typically elicit a type-2 immune response that is orchestrated by T_H2 cells and the canonical type-2 cytokines (IL-3, IL-4, IL-5, IL-9 and IL-13). This response is initiated at the tissue site of helminth exposure when antigen presenting cells (APCs), including dendritic cells (DCs) and basophils, receive signals from helminth products (presumably through as-yet-unidentified pattern recognition receptors) as well as from cytokines released by local epithelial cells that instruct the induction of a type-2 response. The activated APCs migrate to draining lymph nodes where they stimulate the differentiation of antigen-specific naïve $CD4^+$ T cells into T_H2 cells. Effector T_H2 cells,

primed for the secretion of type-2 cytokines, home to the site of infection and direct the recruitment and activation of several populations of innate effector cells that further amplify the type-2 response.

Effector functions that promote helminth clearance

The effector functions of the type-2 response are specialized for the challenge of killing and expelling large, multicellular pathogens. T_H2-derived IL-4 activates antibody class-switching to immunoglobulin (Ig)E by plasma cells. In parallel, IL-3, IL-5 and IL-9 promote the recruitment of basophils, eosinophils and mast cells, respectively. These granulocytes are activated by the cross-linking of Fc-epsilon receptors by IgE, stimulating the release of soluble proteins and small molecules that mediate the direct killing of IgE-coated helminths.

Type-2 cytokines also stimulate enhanced mucus production, smooth muscle contractility, and epithelial cell turnover to promote the expulsion of helminths dwelling in the intestinal lumen (Khan and Collins, 2004). These physiologic functions of type-2 immunity can be sufficient to clear intestinal helminths in the absence of effective parasite killing, as evidenced by the expulsion of live worms. A variety of intestinal nematodes elicit smooth muscle hyper-contractility in rodents that is driven by IL-4, IL-13 and/or IL-9 (Zhao et al., 2003; Khan et al., 2003). Goblet cell hyperplasia, a common histopathological feature of intestinal helminth infection (Miller and Nawa, 1979; Caroll et al., 1984; Else and Finkelman, 1998) is also dependent on T_H2 cytokines (Ishikawa et al., 1997; Urban et al., 1998; Townsend et al., 2000; Khan et al., 2001) Mucins, large glycoproteins that constitute the major component of mucus, are upregulated (Shekels et

al., 2001; Karlsson et al., 2000; Olson et al., 2002) and chemically modified (Koninkx et al., 1988; Ishikawa et al., 1993; Soga et al., 2008) during intestinal nematode infections. Finally, helminth infection alters secretions into the mucus layer, including toxic proteins that are ingested by the worms (Herbert et al., 2009). Thus, quantitative and qualitative changes in intestinal mucus promote parasite clearance by multiple mechanisms, and mucolytic agents increase susceptibility to persistent infection (Miller and Huntley, 1982).

Macrophages rapidly accumulate at sites of helminth exposure, where type-2 cytokines, most importantly IL-4 and IL-13, signal through the transcription factor STAT6 to drive an alternatively-activated phenotype (Gordon and Martinez, 2010). Whereas classical macrophages express interferon-induced nitric oxide synthase to facilitate oxidative killing of intracellular microbes, alternatively-activated macrophages (AAMϕ) express suites of genes related to parasite resistance, wound healing, and immune regulation. The molecular mechanisms responsible for these functions *in vivo* are poorly understood. However, AAMϕ have been shown to directly promote T_H2 differentiation (Loke et al., 2000) and eosinophil recruitment (Voehringer et al., 2007), supporting their role as effector cells in the type-2 response.

Mechanisms of tissue repair

Proteases and elastases are critical virulence factors for many helminths, highlighting the requirement for host tissue destruction in multiple life-cycle stages of these parasites, particularly during invasive and migratory larval stages (McKerrow et al., 2006). Thus, host fitness in the setting of helminth infection is dependent on effective

tissue repair (Allen and Wynn, 2011). Compelling evidence that type-2 immunity is intimately tied to tissue repair lies in the finding that the innate type-2 response is activated by sterile, surgical injury (Loke et al., 2007; Seno et al., 2009). AAMϕ are thought to play a particularly important role in wound healing and fibrosis, in part because they express high levels of arginase I, an enzyme that metabolizes L-arginine and drives the production hydroxyproline and polyamines, precursors for collagen deposition (Hesse et al., 2001). During intestinal helminth infection, the stimulation of goblet cell and Paneth cell functions by type-2 cytokines promotes mucosal healing (Finkelman et al., 2004; Steenwinckel et al., 2009).

Regulatory mechanisms that limit inflammatory pathology

Highly adapted relationships between mammalian hosts and helminth parasites have favored an outcome in which chronic, low-pathology infection is facilitated by immunoregulatory networks that dampen destructive inflammatory responses. Thus, the robust type-2 response commonly undergoes gradual attrition during chronic infection characterized by T cell hyporesponsiveness to both parasite and bystander antigens. This phenomenon is at least partially mediated by the immunosuppressive cytokines IL-10 and TGFβ (Maizels, 2003). These cytokines promote regulatory T cell populations, including IL-10-secreting Tr1 cells and peripherally-induced FoxP3$^+$ regulatory T (T_{reg}) cells, that expand during the chronic stage of several helminth infections and can play an important role in limiting T_H1-, T_H2-, and T_H17-driven inflammation. Recent evidence suggests that certain helminth products can interact directly with T cells to promote a regulatory phenotype (e.g., a nematode-derived TGFβ mimic; Grainger et al., 2010) or alternatively

can instruct DCs to induce regulatory T cells (van der Kleij et al., 2002). AAMϕ also constitute a critical component of helminth-associated immune regulation, partially through the modulation of T cell responses (Herbert et al., 2004; Taylor et al., 2006) as discussed in more detail below.

The same mechanisms responsible for quelling helminth-specific inflammatory responses may also contribute to the protective effects of helminth infection in the setting of autoimmunity and atopy. This notion has been most thoroughly explored in the context of murine infection with *S. mansoni*, an informative model of the modified type-2 response.

Murine schistosomiasis provides a model of helminth-mediated immune regulation.

S. mansoni, a blood-dwelling tropical trematode, infects more than 200 million people worldwide (Steinmann et al., 2006). The geographic distribution of infection depends upon the presence of fresh-water snails (*Biomphalaria* species) that act as intermediate hosts, harboring the miracidial stage of the worm's life cycle. Through asexual reproduction, each miracidium gives rise to hundreds of cercariae, a larval form that is shed from the snail and that can penetrate the skin of a definitive mammalian host. Immature worms migrate via the circulation through the lungs, heart, and liver, finally reaching the mesenteric venules as mature, sexually-reproducing mating pairs. Eggs are laid within the mesenteric venules, and must traverse the intestinal wall to pass in the feces and to complete the worm's life cycle. While this endpoint is achieved by approximately one-third of the eggs, the remaining eggs are swept into the portal venous circulation and carried to the liver.

Regulated T_H2 responses are critical for host survival during S. mansoni *infection*

Schistosome eggs are highly immunogenic and toxic to host tissue. Murine schistosomiasis can be associated with mild or severe pathology depending on the quality and kinetics of the immune response to egg antigens. During mild-pathology infection (e.g. of wildtype C57BL/6 or BALB/c mice), there is a moderate T_H1 response to the migrating and adult worms followed by a dramatic shift to a T_H2 response upon egg deposition at five to six weeks post-infection (acute infection). In contrast, severe-pathology infections (e.g. of CBA mice) are associated with unchecked T_H1 and T_H17 responses to egg antigens and reduced survival into the chronic phase (Rutitsky et al, 2008; Rutitsky et al., 2005).

It is imperative that schistosome eggs are sequestered and the damaging inflammatory response tightly controlled to prevent sepsis as the eggs cross into the intestinal lumen. This is accomplished by the formation of type-2 granulomas around the eggs composed of T_H2 cells, eosinophils, and AAMϕ (Pearce and McDonald, 2002; Davies and McKerrow, 2001). The importance of IL-4 in controlling lethal intestinal immunopathology was demonstrated in IL-4-deficient mice (Brunet et al., 1997). AAMϕ were identified as a critical regulatory cell-type downstream of IL-4, as mice with macrophage/neutrophil-specific IL-4Rα deficiency were unable to survive acute infection due to severe liver and intestinal damage (Herbert et al., 2004). These mice exhibited exacerbated T_H1 responses despite intact T_H2 responses.

As eggs accumulate in the liver during chronic infection, IL-13 drives severe fibrosis that ultimately compromises liver function (Mentink-Kane et al., 2003; Wilson et

al., 2007). Therefore, an optimal immune response during chronic schistosomiasis is characterized by the induction of regulatory networks that limit both the apoptotic pathology associated with potent inflammatory responses to egg antigens, and the fibrogenic pathology associated with excessive T_H2-driven granulomatous responses. Tr1 and FoxP3$^+$ T_{reg} cells are important contributors to the generalized down-modulation of both T_H1 and T_H2 reactivity during chronic infection (Hoffman et al., 2000; Hesse et al., 2004). Altogether, these studies reveal critical roles for T_H2 cells, AAMϕ and regulatory T cells in regulating immunopathology during *S. mansoni* infection.

S. mansoni infection limits immunopathology in multiple models of autoimmunity

As mentioned above, *S. mansoni* infection in mice attenuates aberrant T_H1 and T_H17 responses driving inflammatory pathology in a remarkable diversity of organs including the colon (Smith et al., 2007; Moreels et al., 2004), pancreas (Cooke et al., 1999), nervous system (Sewell et al., 2003), and airways (Smits et al, 2007). Studies in both humans and mice have demonstrated that the T_H2 and regulatory T cell responses elicited by schistosome antigens directly antagonize T_H1 and T_H17 responses to unrelated antigens in these disease models. The modulation of innate immune cells, including DCs and macrophages, also contributes to the attenuation of pathogenic T cell responses. This was clearly demonstrated by the attenuation of chemically-induced colitis upon adoptive transfer of schistosome-elicited macrophages (Smith et al., 2007). It is therefore of considerable interest to elucidate the driving mechanisms of both T_H2 and T_{reg} induction by *S. mansoni* and, specifically, to address the contribution of schistosome-elicited macrophages to these responses. Such studies may offer general insights into helminth-

induced modulation of T cell responses, with broad clinical implications for inflammatory diseases, vaccine responses, and immunity to co-infections in helminth-infected individuals.

A large body of evidence has implicated retinoic acid, a potent metabolite of vitamin A, in the induction of both T_H2 and $FoxP3^+$ T_{reg} responses. Therefore, a role for retinoic acid in helminth-associated T cell modulation is potentially of interest.

The vitamin A metabolite retinoic acid (RA) promotes T_H2 and T_{reg} induction.

The immune function of vitamin A is mediated through RA

The importance of vitamin A in immune function is underscored by its original identification as an "anti-infective factor" more than 80 years ago (Green and Mellanby, 1928). In these pioneering nutritional studies, dietary deficiency in vitamin A strongly predisposed to more severe infections. Large-scale vitamin A supplementation campaigns have since demonstrated markedly reduced morbidity and mortality due to infectious diseases in populations at risk for vitamin A deficiency who received supplements (reviewed in Sommer, 2008). Vitamin A is obtained in the diet primarily as retinol, of which more than 90% is stored in the liver. Upon delivery to tissues, retinol can be oxidized intracellularly to yield RA, a hormone-like metabolite that mediates the effects of vitamin A in the immune system through the binding and activation of nuclear retinoid receptors.

RA is a critical regulator of T cell homing and effector function in humans and mice. RA imprints gut-homing capacity on both $CD4^+$ and $CD8^+$ T cells, and promotes the development of T_H2 and T_{reg} responses while inhibiting T_H1 and T_H17 responses

(reviewed in Hall et al., 2011). These functions will be discussed in more detail in Chapter 4. Given the multifactorial regulation RA imposes on T cell responses, its synthesis is likely to be regulated in coordination with helper T cell differentiation during antigenic challenge.

RA synthesis is tightly controlled by the expression of retinal dehydrogenase enzymes

RA synthesis from retinol requires the expression of two classes of enzymes: alcohol dehydrogenases (ADH, which catalyze the oxidation of retinol to retinal) and retinal dehydrogenases (Raldh1-3, which catalyze the oxidation of retinal to retinoic acid) (Duester et al., 2003). While ADHs are ubiquitously expressed, Raldh expression is tightly regulated. Interestingly, recent studies have revealed that subsets of intestinal DCs and macrophages constitutively express high levels of Raldh1 and/or Raldh2 (Coombes et al., 2007; Benson et al., 2007; Sun et al., 2007; Denning et al., 2007). These cells promote the induction of gut-homing $FoxP3^+$ T_{regs} through the local release of RA during antigen presentation, and are thus thought to partially mediate tolerance to oral antigens and the intestinal flora.

It remains unclear what signals in the gut microenvironment promote homeostatic Raldh expression. Furthermore, a major area of study that has not been explored is the regulation of Raldh expression by APCs during infection. Given the prominence of both T_H2 and T_{reg} cells during helminth infection, a potential role for RA synthesis by helminth-elicited APCs is particularly intriguing.

Putting it all together: How might helminth exposure ameliorate IBD?

The two major forms of IBD, ulcerative colitis and Crohn's disease, are chronic inflammatory syndromes driven by aberrant mucosal inflammatory responses against the commensal flora in genetically susceptible individuals (Xavier and Podolksy, 2007). Changes in the composition of microbial species could precipitate this inflammatory state (Nell et al., 2010). However, studies of population genetics as well as animal models have demonstrated a prevailing role for host factors in IBD pathogenesis (Khor et al., 2011). First, the breakdown of mucosal barrier functions such as mucus production allows for increased microbial attachment and translocation at the intestinal epithelium, activating anti-microbial inflammatory responses. Second, a loss of immune tolerance (due to either a disruption in regulatory function or overactive pro-inflammatory function) can initiate an aberrant immune response that ultimately jeopardizes the mucosal barrier. Thus, the modified type-2 response elicited by helminth infection can potentially interrupt IBD pathogenesis on multiple fronts. Wound healing pathways activated by type-2 cytokines may restore mucosal barrier function, while T_H2 and immunoregulatory networks can directly inhibit pathogenic T_H1 and T_H17 responses.

Plan of work

The work presented in the following chapters will explore the mechanisms of T cell regulation during helminth infection, with two broad approaches: 1) To characterize changes in the intestinal immune compartment induced by *T. trichuris* infection in the setting of IBD (Chapter II and III), and 2) To determine the role of RA in the generation of effector and regulatory T cell responses during *S. mansoni* infection in mice (Chapter

IV). In Chapter II we describe an ulcerative colitis patient who achieved clinical remission after infecting himself with *T. trichiura*. Helminth exposure in this patient induced a mucosal type-2 response as well as T cell-derived IL-22 expression, a cytokine implicated in mucosal healing and barrier maintenance. Crypt architecture and mucus production in the inflamed colon were restored, demonstrating that disease remission was associated with mucosal repair. In Chapter III we describe the outcome of experimental *T. trichiura* infection in juvenile rhesus macaques suffering from chronic idiopathic colitis, a putative model for IBD. Symptomatic improvement following helminth exposure was associated with a mucosal T_H2 response and reduced bacterial attachment to the intestinal epithelium. Together, these findings suggest that the activation of type-2 immunity in the colon by *T. trichiura* exposure promotes the restoration of mucosal barrier functions, thereby reducing bacterial stimulation that drives chronic inflammtion in the setting of IBD. In Chapter IV we demonstrate a critical role for RA in the induction of helminth-elicited type-2 immunity. RA synthesis was found to be a selective function of AAMϕ during *S. mansoni* infection, shedding new light on the role of AAMϕ in T cell regulation and tissue repair.

Chapter V outlines further studies that have been initiated to test specific hypotheses generated by this work. The role of T_H2- and IL-22-activated mucosal barrier functions in helminthic therapy will be investigated in a clinical trial of *T. suis* therapy in ulcerative colitis patients and a larger study of *T. trichiura* infection in colitic juvenile macaques. Furthermore, the contribution of AAMϕ-derived RA in murine models of helminthic therapy will be explored using a mouse strain genetically engineered for cell-

specific, inducible deletion of Raldh2. We discuss the implications of this work for the development of novel therapies for IBD.

CHAPTER II

THERAPEUTIC *TRICHURIS TRICHIURA* INFECTION IN AN ULCERATIVE COLITIS PATIENT

ABSTRACT

UC is less common in countries endemic for helminth infections, suggesting that helminth colonization may have the potential to regulate intestinal inflammation in inflammatory bowel diseases. Indeed, therapeutic effects of experimental helminth infection have been reported in both animal models and clinical trials. Here, we provide a comprehensive cellular and molecular portrait of dynamic changes in the intestinal mucosa of an individual who infected himself with *T. trichiura* to treat his symptoms of UC. Tissue with active colitis had a prominent population of mucosal T_H cells that produced the inflammatory cytokine IL-17 but not IL-22, a cytokine involved in mucosal healing. After helminth exposure, the disease went into remission, and IL-22-producing T_H cells accumulated in the mucosa. Genes involved in carbohydrate and lipid metabolism were up-regulated in helminth-colonized tissue, whereas tissues with active colitis showed up-regulation of proinflammatory genes such as *IL-17*, *IL-13RA2*, and *CHI3L1*. Therefore, *T. trichiura* colonization of the intestine may reduce symptomatic colitis by promoting goblet cell hyperplasia and mucus production through T_H2 cytokines and IL-22. Improved understanding of the physiological effects of helminth infection may lead to new therapies for IBD.

INTRODUCTION

UC is characterized by chronic inflammation of the colonic mucosa. Although the etiology of UC is poorly understood, it is believed that an impaired intestinal epithelial barrier, together with defects in mucosal immune regulation, favors the development of pathogenic T_H17 cells (Xavier and Podolsky, 2007) probably in response to commensal microbiota (Littman and Rudensky, 2010). Treatment of severe UC is usually through administration of immunosuppressive drugs, whose long-term use is limited by adverse side effects, including increased risk of opportunistic infection. Furthermore, up to 30% of patients develop disease refractory to treatment within three years of diagnosis, necessitating colectomy.

IBD is most prevalent in Northern Europe, the United Kingdom and North America (Loftus Jr., 2004) and historically rare in regions of endemic helminth infection such as Asia, Africa and Latin America (Loftus Jr., 2004; de Silva et al., 2003). However, the incidence of UC is rising in countries like Japan, South Korea and Singapore and indeed South Asians that have moved from the Indian subcontinent to the United Kingdom actually have a higher rate of UC than the Caucasian population (Goh and Xiao, 2009). This has raised the hypothesis that helminths may protect against the pathologic inflammation underlying IBD as a bystander effect of their ability to modulate the immune system in order to survive within mammalian hosts (Maizels and Yasdanbakhsh, 2003; Elliott et al., 2007). Studies of colitis in mice (Elliott et al., 2007) as well as in clinical trials (Summers et al., 2005a; Summers et al., 2005b) have suggested that helminth infection can prevent and/or treat IBD. A randomized placebo-controlled trial with *T. suis* ova as therapy for UC indicated a therapeutic improvement in

a disease activity index (Summers et al., 2005a), but the mechanism of action was not characterized. A more complete understanding of this phenomenon could lead to the development of new therapeutics, for example, through infection with live helminths, identification of helminth-derived molecules with immunosuppressive effects, and/or identification of biological pathways activated by helminths that can be targeted through conventional approaches. Because helminth infections can themselves cause inflammation and colitis when immunoregulatory networks are already disrupted (Schopf et al., 2002; Wilson et al., 2010), it is important to understand the molecular pathways that regulate the balance between inflammation and immunity in the intestinal mucosa if live helminths are used as therapeutic agents.

The immune response to helminth infection is typically characterized by a T_H2 response, including the induction of eosinophilia and of macrophages activated by T_H2 cytokines, termed alternatively activated macrophages. T_H2 cytokines such as IL-4 and especially IL-13 contribute to wound healing and tissue remodeling (Wynn, 2007) and may also promote epithelial integrity and mucosal healing. IL-22, an IL-10 cytokine family member, also promotes wound healing, proliferation, and anti-apoptotic pathways in intestinal epithelial cells (Eyerich et al., 2010; Pickert et al., 2009) and furthermore up-regulates anti-microbial peptide expression and mucus production (Zheng et al., 2008; Cells et al., 2009; Liang et al., 2006; Sugimoto et al., 2008). IL-22 can be protective in animal models of colitis, partially attributable to the induction of epithelial wound healing and mucus production (Pickert et al., 2009; Sugimoto et al., 2008; Zenewicz et al., 2008). Although IL-22 was originally described as a cytokine produced by T_H17 cells, it is now recognized that IL-22 expression by T_H cells can be induced independently of

IL-17 expression (Trifari et al., 2009; Duhen et al., 2009). Therefore, regulation of the production of T_H2 cytokines and IL-22 could be a potent avenue for influencing the course of colitis.

Here, we have analyzed the colonic mucosa from a UC patient who obtained symptomatic relief after infecting himself with the nematode parasite *T. trichiura*. It is estimated that almost a billion people worldwide are infected with *T. trichiura,* with the highest prevalence in Central Africa, southern India and Southeast Asia (de Silva et al., 2003; Compton et al., 1999). In contrast to *Trichuris suis* ova, this human parasite establishes chronic, not transient, colonization. Genome-wide transcriptional profiling, flow cytometric evaluation of the immune response, and histological analyses were carried out on biopsy samples collected during active colitis and during stable disease remission associated with helminth infection.

RESULTS

Clinical course and histopathology of UC in the setting of *T. trichiura* infection.

We followed the disease course of a 35 year-old man diagnosed with UC in 2003 (Figures 1 and S1; supplementary figures are included at the end of the chapter). His initial disease was severe and refractory to mesalamine agents, mercaptopurine, and high dose steroids. In 2003, his sigmoid colon had extensive ulceration of the mucosal epithelium, with few epithelial-lined mucosal glands remaining (Figures 2A and S2). Neutrophils heavily infiltrated the glands (Figure 2B) and the surrounding lamina propria, forming crypt abscesses. In early 2004, the immunosuppressant cyclosporine or

Figure 1. Overview of clinical course and analyses of a UC patient.

(A) Time course of UC disease severity (red showing colitis and yellow showing remission) in relation to *T. trichiura* infection, biopsy collection, and analyses of biopsy tissue by transcriptional profiling and flow cytometry. The subject ingested parasite ova twice in 2004 and again in August 2008. Histopathology slides of biopsies collected in August 2003 and October 2005 were reviewed. In January 2007, biopsies were collected for RNA analysis during a flare of proctitis. When the subject reverted to pan colitis in July 2008, biopsies were collected for flow cytometry and RNA analysis. In March of 2009, a colonoscopy demonstrated mucosal healing and biopsies were again collected for flow cytometry and RNA analysis.

(B) Examples of gross pathology seen upon endoscopic examination. In 2007, worms were observed in the cecum, ascending colon and transverse colon, while the sigmoid colon was normal and the rectum exhibited signs of proctitis. In 2008, worms were observed only in the ascending colon and not in the transverse colon; however, the remainder of the colon exhibited signs of severe colitis. In 2009, worms were mainly observed in the ascending colon, and intact mucosa was observed in the remainder of the colon with few signs of inflammation.

FIGURE 1

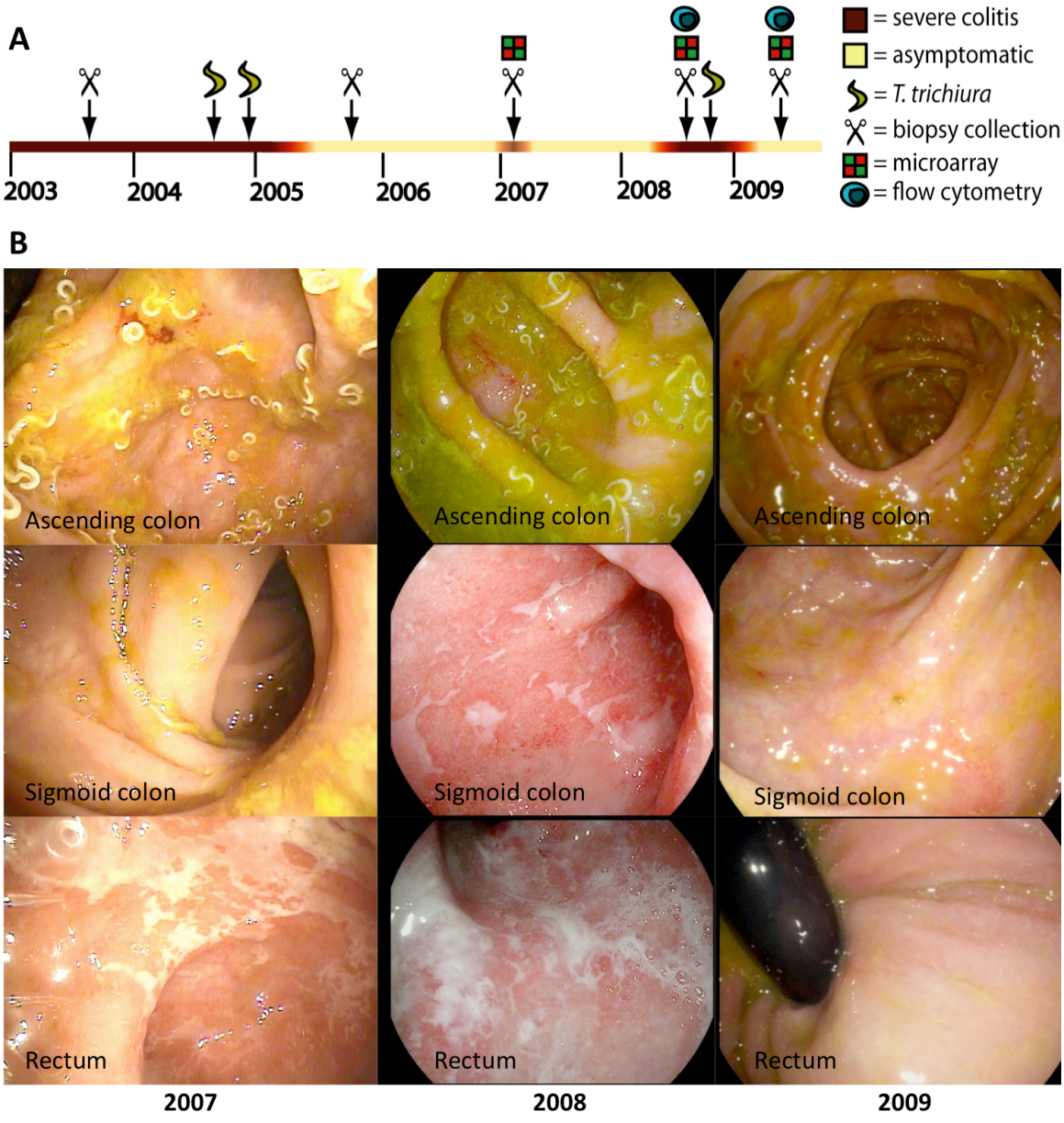

A
2003
2004
2005
2006
2007
2008
2009
= severe colitis
= asymptomatic
= T. trichiura
= biopsy collection
= microarray
= flow cytometry
B
Ascending colon
Ascending colon
Ascending colon
Sigmoid colon
Sigmoid colon
Sigmoid colon
Rectum
Rectum
Rectum
2007
2008
2009

Figure 2. Distinct inflammatory infiltrates characterize colitis-affected and helminth-exposed mucosal tissues. Tissue sections of colon biopsies stained with hematoxylin and eosin.

(A) Sigmoid colon in 2003 showed signs of severe colitis including ulcerated epithelium (UE) and crypt abscesses (CA).

(B) High powered view of the sigmoid colon in 2003 showing prominent neutrophils (N) infiltration of glands and a crypt abscess (CA).

(C) Ascending colon in 2005 with cross section of a *T. trichiura* worm (W), prominent eosinophils (E) and plasma cells (P) as well as goblet cell (G) hyperplasia.

(D) Sigmoid colon in 2005 showed restoration of glands (gl) and goblet cells (G).

(E) Ascending colon in 2007 with cross section of a *T. trichiura* worm (W), with prominent eosinophils (E) and goblet cells (G).

(F) High powered view of the ascending colon in 2007 showing prominent eosinophilia (E) and goblet cells (G).

(G) Proctitis in the rectum in 2007 showed lymphoid aggregates (LA) and neutrophil (N) infiltration into the glands.

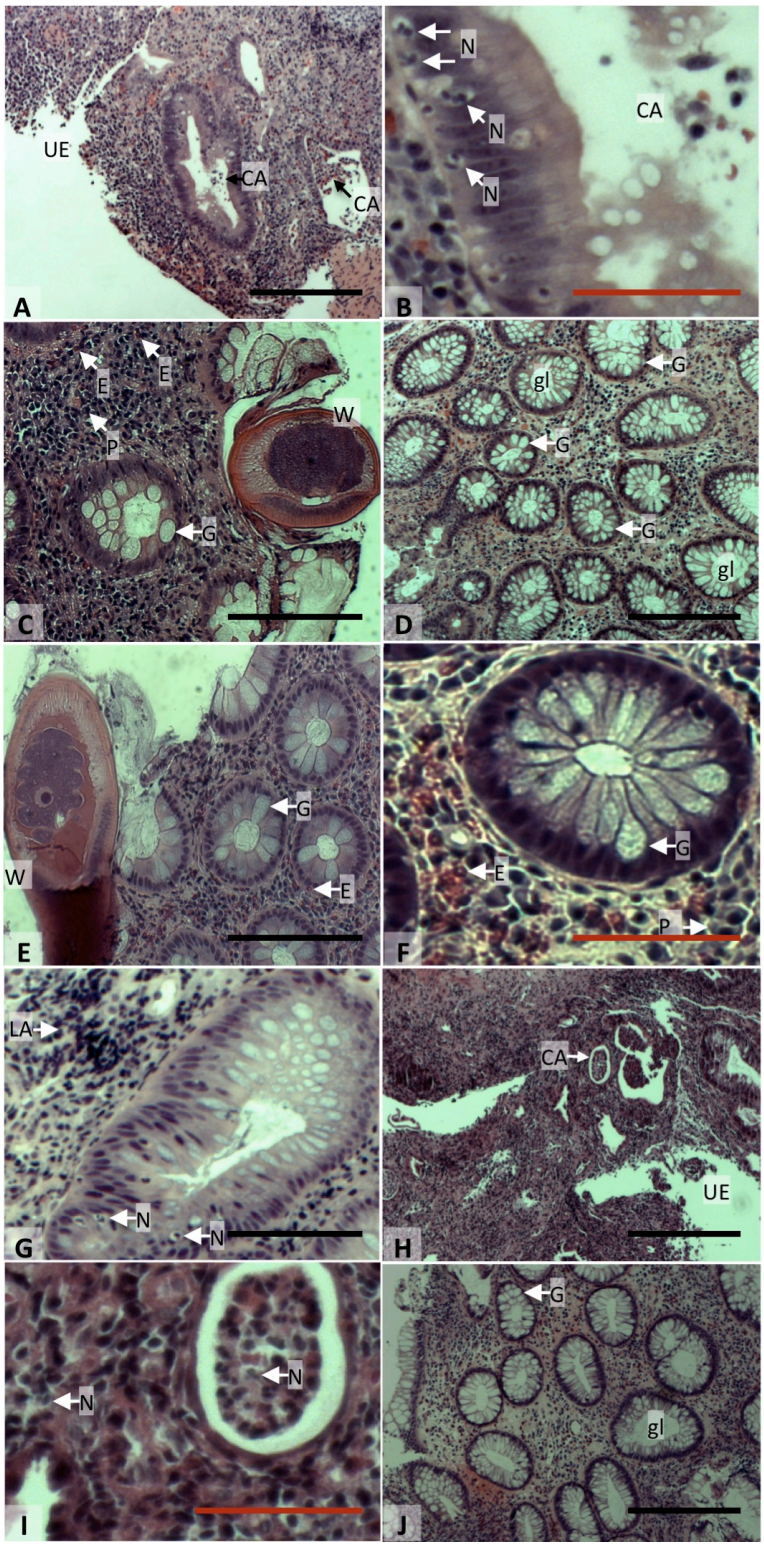
(H) Severe colitis in the sigmoid colon in 2008 showing loss of glands and ulcerated epithelia (UE) and crypt abscess (CA).

(I) High powered view of sigmoid colon in 2008 showing pronounced neutrophil infiltration.

(J) Sigmoid colon in 2009 showed restoration of glands (gl) and goblet cells (G).

Black scale bars, 100 μm; red scale bars, 50 μm.

FIGURE 2


UE
CA
CA
A
N
N
N
CA
B
E
E
P
W
G
C
G
gl
G
G
gl
D
W
G
E
E
G
P
F
LA
N
N
G
CA
UE
H
N
N
I
G
gl
J

colectomy was advised. Instead, the patient chose to infect himself with *T. trichiura* eggs obtained in Thailand. He ingested 500 *in vitro*-germinated eggs in late 2004 and an additional 1000 eggs three months later. His symptoms improved in the following months and, by mid-2005, he was symptom-free and required no treatment for UC.

He periodically took 5-aminosalicylates either topically or by mouth to control symptomatic flares, although he was generally not under any medical therapy. In October 2005, *T. trichiura* worms (Figure 2C) were observed in the cecum, ascending colon, and transverse colon. Despite a prominent infiltration of eosinophils and plasma cells in the lamina propria of these tissues, the mucosa was intact with no significant epithelial cell loss and the glands appeared normal (Figures 2C and S2). In contrast to 2003, no ulceration or neutrophil infiltration was observed in the sigmoid colon, and glands demonstrated moderate goblet cell hyperplasia and mucus hypersecretion (Figure 2D).

A brief flare of symptoms in 2007 warranted a colonoscopy. The ascending colon continued to harbor a heavy worm burden (Figures 1B and S1). This tissue showed no ulceration, with normal glands and many macrophages, eosinophils, and plasma cells (Figure 2, E and F), similar to the previous colonoscopy (Figures 2C and S2). The histopathology of the sigmoid colon was essentially unchanged from 2005. Consistent with the flare of proctitis, macroscopic signs of colitis were apparent in the rectum (Figure 2G). One biopsy contained a predominantly neutrophilic infiltrate in the lamina propria, with cryptitis, a single crypt abscess, and several prominent lymphoid aggregates (Figure 2G). In contrast, other biopsies showed minimal infiltration of the lamina propria, intact epithelium, and goblet cell hyperplasia.

After three years of nearly complete disease remission, the patient's symptoms began to deteriorate in mid-2008, paralleling a decline in stool egg counts from an extremely high number (>15,000 eggs per gram) to more moderate numbers (<7,000 eggs per gram). There was active colitis in both the ascending and sigmoid colons (Figures 1B and S1). The ascending colon remained colonized by worms (Figure 1B) and continued to show minimal signs of chronic colitis (Figure S2). In the sigmoid colon (Figures 2H and S2), evidence for much more severe colitis was observed. Multiple crypt abscesses accompanied prominent neutrophilic infiltrates (Figure 2I). Changes in tissue architecture were apparent, including a loss of glands and distortion of the epithelial layer (Figure 2H). Ulceration and granulation tissue indicated there was chronic inflammation in this tissue.

The patient chose to infect himself again, this time with 2000 *T. trichiura* eggs. His symptoms improved in the following months, and he required no other medication. He had another colonoscopy in early 2009. Large numbers of worms were observed in the ascending colon (Figure 1B), and beyond an eosinophilic infiltrate in the lamina propria, no signs of colitis were present. The sigmoid colon still demonstrated mild colitis, with a few scattered neutrophils in the lamina propria and minor crypt distortion. However, goblet cell numbers were restored and no ulceration was noted (Figure 2J). Thus, renewed *T. trichiura* colonization was again associated with improvement in histopathologic findings, including normalization of tissue architecture and recovery of epithelial integrity.

Characterization of T_H cell responses in the colonic mucosa.

Biopsies collected during the 2008 and 2009 colonoscopies were analyzed by flow cytometry for intracellular IL-17, IL-22, IL-4, interferon-γ (IFNγ), and tumor necrosis factor-α (TNFα) (Figure S3) production. We examined the relative proportions of mono- and poly-cytokine producing $CD4^+$ T_H cells (Figure 3A). In 2008, during the relapse of symptomatic colitis, 70% of cytokine-producing $CD4^+$ T_H cells from the severely-inflamed sigmoid colon expressed only IL-17 (Figure 3 and S3) while fewer than 1% expressed IL-22. In contrast, biopsies from the ascending and transverse colon, in which epithelial integrity and gland structure remained intact, had more IL-22^+ cells (Figures 3 and S3).

Analysis of mucosal T_H cell responses in 2009, during the period of remission after re-infection with *T. trichiura*, revealed that IL-17 expression by T_H cells in the sigmoid colon was comparable to that of 2008 (Figure 3A). Furthermore, the IL-17 response was more prominent in the ascending and transverse colon in 2009 than in 2008. However, a significant population of IL-22^+ T_H cells was now observed in the sigmoid colon (Figure 3, A and B), such that the proportion of IL-22^+ T_H cells was similar in the sigmoid and ascending colon. As expected, IL-4^+ T_H cells were also increased in the helminth-colonized ascending colon (Figures 3 and S3). Thus, despite the continued presence of $CD4^+$ IL-17^+ T_H cells, symptomatic remission associated with a reduction in infiltrating neutrophils and restoration of tissue architecture was characterized by the presence of $CD4^+$ IL-22^+ T_H cells.

Because the protective effect of IL-22 in mouse models of colitis is linked with the stimulation of mucus production by goblet cells (Sugimoto et al., 2008), we used the

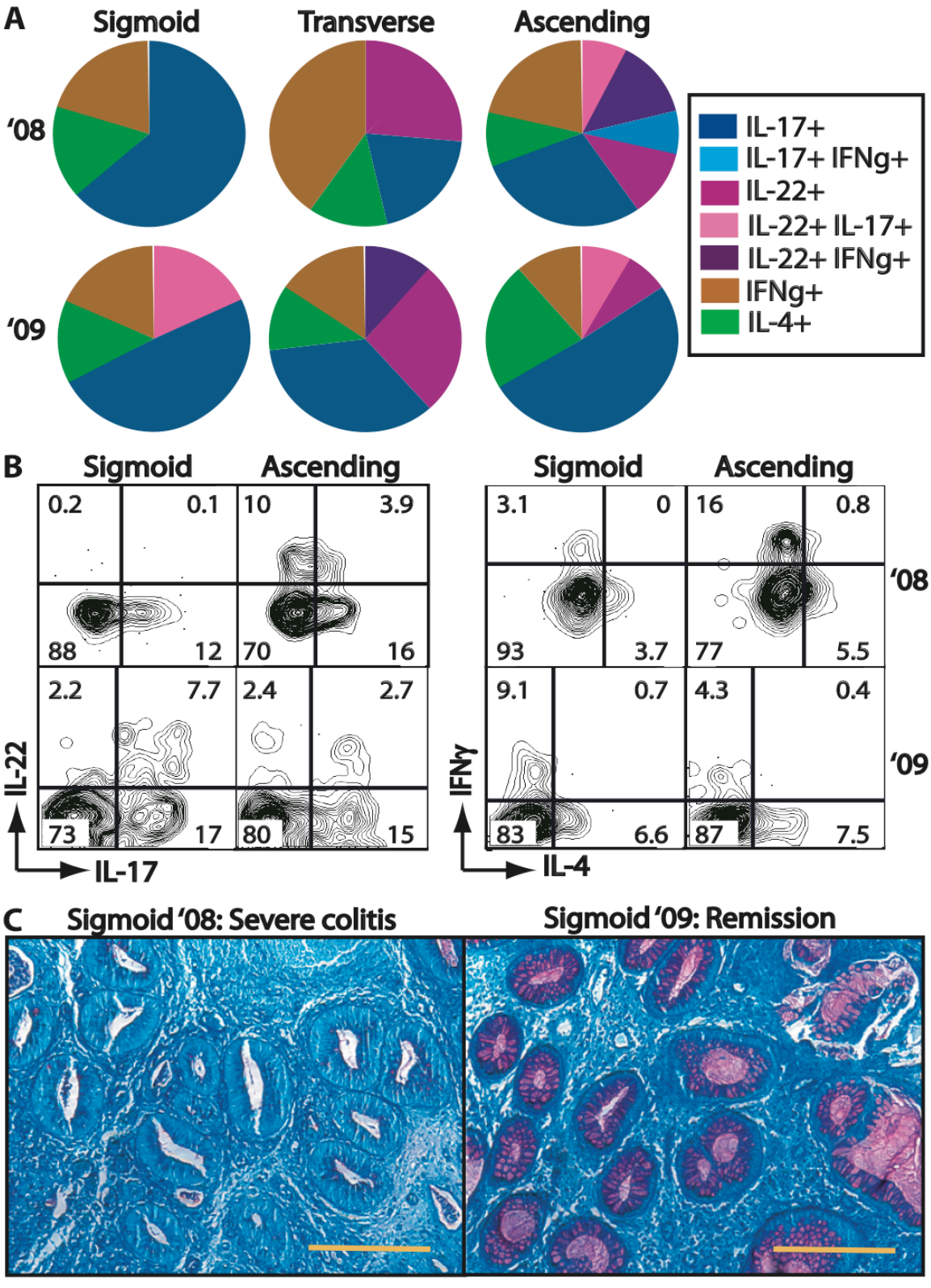
Figure 3. Induction of IL-22 expression in T_H cells of the sigmoid colon is associated with restoration of mucus production. Lymphocytes from colon biopsies collected in 2008 and 2009 were isolated and analyzed by flow cytometry for intracellular cytokine expression after a 5-hour PMA/ionomycin stimulation *ex vivo*.

(A) Visualization of the combinations of cytokines expressed by $CD4^+$ T cells from colon biopsies with pie charts in which each slice represents a different cytokine combination. The flow cytometric gating strategy used to generate these charts is shown in Figure S3. Boolean gates were created for cytokine-positive cells and used to divide these cells into distinct populations corresponding to the patterns of cytokines they are producing. These results were then graphed using the SPICE software to generate pie charts representing the proportion of cytokine-expressing $CD4^+$ T cells that express combinations of IL-17, IL-22, IFNγ and/or IL-4. Cells that are not producing any the these cytokines are not represented in the pie charts.

(B) Flow cytometry bivariate contour plots showing the frequencies of CD4+ T cells expressing IL-17, IL-22, IFNγ and IL-4 from biopsy samples taken from the sigmoid colon and ascending colon in 2008 and 2009.

(C) Mucus production in the sigmoid colon was visualized by the periodic acid Schiff (PAS) stain. Scale bars, 50 μm.

FIGURE 3


A
Sigmoid
Transverse
Ascending
'08
'09
IL-17+
IL-17+ IFNg+
IL-22+
IL-22+ IL-17+
IL-22+ IFNg+
IFNg+
IL-4+
B
Sigmoid
Ascending
Sigmoid
Ascending
0.2
0.1
10
3.9
88
12
70
16
3.1
0
16
0.8
93
3.7
77
5.5
'08
2.2
7.7
2.4
2.7
9.1
0.7
4.3
0.4
73
17
80
15
83
6.6
87
7.5
'09
IL-22
IL-17
IFNγ
IL-4
C
Sigmoid '08: Severe colitis
Sigmoid '09: Remission

periodic acid-Schiff (PAS) stain to determine that although virtually no mucus was detectable in the sigmoid colon in 2008, ample mucus production was present in 2009 (Figure 3C). A role for TNFα in UC pathogenesis has been suggested by the positive outcomes of several clinical trials with the anti-TNF antibody infliximab (Wilhelm et al., 2008). Several types of immune cells, notably activated macrophages and T cells, express TNFα. Although we found a higher frequency of $TNF\alpha^+$ T_H cells in colitis-affected tissue compared to helminth-exposed tissue in 2008 (Figure S3), remission was not associated with the suppression of TNFα responses. The inflammatory response to re-infection with *T. trichiura* was characterized by a high frequency of $TNF\alpha^+$ T_H cells (Figure S3). In summary, active colitis was associated with monocytokine-producing $CD4^+$ IL-17^+ T_H cells, whereas helminth colonization and disease remission was characterized by the presence of IL-22^+ T_H cells in the colonic mucosa. High frequencies of mucosal IL-17^+ and $TNF\alpha^+$ T_H cells were associated with active colitis in 2008, but these cells persisted during symptomatic remission in 2009.

Characterization of T_H cell responses in the peripheral blood.

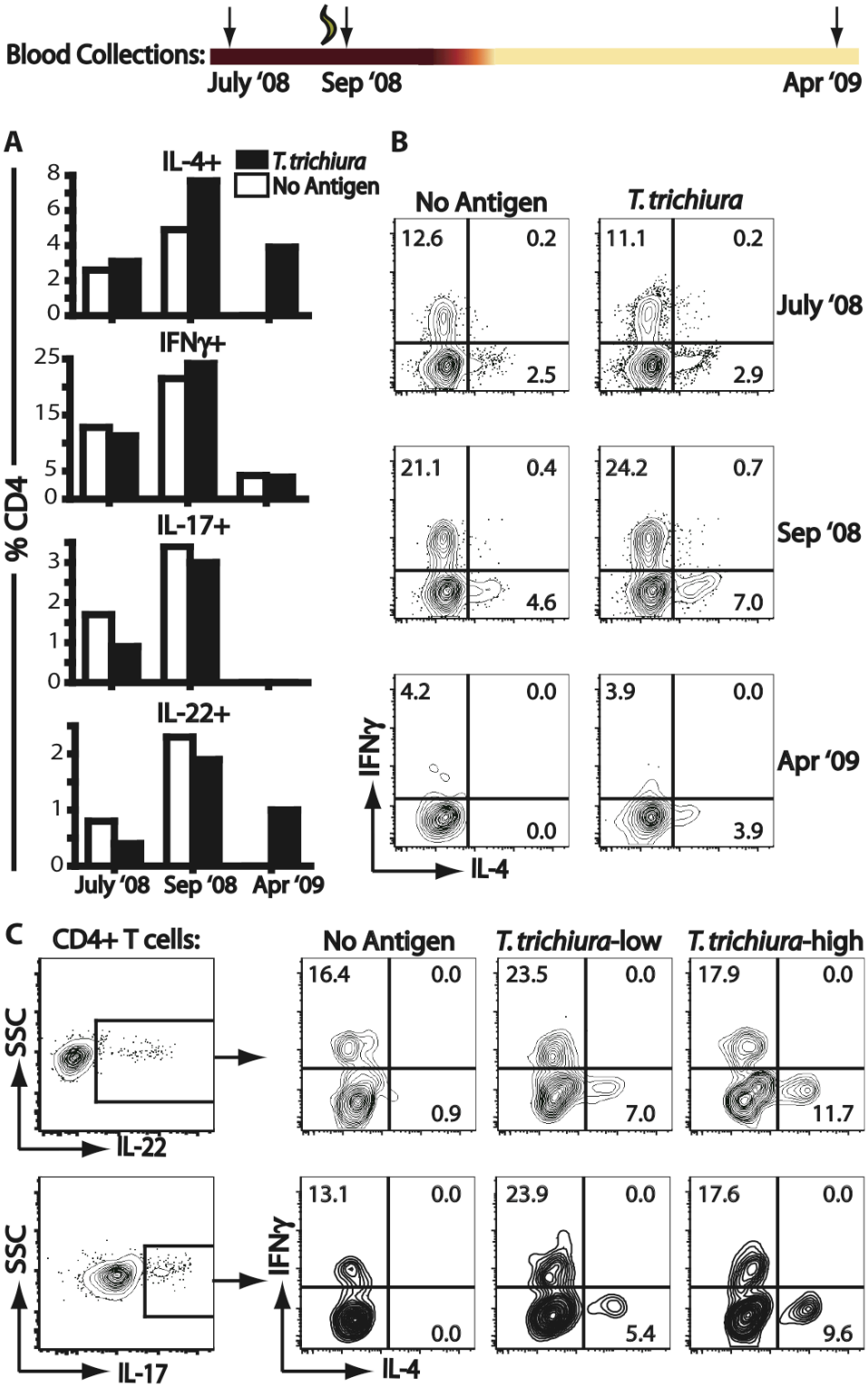
T cell responses in the peripheral blood were also analyzed by flow cytometry at three time-points: mid-2008, during UC relapse; late 2008, immediately following re-infection with *T. trichiura*; and early 2009, during remission (Figure 4). Isolated peripheral blood mononuclear cells (PBMC) from each time-point were cryopreserved at the time of venipuncture and later cultured in parallel in the presence of *T. trichiura* antigen prepared from an adult worm segment extracted from a biopsy. After 96 hours in culture with medium alone, T_H cells from the 2008 time points showed signs of non-

Figure 4. IL-4$^+$ T_H cells characterize *T. trichiura*-specific responses in the peripheral blood. Cropreserved peripheral blood mononuclear cells (PBMC) collected at three time points (marked with arrows) were thawed and cultured in parallel for 96 hours in the presence or absence of a homogenate prepared from worm fragments collected during colonoscopy. After culture, cells were assayed by flow cytometry for intracellular cytokine expression following a 5-hour PMA/ionomycin stimulation.

(A, B) Percentage of CD4$^+$ T cells that produce cytokines in response to *T. trichiura* antigen stimulation compared to cells cultured in media alone are shown as histograms **(A)** or bivariate flow cytometry contour plots **(B)**.

(C) Flow cytometry gating strategy to illustrate an expansion of *T. trichiura* antigen-specific IL-4$^+$ cells within both the IL-22$^+$ and IL-17$^+$ CD4$^+$ T cell subsets from PBMC that were collected after reinfection (September 2008). Worm antigen was added at 100μg/mL (high) or 25μg/mL (low). SSC, side scatter.

FIGURE 4


Blood Collections:
July '08
Sep '08
Apr '09
A
IL-4+
T. trichiura
No Antigen
IFNγ+
IL-17+
IL-22+
% CD4
July '08 Sep '08 Apr '09
B
No Antigen
T. trichiura
12.6
0.2
2.5
11.1
0.2
2.9
July '08
21.1
0.4
4.6
24.2
0.7
7.0
Sep '08
4.2
0.0
0.0
3.9
0.0
3.9
Apr '09
IFNγ
IL-4
C
CD4+ T cells:
SSC
IL-22
No Antigen
T. trichiura-low
T. trichiura-high
16.4
0.0
0.9
23.5
0.0
7.0
17.9
0.0
11.7
SSC
IL-17
13.1
0.0
0.0
23.9
0.0
5.4
17.6
0.0
9.6
IFNγ
IL-4

specific activation, expressing many cytokines in the absence of stimulation with *T. trichiura* antigen (Figure 4, A and B). This could reflect an acute inflammatory response to helminth infection as well as ongoing colitis-associated inflammation. In contrast, T cells collected during remission (several months after re-infection) did not shown signs of non-specific activation. Culture with *T. trichiura* antigen selectively promoted expansion of IL-4^+ T_H cells in PBMC that were collected following re-infection (Figure 4, A and B). Small numbers of IL-22^+ T_H cells could also be detected in 2009 that were specific to *T. trichiura* antigen (Figure 4A). A circulating population of IL-4^+ IL-17^+ T_H cells was recently identified in patients with asthma (Cosmi et al., 2010), a condition also associated with chronic T_H2-driven inflammation. Similarly, IL-22^+ and IL-17^+ T_H cells that co-expressed IL-4 in response to *T. trichiura* antigen were present in PBMC after re-infection (Figure 4C). Such cells were not observed in PBMC from a healthy control, suggesting that the co-expression of IL-4 with IL-22 and IL-17 may represent a signature in the peripheral blood of mucosal T_H2-associated inflammation.

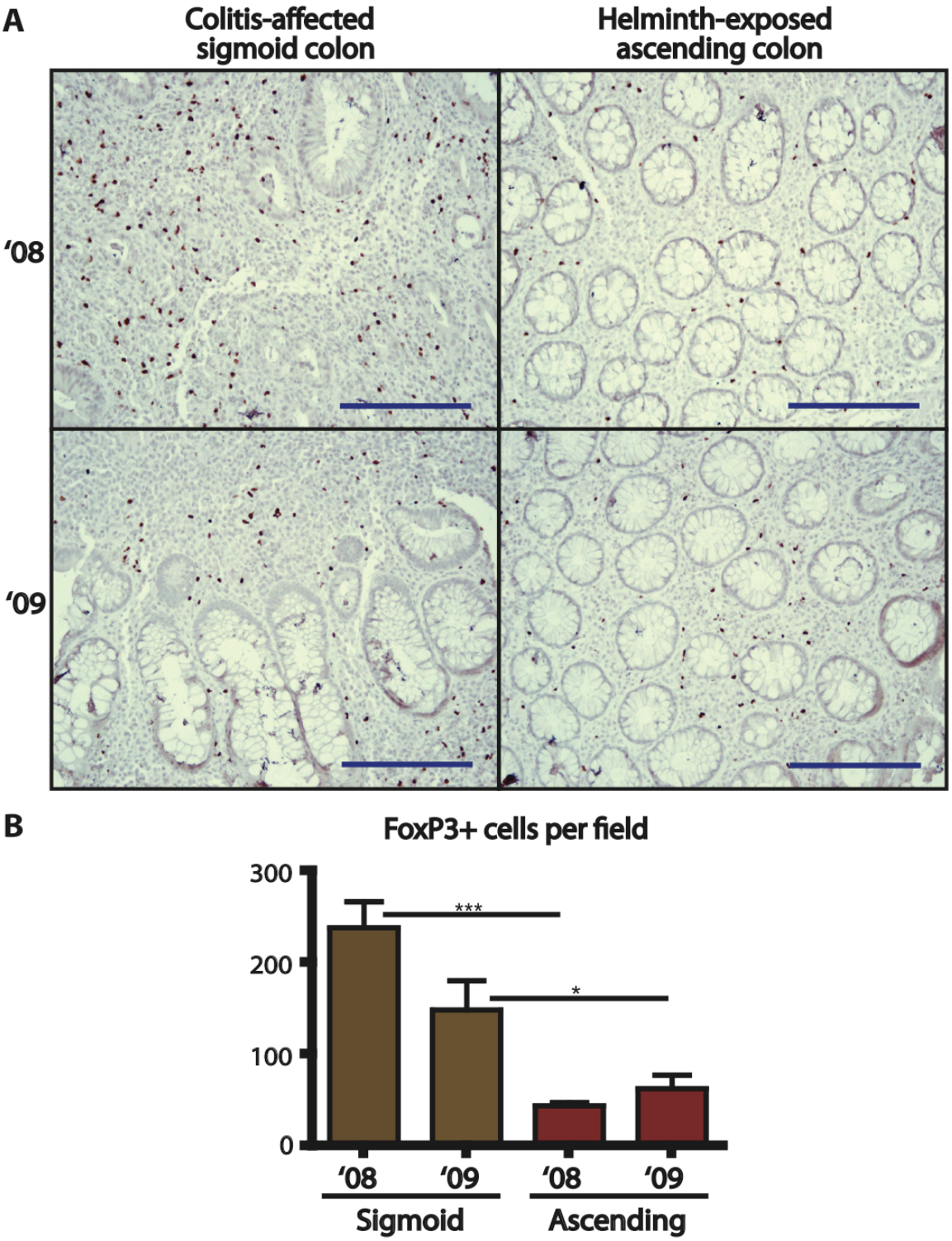
Because $FoxP3^+$ T_{regs} have been implicated in helminth-mediated immune regulation (Maizels and Yasdanbakhsh, 2003), we quantified $FoxP3^+$ cells in the colonic mucosa by immunohistochemistry (Figure 5). $FoxP3^+$ cells were more abundant in the colitis-affected tissue than in the helminth-colonized tissue at both time-points (Figure 5). These results suggest that the presence of T_{regs} in the mucosal tissues is driven predominantly by inflammation rather than helminth colonization, consistent with observations that a high number of T_{regs} is a marker of immune activation in the intestinal mucosa (Loke et al., 2010).

Figure 5. Detection of FoxP3 expression by immunohistochemistry shows that FoxP3$^+$ cells are more abundant in colitis-affected tissue compared to helminth-exposed tissue.

(A) Representative low powered immunohistochemistry images of FoxP3 staining on biopsies collected from the sigmoid colon and the ascending colon in 2008 and 2009. Brown dots are nuclear stained Foxp3+ cells. Scale bars, 50 μm.

(B) Histograms showing the differences in the average number of FoxP3$^+$ cells per field of view between the sigmoid colon and the ascending colon in 2008 and 2009. (*** $P < 0.0005$, * $P < 0.05$).

FIGURE 5


A
Colitis-affected sigmoid colon
Helminth-exposed ascending colon
'08
'09
B
FoxP3+ cells per field
300
200
100
0
***
*
'08
'09
'08
'09
Sigmoid
Ascending

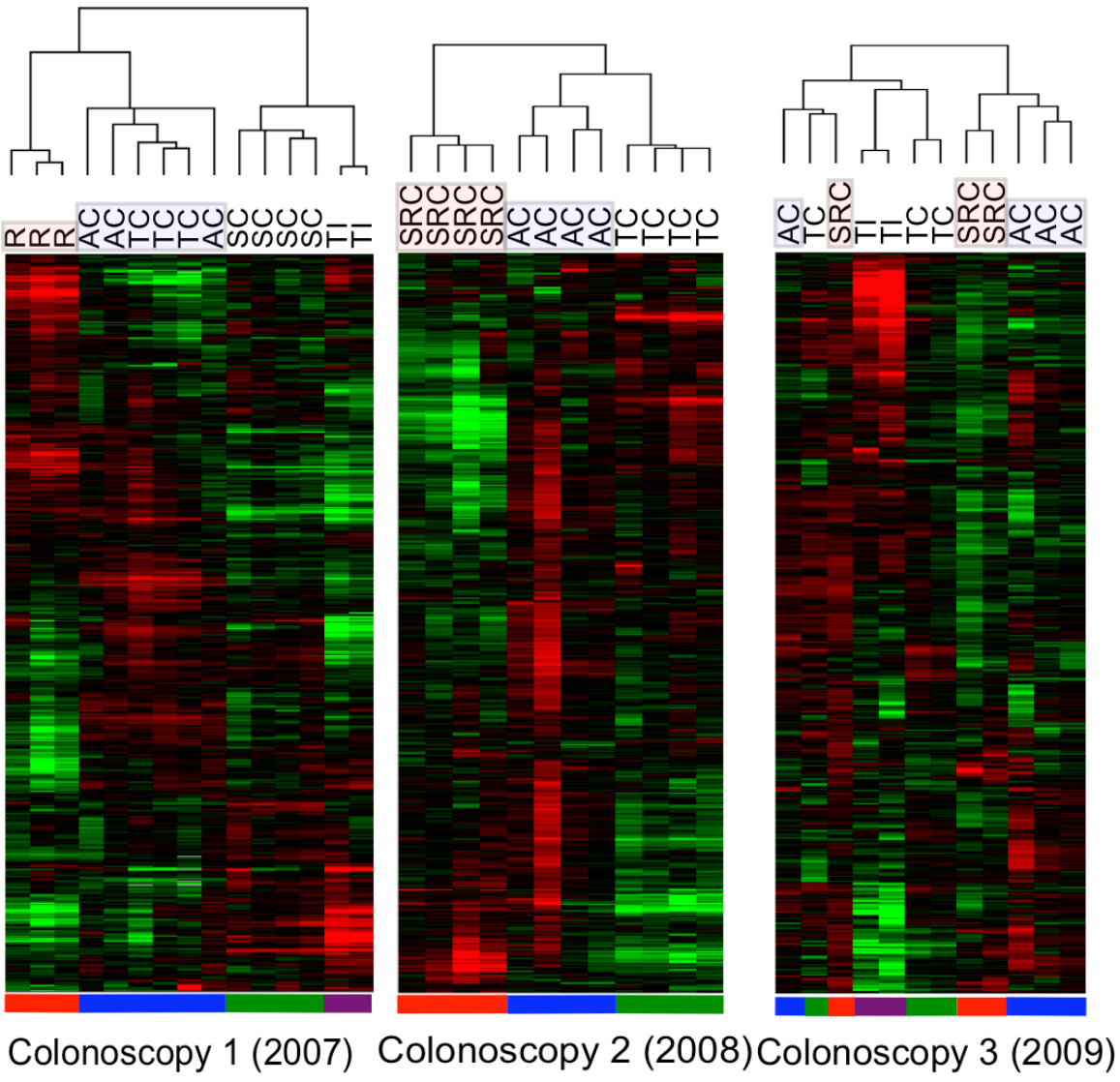
Transcriptional profiling analysis of mucosal responses.

To identify molecular signatures that are associated with helminth colonization and colitis, we performed gene expression profiling on biopsies collected from colonoscopies in 2007, 2008, and 2009. Each biopsy fragment was treated as an independent sample. In 2007, samples were obtained from the terminal ileum (n = 2), the ascending colon (n = 3), the transverse colon (n = 2), the sigmoid colon (n = 4), and the rectum (n = 3). In 2008, samples were obtained from the ascending colon (n = 4), the transverse colon (n = 4), and the sigmoid colon (n = 4). A sample with a worm and its surrounding tissue was also analyzed (n = 1). Finally, in 2009, samples were obtained from the terminal ileum (n = 3), the ascending colon (n = 5), the transverse colon (n = 3), and the sigmoid colon (n = 3).

Unsupervised hierarchical clustering analysis was performed on the three datasets (Figures 6 and S4) to determine whether gene expression levels in different anatomical locations were related. In the first colonoscopy (in 2007), the samples from the ascending colon and transverse colon clustered together, reflecting a similar response to worm colonization in both regions. The samples from the rectum also clustered together strongly because of the common expression of a group of inflammatory genes reflecting the active colitis flare in these tissues at that time (Figures 6 and S4). The terminal ileum had a distinct profile (probably because it is part of the small intestine), but it also had a profile more similar to the normal sigmoid colon than either the colonized or inflamed regions of the large intestine. In 2008, worms were not found in the transverse colon and at this time this region had a profile quite distinct from the ascending colon, which remained colonized by worms. The entire sigmoid colon at this time was heavily

Figure 6. Transcriptional profiling analysis of biopsy fragments from different regions of the colon in three different colonoscopies. Gene expression patterns of biopsy fragments from the terminal ileum (TI), ascending colon (AC), transverse colon (TC), sigmoid colon (SC), rectum (R), or rectosigmoid colon (SRC) taken during a proctitis flare (2007), severe colitis (2008), and symptomatic remission (2009). Hierarchical clustering analysis was used to organize genes and samples. Each row represents an individual gene and each column an individual biopsy fragment from a specific region. Black indicates median level of expression; red, greater than median expression; and green, less than median expression. Horizontal bars at the bottom of the figure indicate the clustering or dispersal of the samples affected by colitis (red), helminth colonization (blue), normal (green) and from the uninvolved terminal ileum (purple). Note that in 2009, the horizontal bars of the same colors are not clustered.

FIGURE 6


R R R AC AC TC TC TC AC SC SC SC SC TI TI
SRC SRC SRC SRC AC AC AC AC TC TC TC TC
AC TC SRC TI TI TC TC SRC SRC AC AC AC
Colonoscopy 1 (2007)
Colonoscopy 2 (2008)
Colonoscopy 3 (2009)

inflamed and these samples clustered tightly together. In 2009, when the colon was no longer inflamed and the patient was in remission, the clustering of expression profiles by anatomical location was much less pronounced. Although the terminal ileum remained very distinct, samples from ascending colon, transverse colon and sigmoid colon were interspersed in different branches of the tree [Figure 6: colonoscopy 3 (2009)].

Supervised two-way comparisons were then made between biopsies from the helminth-colonized areas and colitis-affected areas to identify genes that were differentially expressed as a result of colonization (Table 1) or colitis (supplementary tables available at www.sciencetranslationalmedicine.org). In 2007 and 2008, the genes that were differentially expressed between areas of helminth colonization and colitis were enriched by gene ontology analysis for the biological processes of carbohydrate, lipid, fatty acid and steroid metabolism for helminth colonization (Figure 7A) and immunity and defense for colitis (Figure 7B). These results suggest that, whereas colitis is associated with immune-mediated responses, helminth colonization may be associated with increased carbohydrate and steroid metabolism. A large number of genes (Table 1) upregulated in helminth-colonized tissues are uncharacterized, revealing the rudimentary extent of our understanding of the mucosal response to helminth colonization. In 2009, the uninflamed colon had very few (< 5) genes that were significantly differentially expressed between the ascending colon (helminth colonized) and the sigmoid colon (previously inflamed) tissues. When we conducted multi-class significance analysis of microarrays (SAM) analysis to include comparison with biopsy tissues that are unaffected by colitis or helminth colonization, we found that the genes that are most differentially expressed between regions of the colon in 2007 and 2008 were still

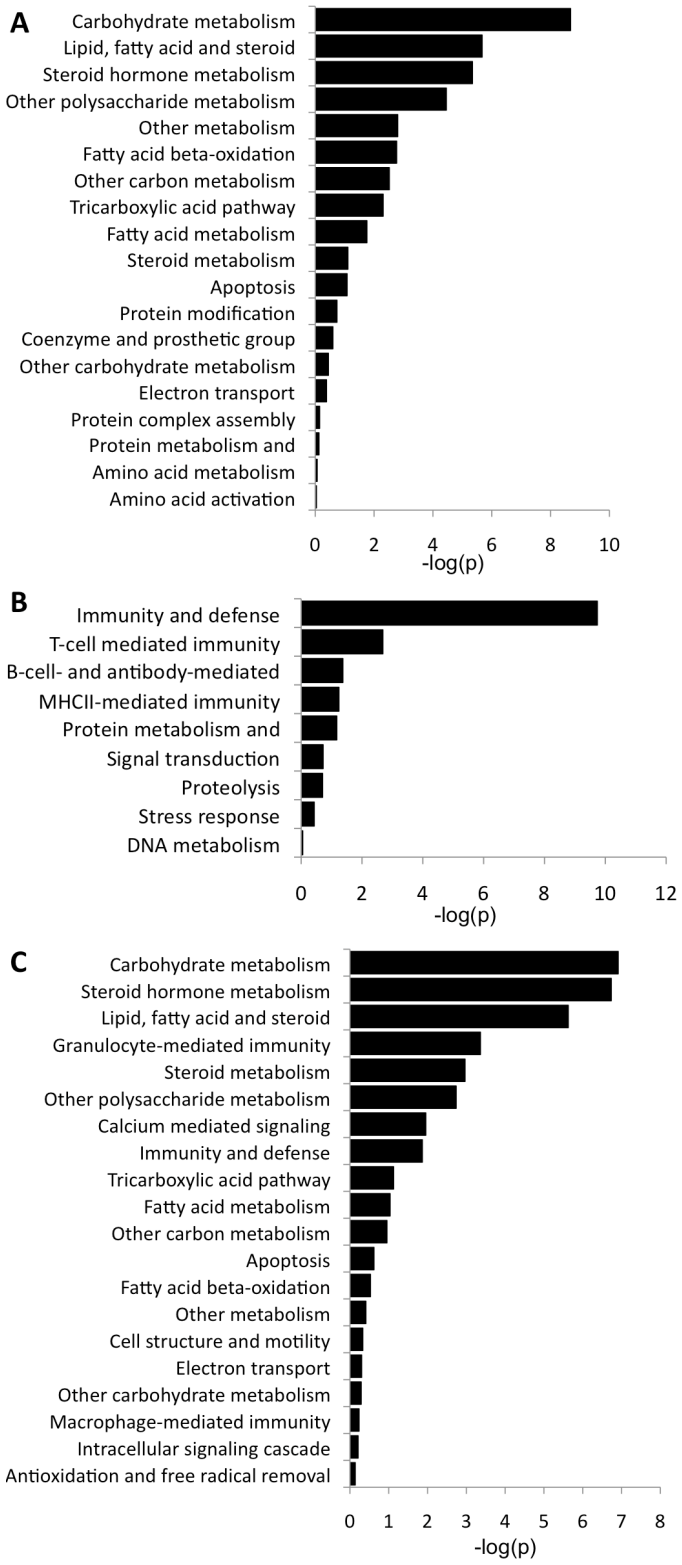
Figure 7. Gene ontology (GO) analyses to identify biological processes that are induced in helminth-colonized and colitis-affected regions of the colon.

(A) Biological processes induced in helminth-colonized tissues as determined by GO analysis of genes that are significantly upregulated in these tissues in 2007. X-axis indicates the level of statistical significance [as -log(p)] in enrichment for the indicated biological process.

(B) Biological processes induced in tissues with active colitis as determined by GO analysis of genes significantly upregulated in these tissues in 2007.

(C) Biological processes of genes differentially expressed between regions with active colitis, helminth colonization, and normal appearance as determined by multi-class analysis using the statistical analysis of microarrays (SAM) analysis from samples in 2008.

FIGURE 7


A
Carbohydrate metabolism
Lipid, fatty acid and steroid
Steroid hormone metabolism
Other polysaccharide metabolism
Other metabolism
Fatty acid beta-oxidation
Other carbon metabolism
Tricarboxylic acid pathway
Fatty acid metabolism
Steroid metabolism
Apoptosis
Protein modification
Coenzyme and prosthetic group
Other carbohydrate metabolism
Electron transport
Protein complex assembly
Protein metabolism and
Amino acid metabolism
Amino acid activation
0 2 4 6 8 10
-log(p)
B
Immunity and defense
T-cell mediated immunity
B-cell- and antibody-mediated
MHCII-mediated immunity
Protein metabolism and
Signal transduction
Proteolysis
Stress response
DNA metabolism
0 2 4 6 8 10 12
-log(p)
C
Carbohydrate metabolism
Steroid hormone metabolism
Lipid, fatty acid and steroid
Granulocyte-mediated immunity
Steroid metabolism
Other polysaccharide metabolism
Calcium mediated signaling
Immunity and defense
Tricarboxylic acid pathway
Fatty acid metabolism
Other carbon metabolism
Apoptosis
Fatty acid beta-oxidation
Other metabolism
Cell structure and motility
Electron transport
Other carbohydrate metabolism
Macrophage-mediated immunity
Intracellular signaling cascade
Antioxidation and free radical removal
0 1 2 3 4 5 6 7 8
-log(p)

enriched for the biological processes of carbohydrate and steroid metabolism as well as for granulocyte-mediated immunity (Figure 7C).

After completing analysis of the microarray results from the first colonoscopy (2007), we conducted real-time PCR analysis to measure expression of genes previously identified to be important in helminth immunity and mucosal responses (Figures 8A and S5) as well as to verify genes identified by our microarray analysis (Figure 8B). Helminth-colonized ascending colon expresses significantly ($P < 0.05$) higher levels of transcripts for IL-4, IL-25, and resistin-like molecule beta (RELMβ) than the rectum. By contrast, IL-17 (Figure 8A) and TNF (Figure S5) were elevated in the rectum, which had active proctitis. In addition to the well-established inflammatory cytokines (for example, IL-1β, IL-6, and IL-8), this tissue also revealed strikingly high expression of the decoy IL-13 receptor alpha 2 (IL-13RA2) and Chitinase 3 like 1 (CHI3L1) (Figure 8, B and C). The chemokine CCL20 was more highly expressed in the helminth-colonized tissue (in the ascending and traverse colon) whereas CXCL1 and CXCL13 were more expressed in the rectal region with proctitis (Figure 8B). Helminth-colonized tissues also expressed much higher levels of metabolic enzymes such as ethanolamine kinase 1 (ERHK1) and hyaluronan synthase 3 (HAS3).

We conducted additional real-time PCR analysis of biopsies taken from 2008 and 2009 to examine dynamic changes of specific genes (Figures 8C and S6). We also compared RNA expression in these biopsies to that found in biopsies ($n = 6$) taken from a healthy individual. The improvement in inflammatory conditions for the colitis-affected regions in 2009 was most strikingly associated with decreased expression of IL-8, IL-17A, IL-13RA2, and CHI3L1 (Figures 8C and S6). In contrast, expression of

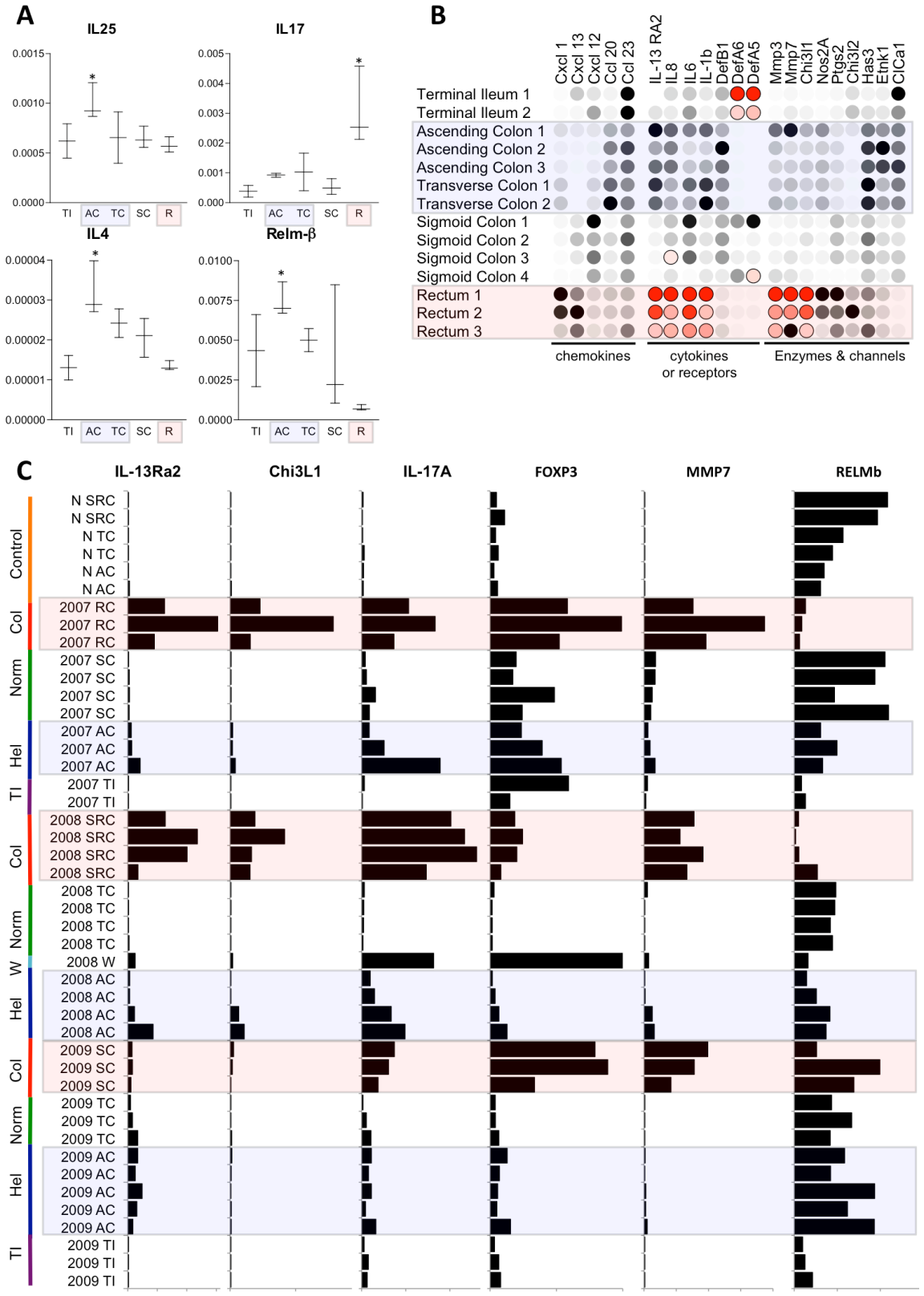
Figure 8. Real-time PCR validation and analysis of differentially expressed genes in biopsy fragments. Samples from helminth-colonized tissue are shaded in light blue and samples from tissue with active colitis are shaded in red. TI = terminal ileum, AC = ascending colon, TC = transverse colon, SC = sigmoid colon, SRC = rectosigmoid colon, W = worm granuloma, and R = rectum.

(A) Boxplot showing the minimum, maximum, and median values of real-time PCR measurements of selected proinflammatory mediators from biopsy fragments collected in 2007. * $P < 0.05$

(B) Real-time PCR verification of selected genes from the microarray profiling in 2007. Each individual spot shows the relative expression level of a gene from a particular sample in relative shades of black and red. Darker spots reflect higher expression. Red spots (with borders) reflect 10 fold greater levels of expression than black spots and are used when there are logarithmic level differences in expression.

(C) Real-time PCR measurement of dynamic changes in selected proinflammatory mediators from samples taken in 2007, 2008, and 2009, and compared to samples collected from a normal individual (N) as indicated by an orange vertical bar (Control). Red vertical bar (Col) indicates samples taken from regions with active colitis (also shaded in red) and blue vertical bars (Hel) indicates samples taken from helminth-colonized tissues (also shaded in blue). Tissues with normal appearance (Norm) are indicated in green and samples from the terminal ileum (TI) are indicated in purple. A sample that came from a worm surrounded by granulomatous tissue (W) is shown in light blue.

FIGURE 8


A
IL25
IL17
IL4
Relm-β
TI
AC
TC
SC
R
B
Cxcl 1
Cxcl 13
Cxcl 12
Ccl 20
Ccl 23
IL-13 RA2
IL8
IL6
IL-1b
DefB1
DefA6
DefA5
Mmp3
Mmp7
Chi3l1
Nos2A
Ptgs2
Chi3l2
Has3
Etnk1
ClCa1
Terminal Ileum 1
Terminal Ileum 2
Ascending Colon 1
Ascending Colon 2
Ascending Colon 3
Transverse Colon 1
Transverse Colon 2
Sigmoid Colon 1
Sigmoid Colon 2
Sigmoid Colon 3
Sigmoid Colon 4
Rectum 1
Rectum 2
Rectum 3
chemokines
cytokines or receptors
Enzymes & channels
C
IL-13Ra2
Chi3L1
IL-17A
FOXP3
MMP7
RELMb
Control
Col
Norm
Hel
TI
W
N SRC
N TC
N AC
2007 RC
2007 SC
2007 AC
2007 TI
2008 SRC
2008 TC
2008 W
2008 AC
2009 SC
2009 TC
2009 AC
2009 TI

matrix metalloproteinase 7 (MMP7) remained high in the sigmoid colon tissues for 2009, indicating that the process of tissue repair was ongoing. Also, expression of RELMβ was significantly (P = 0.0002) reduced under conditions of colitis, relative to helminth-colonized tissues and healthy tissues. Helminth-colonized tissues consistently expressed proinflammatory genes also, although in lower levels than in the colitis-affected regions. These dynamic changes in gene expression provide a molecular picture of inflammation during colitis, tissue recovery, wound healing, and helminth colonization.

DISCUSSION

Helminthic therapy has generated substantial interest clinically, in large part due to the successful outcomes of clinical trials showing a positive effect of *Trichuris suis* ova treatment on IBD (Summers et al., 2005a; Summers et al., 2005b) and a growing body of literature demonstrating the ability of helminths to suppress pathologic inflammation in animal models of autoimmunity (Maizels and Yazdanbakhsh, 2003; Elliott et al., 2007). However, the mechanisms that underlie the benefits of helminth exposure are unknown. We found that intestinal tissue of the colon with active colitis contains mono-cytokine-producing IL-17^+ T_H cells in the lamina propria, whereas intestinal tissue that has been colonized by helminths or has undergone mucosal healing contains polycytokine-producing IL-22^+ T_H cells, supporting a protective role for IL-22 in maintaining mucosal integrity. Although it is difficult to draw firm conclusions from the analysis of a single individual, these findings have generated hypotheses that can be tested through future functional studies in mouse models or with larger clinical cohorts.

We hypothesize that the presence of *T. trichiura* in the intestinal epithelium activates a T_H2 response as well as IL-22^+ T_H cells in order to expel the parasites through increased epithelial cell turnover, goblet cell hyperplasia and increased mucus production in the entire colon (Artis and Grencis, 2008), which has the bystander consequence of reducing the pathology associated with UC and leading to symptomatic relief. In this subject, the inflammatory response induced by *T. trichiura* colonization was associated with the repair of the colonic epithelium and glands, and a striking restoration of mucus production.

Whereas goblet cell hyperplasia is a characteristic of helminth infection, loss of goblet cells and mucus production is commonly observed in the diseased tissue of UC patients (Xavier and Podolsky, 2007). Mucus plays a key role in maintaining the protective mucosal barrier (Gibson and Muir, 2005). Some mucin gene variants confer a predisposition to UC (Kyo et al., 1999; Kyo et al., 2001). Furthermore, mucin-deficient mice develop spontaneous colitis (Van der Sluis et al., 2006; An et al., 2007; Heazlewood et al., 2007). Indeed, the delivery of phosphatidyl rich phospholipids into the lumen of UC patients has been clinically tested with positive results (Gibson and Muir, 2005; Stremmel et al., 2005; Stremmel et al., 2010), with the hypothesis that it improves the barrier function of the mucus layer. Gene ontology analysis of our microarray data revealed that carbohydrate metabolism pathways were highly upregulated in helminth-exposed compared to colitis-affected tissue. This may reflect goblet cell hyperplasia in helminth-colonized tissues; indeed, expression of mucin 1 and mucin 4 are upregulated after helminth colonization (Figure S4).

IL-22 induces intestinal mucus production in mouse goblet cells, and this effect likely partially underlies the ability of IL-22 to suppress colitis. IL-13 and IL-10 (Schopf et al., 2002; Artis and Grencis, 2008) also promote mucus production during helminth infection and may contribute to the effect observed in this subject. Hence, the coordinated immune response activated to expel the nematode parasite and to protect the host from the intestinal damage that is caused by the invasion of the epithelium by the parasite, may also promote mucosal healing at a distal site affected by colitis. However, it is also possible that the appearance of IL-22$^+$ T_H cells in the intestinal mucosa is an indicator of mucosal healing and would occur independently of helminth infection in individuals with UC who spontaneously go into remission. Excess IL-13 production is itself pathogenic in a mouse model of UC (Fuss et al., 2008). It is possible that helminth infection may exacerbate UC symptoms in some patients by elevating the T_H2 response in the intestinal mucosa. If helminthic therapy becomes widely used, it will be particularly important to separate patients into groups that may respond, may not respond, or may suffer disease exacerbation from infection.

Transcriptional profiling may distinguish among patients with UC, Crohn's disease, and irritable bowel syndrome (von Stein et al., 2008), as well as predict responses to TNF antibody treatments (Arijs et al., 2009). Many of the genes identified in these previous studies as associated with UC were also found by us to be upregulated in colitis-affected tissues (for example, IL-13RA2, CHI3L1, MMP3, MMP7, PTGS2) as well as established and expected inflammatory mediators (for example, IL-1B, IL-6, IL-8, NOS2A, CXCL1 and CXCL13), validating our approach. In our comparison of mucosal inflammatory responses to helminth colonization with inflammatory responses

during UC, we found that many of the genes associated with helminth colonization (Table 1) have unknown functions. IL-13RA2, which we found to be increased with colitis and *T. trichiura* colonization (Figure 7), has been associated with fibrosis during chronic colitis, airway inflammation, and schistosomiasis (Wilson et al., 2010; Wynn, 2007; Fichtner-Feigl et al., 2006; Fichtner-Feigl et al., 2008). Whether IL-13RA2 plays a protective or pathogenic role in intestinal inflammation remains to be established. Increased expression of CHI3L1 (also known as YKL-40) has been associated with IBD (Mizoguchi, 2006) and airway inflammation (Ober and Chupp, 2009), where it may be involved in extracellular matrix remodeling. Notably, we found that the expression of RELMβ was inversely correlated with expression of IL-13RA2 and CHI3L1, being suppressed in tissues with active colitis. RELMβ is a protein produced by goblet cells that promotes expulsion of gastrointestinal parasites (Artis et al., 2004; Herbert et al., 2009) and reduces the severity of trinitrobenzene sulfonate-induced colitis in mice (Krimi et al., 2008).

Although this case study suggests that *T. trichiura* infection can alleviate symptoms of UC, infection itself can also cause intestinal inflammation and colitis that mimics IBD (Schopf et al., 2002; Wilson et al., 2010). Indeed, proinflammatory cytokines and mediators were upregulated in helminth-colonized regions of the colon, although at lower amounts than in regions with active colitis. Heavy worm infestation, especially in children, can cause dysentery and rectal prolapse and lead to growth retardation, secondary anemia, and reduced cognitive function. The specific conditions in which *T. trichiura* infection may lead to any therapeutic benefit are currently unclear, and it is possible that infection can exacerbate existing conditions. Nonetheless, the

phenomenon of helminth-mediated immune modulation is now well established (Maizels and Yazdanbakhsh, 2003; Elliott et al., 2007). Hence, the identification of the mechanisms of these helminth-induced mucosal responses could provide new therapeutic targets for IBD.

MATERIALS AND METHODS

Biopsy collection and endoscopic analysis. Four to five pinch biopsies were collected from each different anatomical location (terminal ileum, ascending colon, transverse colon, sigmoid colon or rectum) during ileocolonoscopies in 2007, 2008 and 2009. Two biopsies were sent for histology, while the other biopsies were separated into fragments for either immediate flow cytometry analysis or snap frozen for RNA extraction in Trizol reagent (Invitrogen).

Histopathology and immunohistochemistry. Colon biopsy specimens were fixed in 10% buffered formalin and embedded in paraffin. Tissue sections were washed with xylene and re-hydrated. Hematoxylin and eosin stains were carried out according to standard procedures. The Periodic Acid Schiff's stain was carried out with kit components from American MasterTech, according to the manufacturer's instructions. A mouse monoclonal antibody (clone 236A/E7, Abcam) was used for detection of FoxP3 by immunohistochemistry. Antigen retrieval was achieved by placing slides (in sodium citrate buffer) in a pressure cooker for 20 minutes. Sections were incubated with primary antibody (10 μg/mL in TBS, 2% BSA) for one hour at room temperature. Primary antibodies were detected with anti-mouse antibodies conjugated to horseradish

peroxidase (DAKO Envision kit) and developed with 3,3'-diaminobenzidine. Data was analyzed with the t-test using GraphPad Prism software.

Flow cytometry on colon biopsies and peripheral blood. Colon biopsy specimens were treated with 0.25 mg/ml collagenase type II (Sigma-Aldrich) for 30 minutes with constant shaking at room temperature. Digested tissue was dispersed over a 70-micron nylon mesh filter. Cell suspensions were washed twice with RPMI containing 15% fetal calf serum. Whole blood was collected into ACD-containing tubes (BD Biosciences) and PBMC were isolated by density centrifugation. For stimulation of PBMC with *T. trichiura* antigen, a homogenate was prepared from an adult worm fragment collected at biopsy. Cryopreserved PBMC from each timepoint were thawed and cultured in parallel for 96 hours in the presence or absence of homogenate at 100μg/mL (high) or 25μg/mL (low). Biopsy cells and PBMC ($1x10^6$) were resuspended in 200 μl of complete R-10 [RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum (Hyclone), 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine], and stimulated with phorbol myristate acetate (10 ng/ml) and ionomycin (1 μg/ml) in the presence of brefeldin A (GolgiPlug, BD Pharmingen) for five hours at 37°C. Unstimulated control samples were cultured with brefeldin A only. For biopsy cells, amphotericin B (Gibco) was also added to the culture media. Cell surface staining and intracellular cytokine staining were performed with Fix/Perm and PermWash solutions from BD and eBioscience, according to the manufacturer's instructions. Staining antibodies are listed in Table S1. Pie charts were generated using SPICE software (provided by M. Roederer).

Microarray and real-time PCR analysis of biopsy fragment samples. Total RNA was extracted from biopsy fragments and amplified with an RNA amplification kit (Ambion). Cy5-labeled experimental samples were hybridized against a Cy3-labeled reference consisting of an equal quantity of pooled, amplified RNA from all the experimental samples from that experiment. Two-color hybridizations were performed on Human Exonic Evidence-Based Oligonucleotide (Invitrogen) microarrays (69) printed in-house at the UCSF Center for Advanced Technologies. Arrays were scanned using a GenePix 4000B scanner and GenePix PRO Version 4.1 (Axon Instruments/ Molecular Devices). The Spotreader program (Niles Scientific) was used for array gridding and image analysis. The resulting files were uploaded to Acuity Version 4.0 (Molecular Devices), where the raw data were log transformed and filtered for retention of spots that were of high quality and for removal of nonhuman control spots. Expression profiling data were filtered further (for microarray spots with data in at least 80% of the arrays) before undergoing unsupervised hierarchical clustering analysis and then visualized using Treeview. Filtered datasets were also analyzed for statistically significant genes (either through two comparisons or multi-class analysis) using the Significance Analysis of Microarrays (SAM) software Version 2.23A (70). Gene ontology and pathway analyses were performed using Protein Analysis Through Evolutionary Relationships (71). For real-time polymerase chain reaction (PCR) analysis, 100 ng - 1 μg of RNA from each sample was reverse transcribed and the resulting cDNA was used in quantitative real-time PCR reactions, with SYBR green labeling. Most of the PCR reactions used primers designed (in-house) to span introns so that amplification of genomic DNA could be avoided, whereas IL-17 and FoxP3 mRNA were measured using

Taqman probes (Applied Biosystems). All values were normalized to β-actin values. Data was analyzed with the t-test using GraphPad Prism software.

Figure S1. Examples of photographs taken during endoscopic examinations in 2007, 2008, and 2009 from different regions of the colon. In 2007, worms were observed in the cecum, ascending and transverse colons, while the sigmoid colon was normal and the rectum exhibited signs of proctitis. In 2008, worms were only observed in the ascending colon and not in the transverse colon, while the remainder of the colon exhibited signs of widespread colitis. In 2009, worms were mainly observed in the ascending colon, while intact mucosa was observed in the remainder of the colon with few signs of inflammation.

SUPPLEMENTARY FIGURE 1

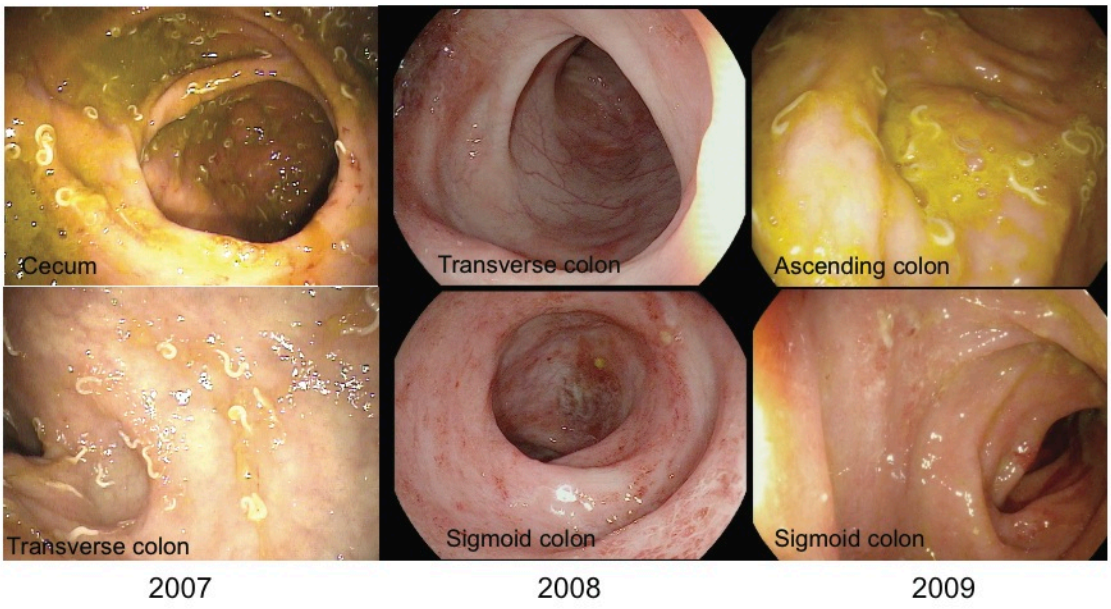


Figure S2. Examples of histopathology images from colitis-affected and helminth-exposed mucosal tissues. Tissue sections of colon biopsies stained with hematoxylin and eosin.

(A) Severe colitis in the sigmoid colon in 2003 showing ulceration and crypt abscess.

(B, C) Sigmoid colon in 2005, after *T. trichiura* colonization, showing restored glands and goblet cells.

(D, E, F) Sigmoid colon in 2007 showing normal glands and goblet cells.

(G) Ascending colon in 2005 showing prominent eosinophilia.

(H) Transverse colon in 2007 showing prominent eosinophilia even in regions not in direct contact with worms.

(I) Ascending colon in 2007 showing prominent eosinophilia.

(J, K) Proctitis in the rectum in 2007.

(L) Severe colitis in the sigmoid colon in 2008 showing ulceration and crypt abscess.

(M) Sigmoid colon in 2009, after the second ingestion of *T. trichiura* ova, showing restoration of goblet cells and gland structure.

(N, O) Longitudinal section of a *T. trichiura* worm (W) with ova (Eggs).

(P) Cross section of a *T. trichiura* worm (W) in the ascending colon in 2009.

SUPPLEMENTARY FIGURE 2

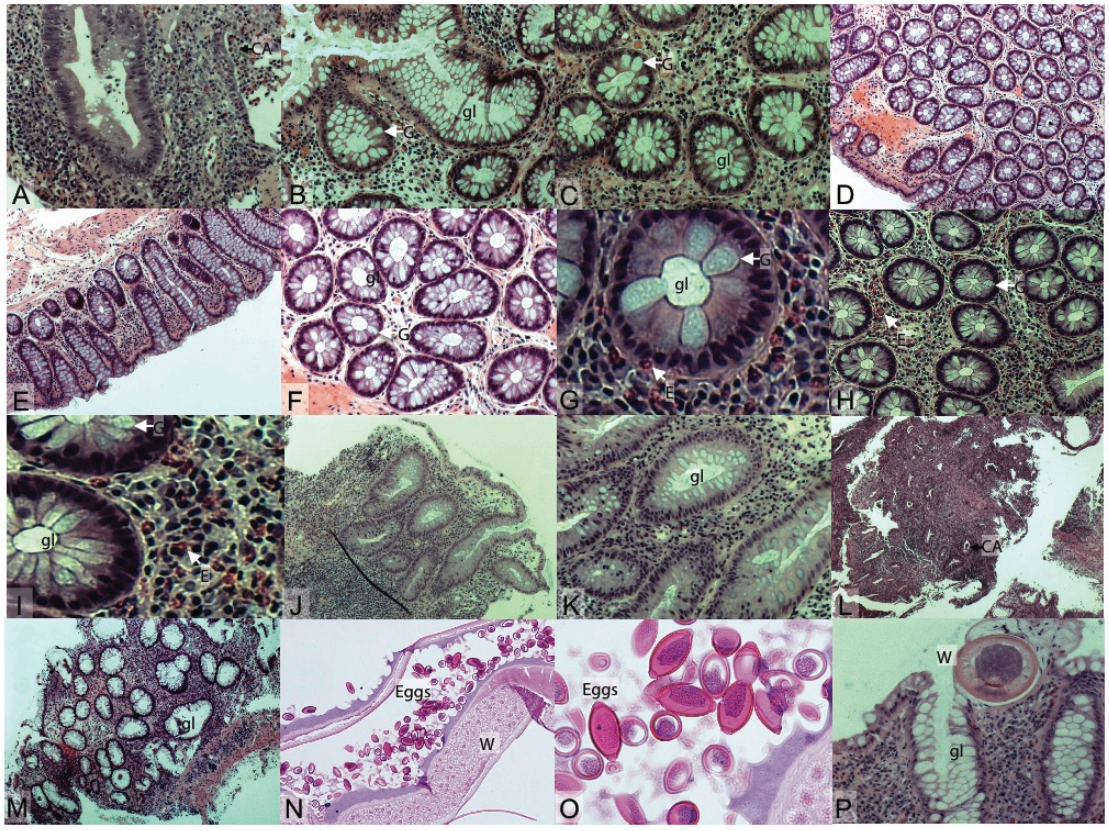


Figure S3. Flow cytometry analysis of mucosal tissues from 2008 and 2009. Intracellular cytokine staining of CD4+ and CD8+ cells was conducted on PMA/Ionomycin stimulated lymphocytes isolated *ex vivo* from biopsies.

(A) An example of the gating strategy used for the detection of live (Amine-Aqua negative), CD3+ lymphocytes, CD4+ and CD8+ cells extracted from colon biopsy tissue for analysis of cytokine-producing T cells.

(B, C) Detection of **(B)** IL-17, IL-22, IL-4, IFNγ and **(C)** TNF expression in CD4+ T cells from biopsy tissues in 2008 and 2009.

SUPPLEMENTARY FIGURE 3

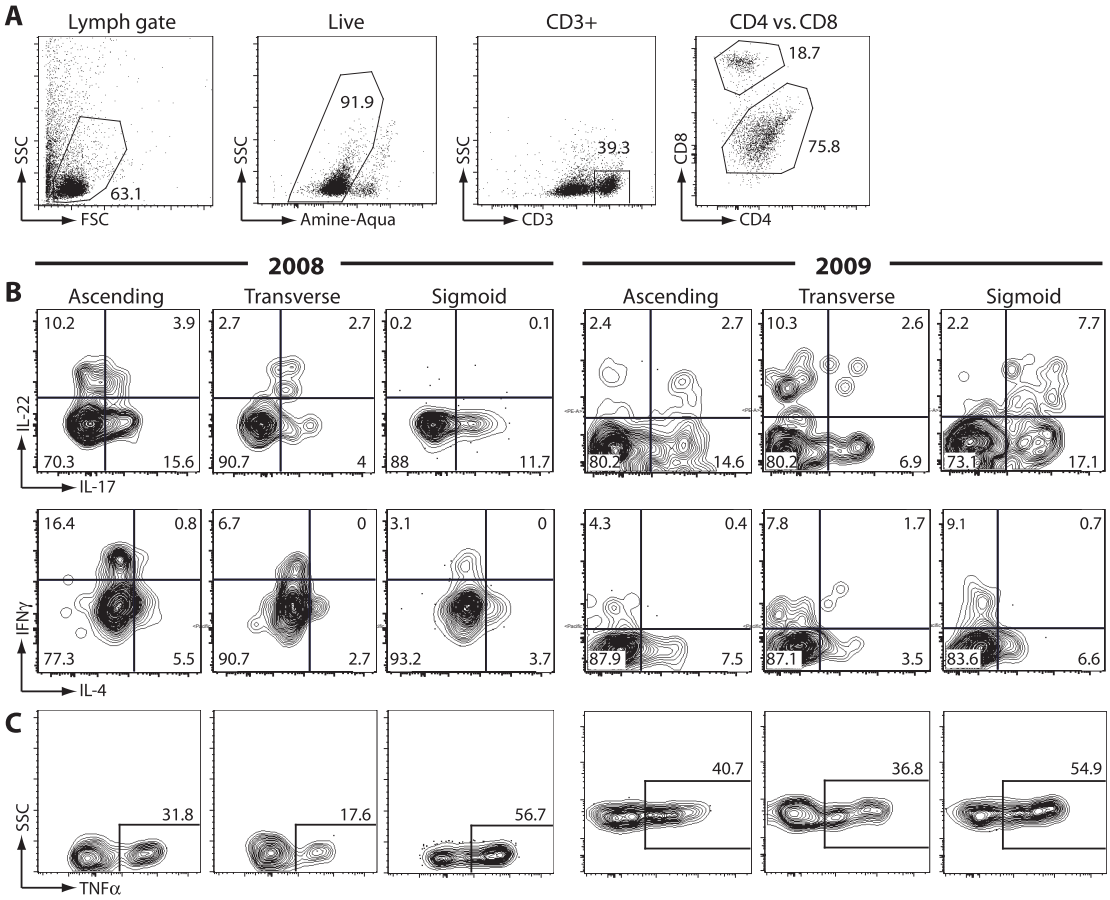


Figure S4. Microarray analysis of biopsy fragments from different regions of the colon. Hierarchical clustering analysis was used to organize genes and samples as shown in Figure 6. The heatmap shows the ratio of hybridization of fluorescent cDNA probes prepared from each experimental mRNA sample to a reference mRNA sample. These ratios are a measure of relative gene expression in each experimental sample, with red indicating upregulated, green indicating downregulated and grey indicating missing or exluded data. The dendogram at the top illustrates the relatedness of gene expression in each sample. Each row represents an individual oligo measurement on the microarray and each column a separate mRNA sample. Clustering analysis was visualized on Treeview software and selected genes of interest from notable clusters that were significantly different between regions of the colon were selected and shown. Horizontal bars at the bottom of the figure indicate samples affected by colitis (red), helminth colonization (blue), normal (green), and from the uninvolved terminal ileum (purple). Terminal ileum (TI), ascending colon (AC), transverse colon (TC), sigmoid colon (SC), rectum (R), or rectosigmoid clon (SRC).

SUPPLEMENTARY FIGURE 4

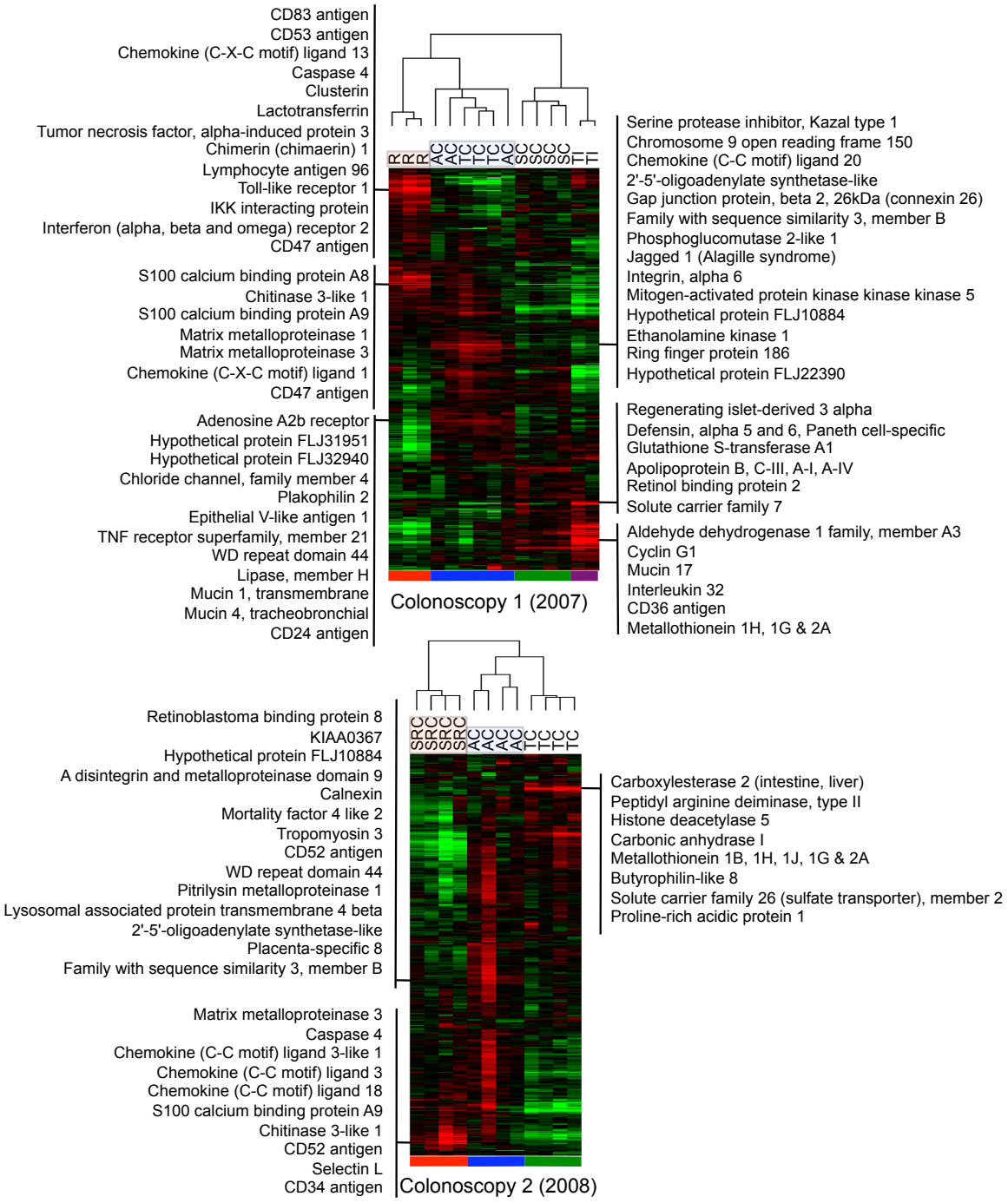


Figure S5. Real-time PCR analysis of inflammatory mediators from biopsy fragments in 2007. Boxplot showing the minimum, maximum, and median values of real-time PCR measurements of selected proinflammatory mediators from biopsy fragments collected in 2007 from the terminal ileum (TI), ascending colon (AC), transverse colon (TC), sigmoid colon (SC), rectosigmoid colon (SRC), worm granuloma (W), and rectum (R).

SUPPLEMENTARY FIGURE 5

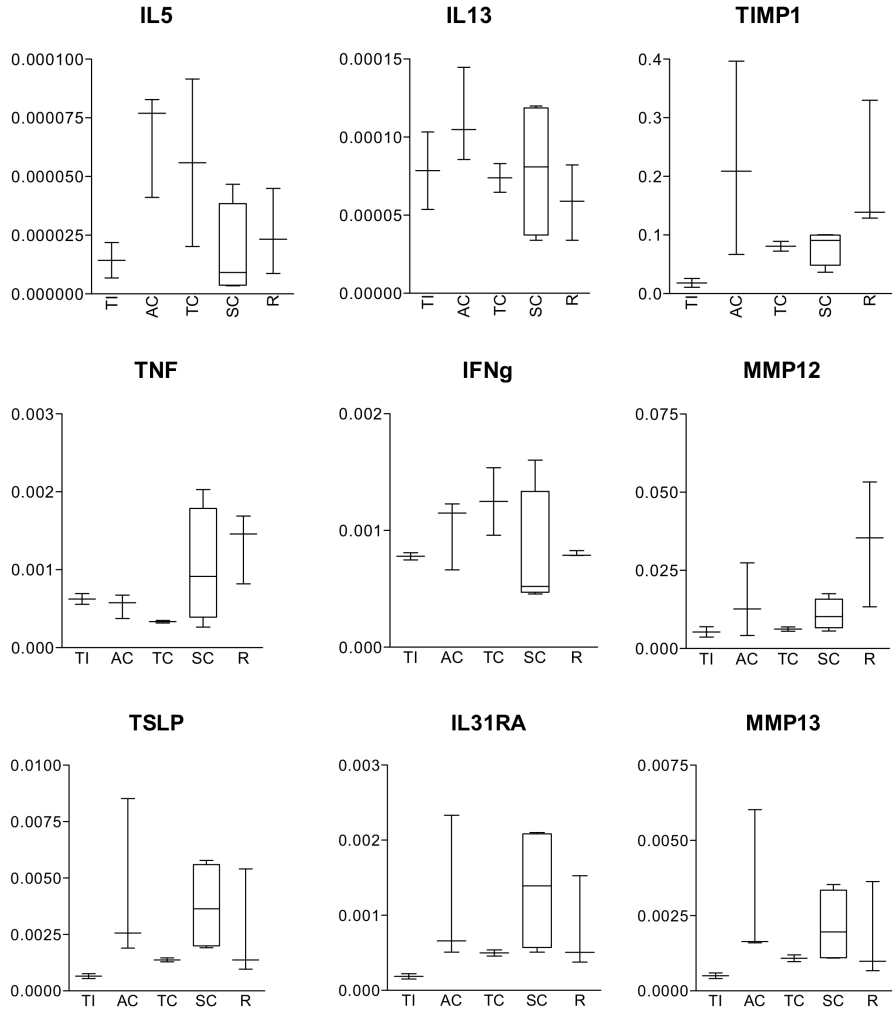
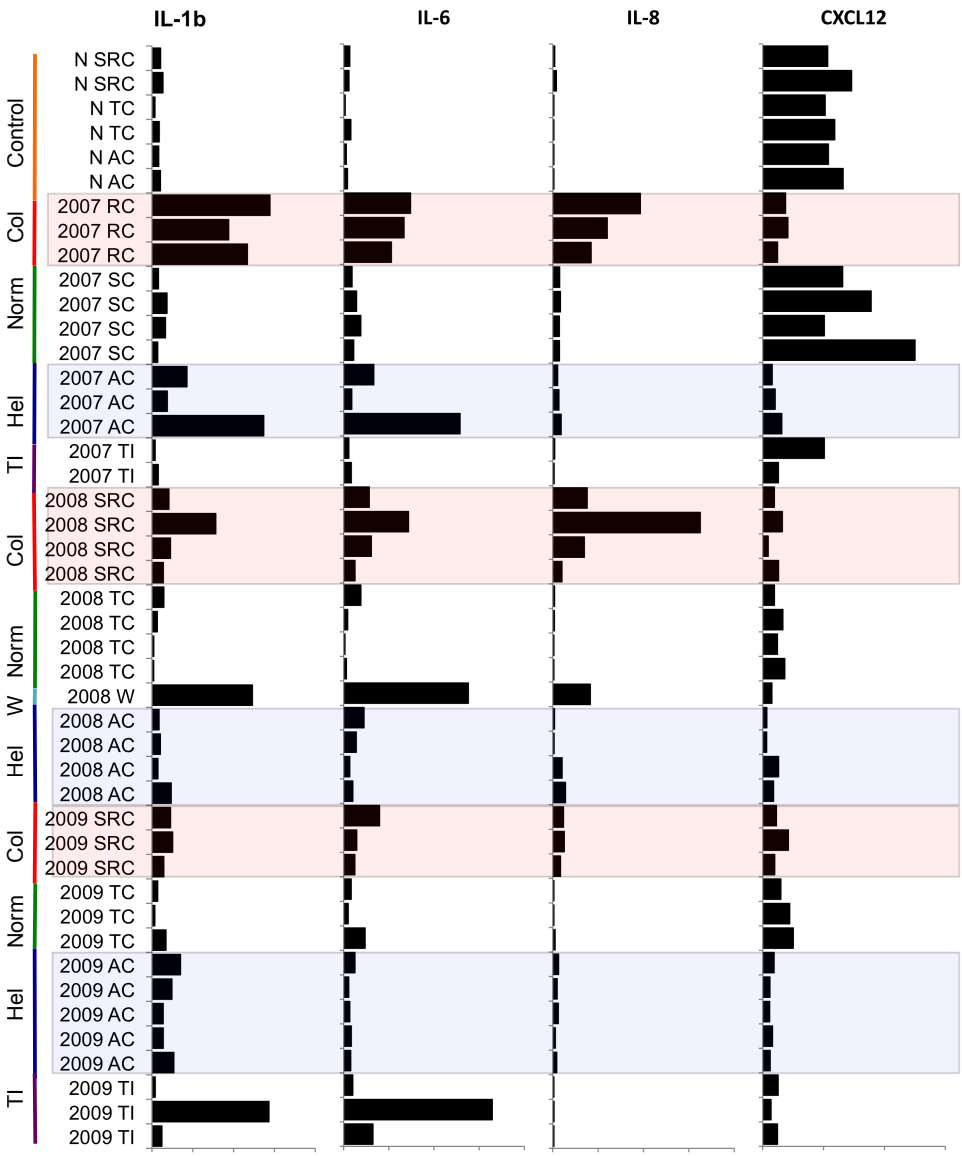


Figure S6. Real-time PCR measurement of dynamic changes in additional proinflammatory mediators in biopsy fragments. RNA from samples taken in 2007, 2008, and 2009 were compared to samples collected from a normal individual (N) as indicated by an orange vertical column (Control). Red vertical bars (Col) indicate samples taken from regions with active colitis (also shaded in red) and blue vertical bars (Hel) indicate samples taken from helminth-colonized tissues (also shaded in blue). Tissues with normal appearance (Norm) are indicated in green and samples from the terminal ileum (TI) are indicated in purple. A sample that came from a worm surrounded by granulomatous tissue (W) is shown in light blue.

SUPPLEMENTARY FIGURE 6



CHAPTER III

HELMINTHIC THERAPY FOR CHRONIC IDIOPATHIC COLITIS IN JUVENILE RHESUS MACAQUES

ABSTRACT

Chronic idiopathic colitis, a common condition at primate research centers that shares clinical and pathological features with human IBD, is the leading cause for monkeys requiring veterinary attention. Here we show that exposure to *Trichuris trichiura* improves clinical outcome, which correlates with the expansion of mucosal T_H2 cells, reduced inflammatory gene expression, and reduced bacterial attachment at the intestinal mucosa. These findings suggest that helminth exposure can improve symptoms of colitis by restoring mucosal barrier function and thus block the attachment of inflammatory bacteria at the colonic epithelium.

INTRODUCTION

Juvenile rhesus monkeys (*Maccaca mulatta*) at primate research centers, especially those housed indoors, frequently suffer from chronic idiopathic colitis (CIC), resulting in progressive weight loss and dehydration secondary to chronic diarrhea (Elmore et al., 1992). As many as 20% of all captive rhesus macaques develop this syndrome and, in this species, it is currently the leading cause of euthanasia not related to research. The affected colons often have severe inflammation that is similar to the major human form of IBD (ulcerative colitis), characterized by lymphocytic infiltration, multifocal crypt abscesses, and occasional mucosal erosion and ulceration (Blackwood et al, 2008; Sestak et al., 2003). Although it has been suggested that CIC may be an informative model for IBD, durable therapy is lacking and a robust immunologic assessment has yet to be made.

In this study, we sought to characterize the mucosal inflammatory response driving CIC, and to determine whether helminth exposure can modulate this inflammatory response attendant with clinical improvement. Flow cytometry and gene expression analyses of colon biopsy samples collected from five CIC subjects revealed molecular signatures of inflammation and tissue injury resembling similar analyses in IBD patients. Oral ingestion of *T. trichiura* eggs induced a persistent T_H2 response in the colonic mucosa of all subjects, and was associated with a favorable clinical response in four out of five subjects. Clinical improvement correlated with the downregulation of inflammatory genes and the upregulation of genes implicated in type-2 immunity. Quantification of bacterial DNA in mucosal biopsies showed a marked increase in bacterial attachment in CIC subjects compared to healthy controls that was significantly

reduced following *T. trichiura* exposure. Altogether, these findings suggest that CIC is an IBD-like inflammatory disease that can benefit from the type-2 immune response and mucosal repair elicited by helminth exposure.

RESULTS

T. trichuris exposure led to clinical improvement in four out of five CIC subjects

We performed colonoscopies on five juvenile macaques with CIC and two healthy, age-matched controls to obtain pinch biopsies for *ex vivo* analysis and nucleic acid isolation. We then gavaged the five CIC subjects with 1000 *T. trichiura* ova obtained from the research subject described in Chapter 2. Fecal consistency (an important clinical parameter for CIC severity; Table 1) was monitored daily. We began to examine the stool for *T. trichiura* ova at 7 weeks post-gavage, at the expected onset of egg deposition. Because ova were never detected in the stool, we concluded that patent infection was not established. However, an improvement in fecal consistency was observed in 4 out of the 5 monkeys (subject 38870 did not respond) following *T. trichiura* exposure (Figure 1), with the most dramatic improvement corresponding to the arrival of mature larvae in the colon at 7 weeks post-gavage. At 14 weeks post-gavage, we again performed colonoscopies on the CIC subjects to collect pinch biopsies.

T. trichuris exposure induced a mucosal T_H2 response

Peripheral blood mononuclear cells (PBMC) and mucosal leukocytes harvested from colon biopsy samples were processed for multiparameter flow cytometry.

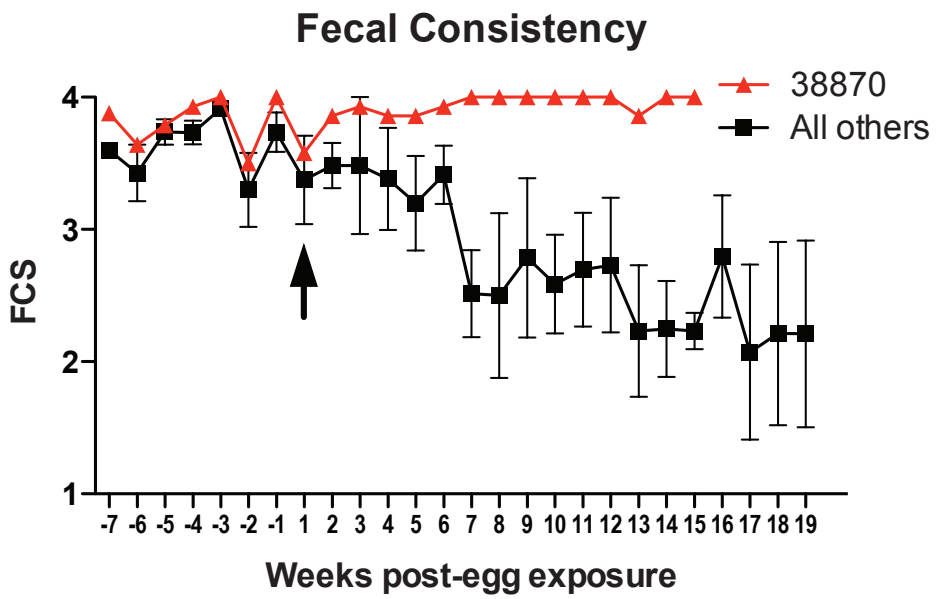
Table 1. Fecal consistency scoring scale

TABLE 1

Score	Description
1	Well-formed, normal
1.5	Normal to semi-solid*
2	Semi-solid to normal
2.5	Semi-solid
3	Semi-solid to liquid
3.5	Liquid to Semi-solid
4	Liquid

Figure 1. Fecal consistency score (FCS) demonstrates symptomatic improvement following helminth exposure. Fecal consistency was monitored daily according to the scoring scale in Table 1. Weekly averages are plotted. The arrow marks the ingestion of *T. trichiura* eggs.

FIGURE 1



Intracellular cytokine production was measured following *ex vivo* polyclonal stimulation, while markers of proliferation (Ki67) and regulatory phenotype (FoxP3) were measured in unstimulated cells. We found that the proportion of IL-4^+, but not IFNγ^+ $CD4^+$ T cells in the colonic mucosa was significantly expanded following *T. trichiura* exposure (Figure 2A), representing a localized T_H2 response that was not reflected in the peripheral blood (Figure 2B). All five CIC subjects demonstrated this response, indicating that the lack clinical improvement in subject 38870 was not due to the absence of helminth exposure or immune recognition. Notably, the proportion of TNF^+ and IL-2^+ $CD4^+$ T cells were also expanded in the colonic mucosa following *T. trichiura* exposure (Figure 2A), suggesting that the type-2 response was not associated with generalized T cell suppression.

The colonic mucosa of CIC subjects showed a high proportion of proliferating $Ki67^+$ $CD4^+$ T cells (average = 28% of $CD4^+$ T cells compared to 4% in healthy controls; Figure 2B) and $FoxP3^+$ $CD4^+$ T_{reg} cells (average = 26% of $CD4^+$ T cells compared to 11% in healthy controls; Figure 3A). The four clinical responders showed a diminished population of proliferating T_H cells following *T. trichiura* exposure (Figure 3A), and three out of four also showed a marked reduction in T_{reg} cells (Figure 3B). In contrast, subject 38870 showed a further expansion of $Ki67^+$ T_H cells and only a minor reduction in $FoxP3^+$ T_{reg} cells. Thus, T_H cell proliferation and the local expansion of T_{reg} cells are indicators of pathogenic inflammation in the colonic mucosa.

Figure 2. *T. trichiura* elicits a localized T_H2 response in the colonic mucosa. Flow cytometric analysis of intracellular cytokines in mucosal leukocytes **(A)** and PBMC **(B)** from healthy controls (Control) and CIC subjects before (Pre-Tt) and after (Post-Tt) *T. trichiura* exposure following *ex vivo* stimulation with PMA and ionomycin in the presence of brefeldin A. Error bars illustrate SEM; *p<0.05, ***p<0.005.

FIGURE 2

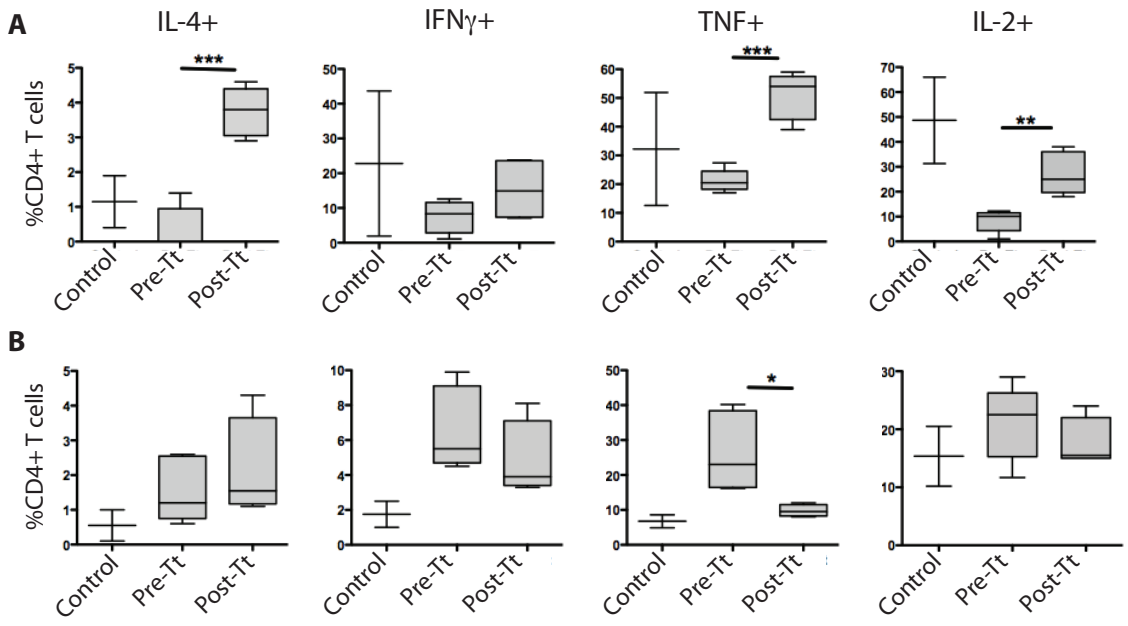
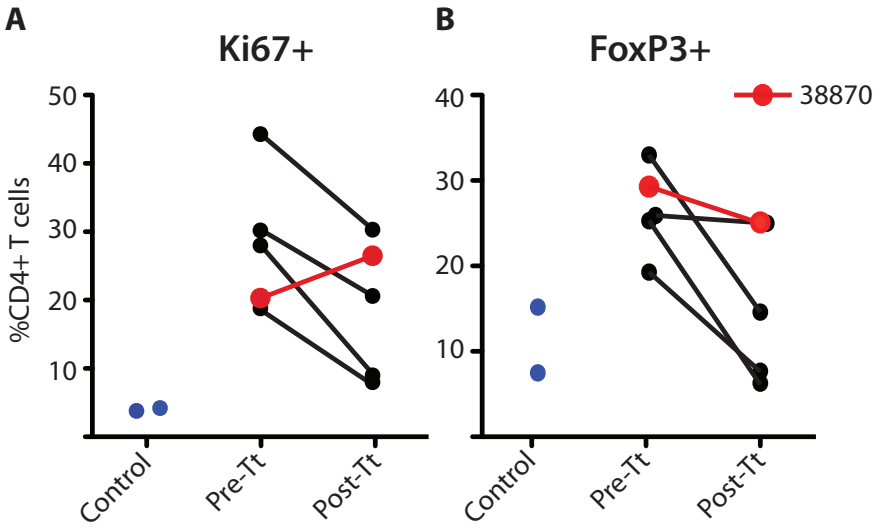


Figure 3. Localized $CD4^+$ T cell proliferation and T_{reg} expansion are associated with symptomatic mucosal inflammation. Flow cytometric analysis of intranuclear Ki67 **(A)** and FoxP3 **(B)** expression in mucosal leukocytes from healthy controls (Control) and CIC subjects before (Pre-Tt) and after (Post-Tt) *T. trichiura* exposure.

FIGURE 3



Clinical improvement paralleled a downregulation of inflammatory genes and the upregulation of type-2 response genes

Whole-genome gene expression profiling analysis was performed on colonic biopsy samples to identify differentially expressed transcripts between control and CIC subjects. We found that 185 transcripts distinguished the inflamed mucosa of CIC subjects (prior to *T. trichiura* exposure) from healthy colon tissue (Figure 4A). The genes upregulated in CIC samples included classical type-1 inflammatory mediators such as inducible nitic oxide synthase (*nos2*), chemokines (*cxcl9*, *cxcl10*, *cxcl11*), and serum amyloid A (*saa1, saa3, saa4*). Genes implicated in mucosal injury and defense, many of which have been identified as IBD-associated genes, were also upregulated in CIC samples. These included regenerative factors (*reg1*, *reg3*), trefoil peptides (*tff1*), and defensins (*mnp2*, *road1*, *road2*). The upregulation of several inflammatory genes in CIC samples was confirmed by RT-PCR (Figure 4B).

Changes in gene expression induced by helminth exposure were also evaluated, and 99 transcripts were found to be significantly differentially expressed following *T. trichiura* exposure (Figure 5A). Notably, many of the IBD-associated genes identified in pre-exposure samples were downregulated following *T. trichiura* exposure. Furthermore, post-exposure samples demonstrated the induction of type-2 response pathways, including IgE signaling (*fcer1a*, *ms4a2*), mast cell activation (*cpa3*, *cma1*), T_H2 and eosinophil recruitment (*ccl17*, *ccl18*, *ccl26*), alternative activation of macrophages (*alox5*, *alox15*), type-2 cytokine signaling (*il5ra*, *il9r*, *postn*) and worm expulsion (*relmb*).

Figure 4. CIC is characterized by type-1 inflammatory gene expression in the colonic mucosa

(A) Microarray analysis of gene expression patterns in colon biopsy fragments. Hierarchical clustering analysis was used to organize genes and samples. Each row represents an individual gene and each column an individual animal subject. Black indicates median level of expression; red, greater than median expression; and green, less than median expression. Colored horizontal bars at the bottom of the figure indicate the clustering of samples collected from healthy controls (Control; orange), CIC subjects who responded to helminth exposure [pre-exposure (Responder: Pre-Tt; light purple) and post-exposure (Responder: Post-Tt; dark purple)], and subject 38870 who did not respond to helminth exposure [pre-exposure (NR: Pre-Tt; light blue) and post-exposure (NR: Post-Tt; dark blue)]. A heatmap of 185 genes that are differentially expressed (B>0) between healthy controls and pre-exposure CIC subjects.

(B) RT-PCR analysis of gene expression in colon biopsy fragments from healthy controls and CIC subjects prior to helminth exposure. Expression is normalized to GAPDH.

FIGURE 4

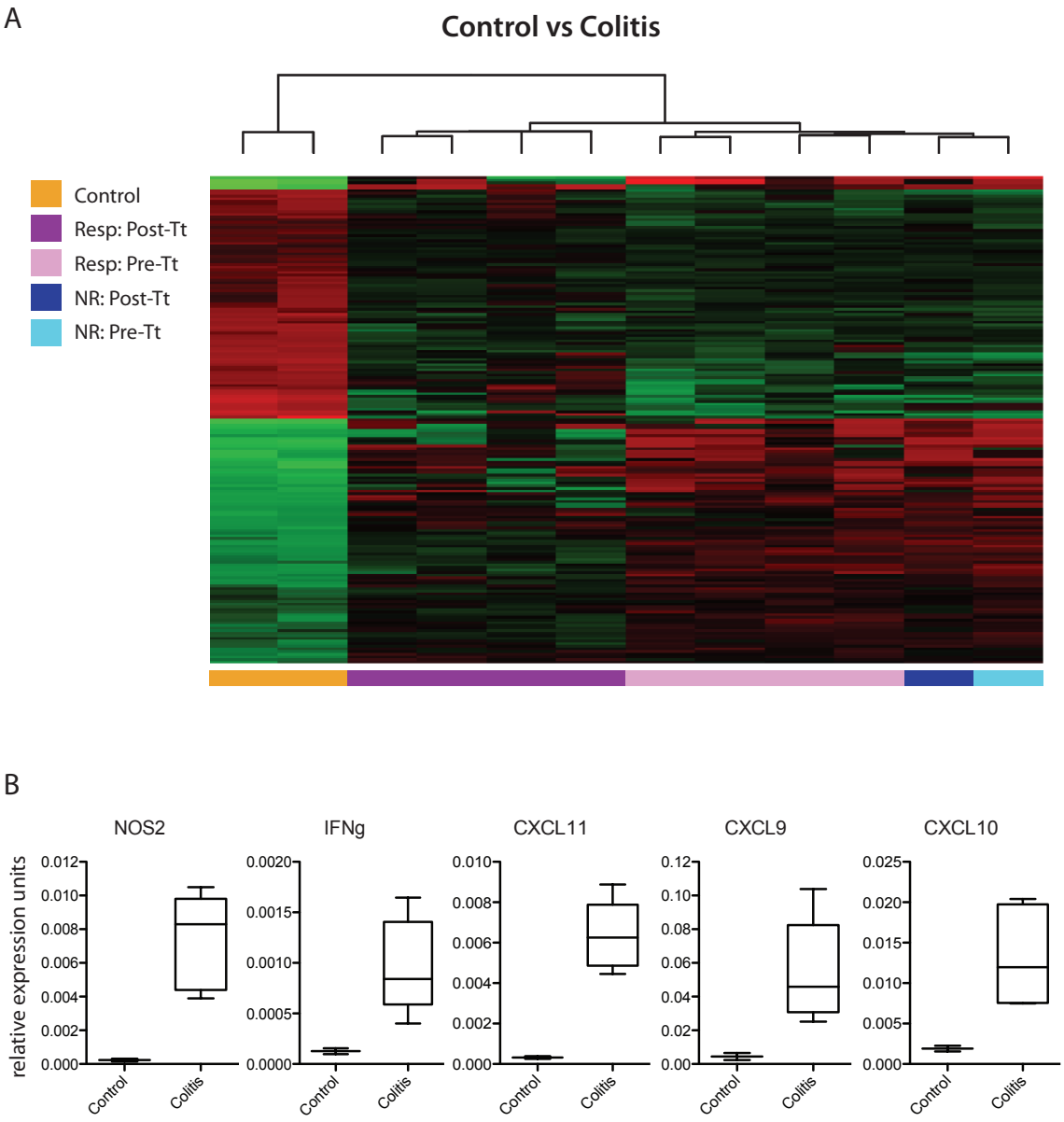
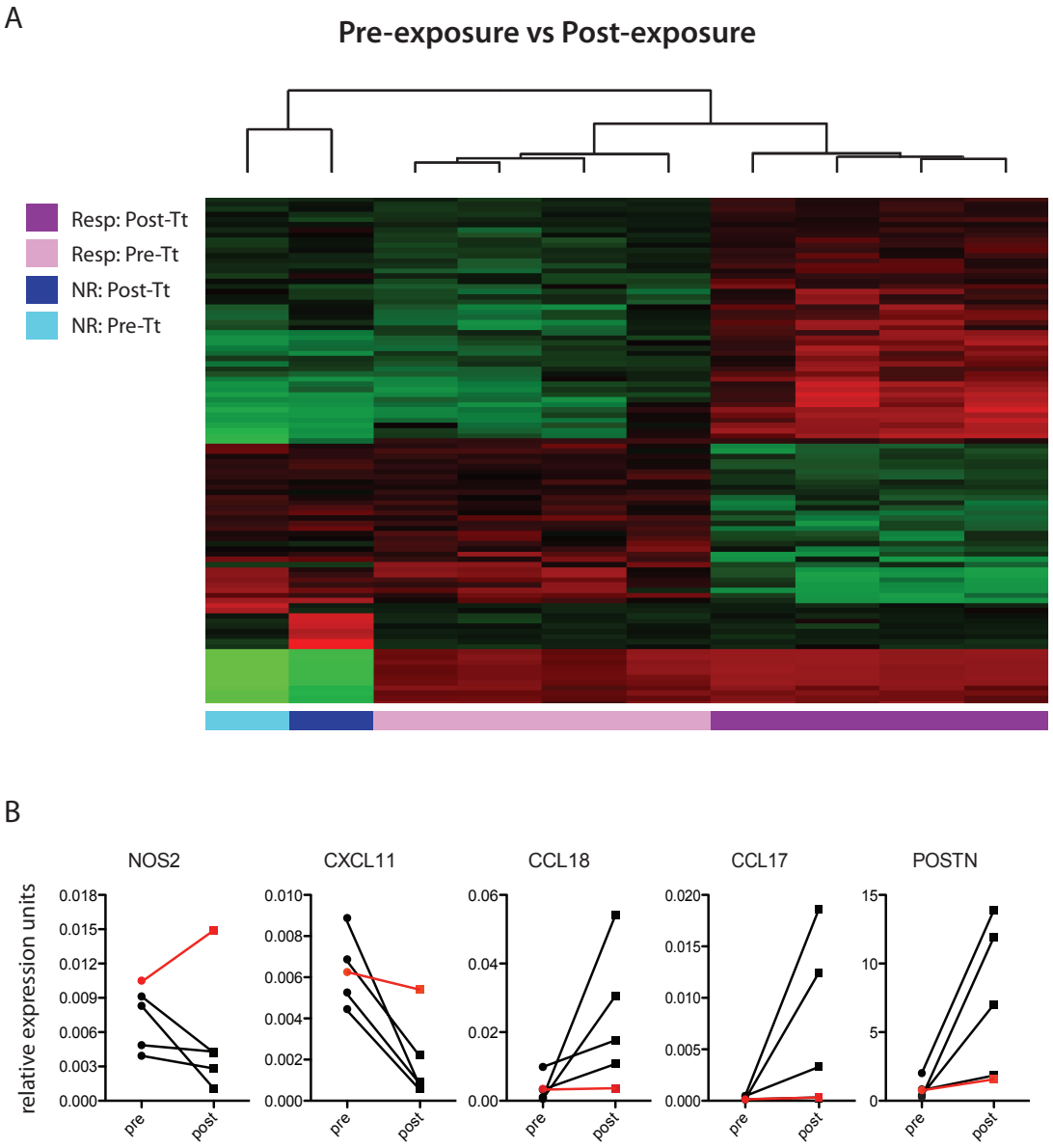

A
Control vs Colitis
Control
Resp: Post-Tt
Resp: Pre-Tt
NR: Post-Tt
NR: Pre-Tt
B
NOS2
IFNg
CXCL11
CXCL9
CXCL10
relative expression units
0.012
0.010
0.008
0.006
0.004
0.002
0.000
0.0020
0.0015
0.0010
0.0005
0.0000
0.010
0.008
0.006
0.004
0.002
0.000
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.025
0.020
0.015
0.010
0.005
0.000
Control
Colitis

Figure 5. Clinical improvement following *T. trichiura* exposure is associated with reduced type-1 inflammatory gene expression and the induction of type-2 response genes

(A) Microarray analysis of colon biopsy fragments, as described in Figure 4. A heatmap shows 99 genes that are differentially expressed (B>0) between pre-exposure and post-exposure CIC samples.

(B) RT-PCR analysis of gene expression in colon biopsy fragments from CIC subjects collected before and after helminth exposure. Subject 38870 is indicated in red. Expression is normalized to GAPDH.

FIGURE 5


A
Pre-exposure vs Post-exposure
Resp: Post-Tt
Resp: Pre-Tt
NR: Post-Tt
NR: Pre-Tt
B
NOS2
CXCL11
CCL18
CCL17
POSTN
relative expression units
0.018
0.015
0.012
0.009
0.006
0.003
0.000
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0.04
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0.020
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0.010
0.005
0.000
15
10
5
0
pre
post

The transcriptional profile of subject 38870 clustered separately from the four clinical responders, primarily driven by a group of immunoglobulin-related transcripts that were present at much lower levels in this subject. 38870 did not demonstrate the shift in gene expression following *T. trichiura* exposure seen in the four responders, resulting in the close hierarchical clustering between pre- and post-exposure samples from this animal. This observation was confirmed by RT-PCR (Figure 5B), as 38870 did not show the same pattern of reduced expression of type-1 inflammatory genes (*nos2*, *cxcl11*) and higher expression of type-2 response genes (*ccl18*, *ccl17*, *postn*) following *T. trichiura* exposure.

Bacterial attachment at the mucosal epithelium was reduced following *T. trichuris* exposure

Chronic inflammation in IBD may be driven by an aberrant immune response against commensal gut bacteria due to changes in bacterial attachment at the intestinal mucosa. Therefore, we quantified the abundance of several bacterial taxa in colon biopsy samples using quantitative PCR for 16S ribosomal RNA genes. Bacterial abundance was greater in biopsies from CIC samples compared to healthy controls for all taxa assayed, demonstrating a non-specific increase in bacterial attachment in these subjects (Figure 6). Bacterial attachment was broadly reduced following *T. trichiura* exposure, suggesting that the defective mucosal barrier was partially restored.

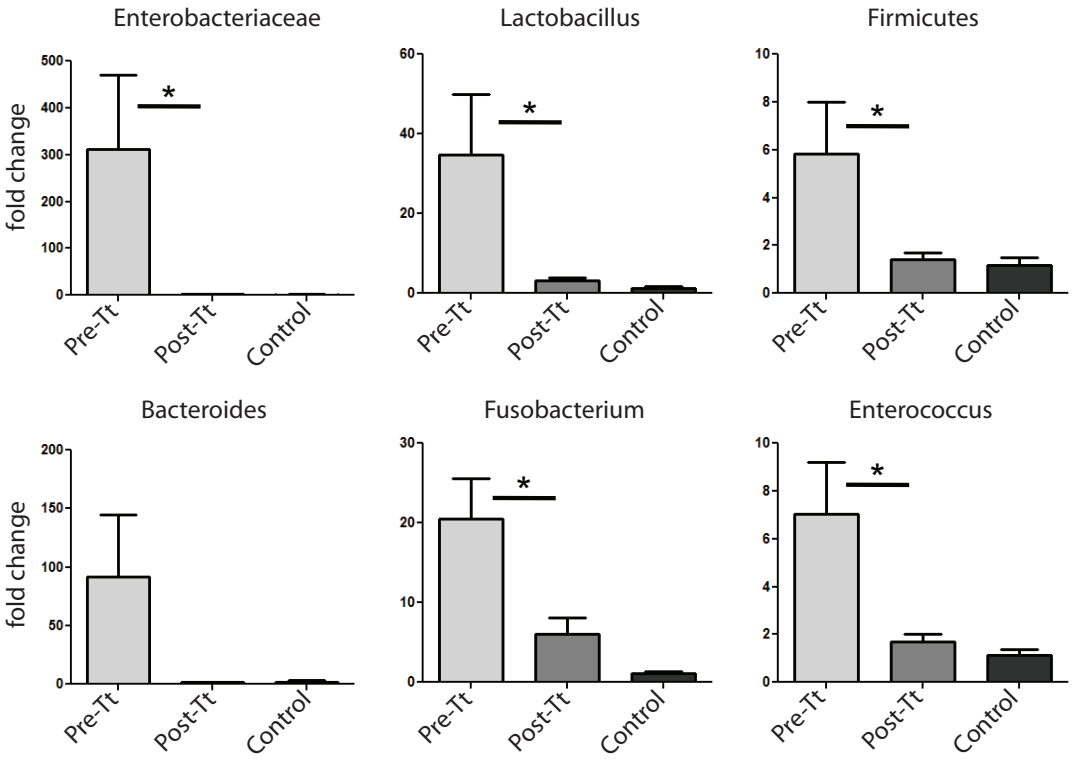
DISCUSSION

The data presented here suggest that CIC in juvenile rhesus macaques is an IBD-like inflammatory disease in which the mucosal barrier is compromised, allowing increased bacterial attachment that contributes to persistent inflammation. This study revealed that *T. trichiura* exposure induces a T_H2-driven type-2 response in the intestinal mucosa of CIC subjects that is associated with symptomatic improvement. Clinical response was inversely correlated with cellular markers of mucosal T cell inflammation, including T cell proliferation and T_{reg} expansion. We propose that *T. trichiura* promotes mucosal healing in the setting of CIC through increased epithelial cell turnover and mucus production, thereby reducing the attachment of immunostimulatory bacteria to the colonic epithelium.

Importantly, differences in gene expression patterns were apparent between the four clinical responders and the single non-responder in this study. The non-responder showed lower expression of immunoglobulin-related genes, indicating a possible difference in the mucosal B cell compartment. For example, the loss of regulatory B cell subsets can precipitate colitis (Shimomura et al., 2008). These findings may contribute to the identification of molecular signatures that predict which IBD patients are most likely to benefit from helminthic therapy. Our findings support the study of CIC as a model for IBD, and provide further insight into the role of type-2 immunity in driving the therapeutic effects of helminthic exposure in the intestinal mucosa.

Figure 6. *T. trichiura* exposure reduces bacterial attachment in the colonic mucosa. DNA harvested from colon biopsies was analyzed by quantitative PCR for the abundance of bacterial taxa based on 16S ribosomal DNA sequence. Abundance is expressed as a fold-change above the sample yielding the lowest signal for each taxa. Error bars illustrate SEM; *p<0.05.

FIGURE 6



MATERIALS AND METHODS

Subject recruitment and clinical monitoring. All animals were housed indoors at the California National Primate Research Center. CIC cases were identified by recurrent episodes of diarrhea (during 45 or more days in a 90 day period) without evidence for (or a history of) known causes of infectious colitis (three negative cultures for bacterial pathogens; negative stool examination and immunofluorescence assays for protozoan and helminthic parasites). The diarrhea was refractory to antibiotic and antiparasitic treatment. Animals were weighed daily and their stool evaluated according to a standardized four-point scale for fecal consistency.

Collection of colonic biopsies and peripheral blood. Animals were fasted for 36 hours prior to colonoscopy, and 30 mL/kg of polyethylene glycol-electrolyte solution (PEG-ES; GoLYTELY brand) was provided twice the day before the procedure. GoLYTELY solution was prepared by mixing 67g of GoLYTELY with 1 liter of citrus-flavored water (Tang, Kraft Foods, Northfield, IL) and was made available for the animals to drink by a hanging bottle in the cage. Blood was collected by venipuncture into citrate tubes prior to colonoscopy. Five pinch biopsies were collected during colonoscopy from the proximal ascending colon. Three biopsies were collected into culture media for *ex vivo* analysis, and two were collected into RNAlater for nucleic acid extraction.

Flow cytometry analysis of biopsies and peripheral blood. Colon biopsy specimens were treated with 0.25 mg/ml collagenase type II (Sigma-Aldrich) for 30 minutes with constant shaking at room temperature. Digested tissue was dispersed over a 70-micron

nylon mesh filter. Cell suspensions were washed twice with RPMI containing 15% fetal calf serum. Whole blood was collected into ACD-containing tubes (BD Biosciences) and PBMC were isolated by density centrifugation. Biopsy cells and PBMC ($1x10^6$) were resuspended in 200 µl of complete R-10 [RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum (Hyclone), 50 U/ml penicillin, 50 µg/ml streptomycin, and 2 mM L-glutamine], and stimulated with phorbol myristate acetate (10 ng/ml) and ionomycin (1 µg/ml) in the presence of brefeldin A (GolgiPlug, BD Pharmingen) for five hours at 37°C. For biopsy cells, amphotericin B (Gibco) was also added to the culture media. Cell surface staining and intracellular cytokine staining were performed with Fix/Perm and PermWash solutions from BD and eBioscience, according to the manufacturer's instructions.

Microarray analysis of biopsy samples. Biopsies were collected into RNAlater (Qiagen) and homogenized in TRIzol (Invitrogen). RNA was collected in the aqueous extraction phase and column purified using an RNeasy kit (Qiagen). Sample preparation, labeling, and array hybridizations were performed according to standard protocols from the UCSF Shared Microarray Core Facilities and Agilent Technologies (http://www.arrays.ucsf.edu and http://www.agilent.com). Total RNA quality was assessed using a Pico Chip on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). RNA was amplified and labeled with Cy3-CTP using the Agilent low RNA input fluorescent linear amplification kits following the manufacturers protocol (Agilent). Labeled cRNA was assessed using the Nandrop ND-100 (Nanodrop Technologies, Inc., Wilmington DE), and equal amounts of Cy3 labeled target were hybridized to Agilent

Rhesus Macaque (V2) whole genome 4x44K Ink-jet arrays (Agilent). Hybridizations were performed for 14 hours, according to the manufacturers protocol (Agilent). Arrays were scanned using the Agilent microarray scanner (Agilent) and raw signal intensities were extracted with Feature Extraction v10.1 software (Agilent). Each dataset was normalized using the *quantile* normalization method (Bolstad et al., 2003) with no background subtraction. A one-way ANOVA linear model was fit to the comparison to estimate the mean M values and calculated moderated t-statistic, B statistic, false discovery rate and p-value for each gene for the comparison of interest. All procedures were carried out using functions in the R package.

Reverse transcription and quantitative PCR analysis of colon biopsies. Tissue samples were homogenized in TRIzol (Invitrogen). RNA was collected in the aqueous extraction phase and DNA was harvested from the interphase and phenol-chloroform organic phase. RNA was column purified using an RNeasy kit (Qiagen). cDNA was generated using an Omniscript Reverse Transcription kit (Qiagen) with oligo-dT primers in the presence of RNasin Plus RNase inhibitor (Promega). DNA was collected by ethanol precipitation and washed according to the manufacturer's instructions. PCR reactions were carried out with Taqman primer/probe sets (Applied Biosystems) in a StepOne Plus machine (Applied Biosystems).

CHAPTER IV

RETINOIC ACID SYNTHESIS AND FUNCTION DURING HELMINTH INFECTION

ABSTRACT

Although the vitamin A metabolite retinoic acid (RA) is critical for T cell function, RA synthesis during pathogen-elicited T cell responses is poorly understood. Here, we show that retinal dehydrogenases (Raldh), required for the synthesis of RA, are induced during retinoid-dependent T_H2 responses elicited by *Schistosoma mansoni* infection, but not during retinoid-independent T_H1 responses elicited by virus infection. Macrophages highly express Raldh2 during *S. mansoni* infection and IL-4 is sufficient to induce Raldh2 expression in macrophages. Thus, the regulation of Raldh enzymes during infection is pathogen specific and reflects differential requirements for RA during effector T cell responses. Specifically, alternatively-activated macrophages (AAMϕ) are an inducible source of RA synthesis during retinoid-dependent T_H2 responses to helminth infection.

INTRODUCTION

Vitamin A (retinol) is a critical factor in protective immunity, as evidenced by the increase in infectious disease morbidity and mortality associated with its deficiency in the diet (Stephensen et al., 2001). The biological activity of vitamin A requires intracellular oxidation of retinol to retinoic acid (RA). The rate-limiting step in RA synthesis is catalyzed by three major isoforms of retinal dehydrogenase (Raldh1-3), a family of tightly regulated enzymes (Duester et al., 2000; Duester et al., 2003; Sima et al., 2009). Antigen presenting cells (APCs) that express Raldh enzymes can direct RA signaling in T cells (Iwata et al., 2004). While homeostatic RA synthesis by APCs is largely restricted to the gut-associated lymphoid tissues (GALT) (Iwata et al., 2004; Sun et al., 2007; Denning et al., 2007; Coombes et al., 2007), it remains unclear whether Raldh expression is inducible during infection.

Elucidating the regulation of RA synthesis by inflammatory APCs is critical for understanding the role of RA signaling in shaping T cell responses *in vivo*. While basal RA signaling is required for general T cell activation (Hall et al., 2011), RA also acts in concert with other signals to mediate inflammatory (DePaolo et al., 2011) and regulatory (Benson, 2007 et al.; Sun et al., 2007; Mucida et al., 2007; Coombes et al., 2007) T cell functions. In the presence of IL-4, a critical mediator of type-2 inflammation, RA favors T_H2 responses in murine (Iwata et al., 2003; Stephensen et al., 2002) and human (Dawson et al., 2006) $CD4^+$ T cells by enhancing the expression of GATA-3 and type-2 cytokines while inhibiting T-bet and IFNγ expression. Accordingly, vitamin A deficiency attenuates eosinophilia, IgE responses, and type-2 cytokine expression *in vivo* (Schuster et al., 2008; Carman et al., 1992; Cantorna et al., 1994). T_H2 cells mediate protective immunity to

helminth parasites that are common in regions of the world where vitamin A deficiency is prevalent (Anthony et al., 2007). However, the importance of RA in the generation of T_H2 responses during helminth infection is not well characterized and the population of cells responsible for RA synthesis in this setting has not been identified.

In this study, we sought to determine whether RA synthesis is a regulated component of immune responses during infection. Based on the substantial evidence that RA promotes T_H2 responses, we hypothesized that Raldh expression is induced in APCs that are activated during T_H2 inflammation. To address this hypothesis, we evaluated RA signaling and Raldh expression in mice infected with the parasitic helminth, *Schistosoma mansoni*, an important human pathogen that provides a well-characterized model of T_H2 inflammation. Deposition of *S. mansoni* eggs in the liver and intestine drives a type-2 granulomatous response characterized by T_H2 cells, AAMϕ, and eosinophils (Davies and McKerrow, 2001). In parallel, and in a model of T_H1 responses, we evaluated mice infected with lymphocytic choriomeningitis virus (LCMV). Vitamin A deficient mice showed severely impaired Th2 but not Th1 responses in the liver, suggesting a role for RA synthesis during T_H2 inflammation at this site. Raldh enzymes were highly expressed by AAMϕ recruited to liver granulomas during *S. mansoni* infection and Raldh2 expression in macrophages was induced by activation with IL-4 but not IFNγ *in vitro*. Thus, our findings demonstrate that helminth-elicited AAMϕ are an inducible source of RA synthesis in the setting of retinoid-dependent T_H2 inflammation and identify IL-4 activation as a selective mechanism for Raldh2 induction in these cells.

RESULTS

Vitamin A is critical for liver T_H2 responses during *S. mansoni* infection

To assess the role of RA synthesis during infection, we first determined whether *S. mansoni*- and LCMV-elicited T cell responses are dependent upon vitamin A. Mice were maintained on a vitamin A deficient (A-) or control (A+) diet beginning at day 10 of gestation. *S. mansoni*-infected mice were analyzed at week 7 post-infection, corresponding to the acute Th2 response elicited by egg deposition, while LCMV (Armstrong strain)-infected mice were analyzed at day 7 post-infection. Infections were timed such that all the mice were analyzed at 15 weeks of age. By this time, serum retinol levels in A- mice were reduced to ~0.35μM, a level defined by the World Health Organization as severe vitamin A deficiency (WHO 2009).

Within the liver of *S.mansoni*-infected mice, eggs are deposited that evoke granulomatous eosinophilic inflammation, a process that is T_H2-dependent. Although the livers of A+ and A- mice showed no differences in the numbers of eggs (Figure 1A), A- mice had significantly smaller granulomas (Figure 1B) and reduced eosinophilic infiltration (Figure 1C), similar to mice genetically deficient in T_H2 responses (IL-$4^{-/-}$, Stat$6^{-/-}$) (Kaplan et al., 1998; Brunet et al., 1997). The diminished granuloma size in A- mice was associated with microvesicular damage in the liver (Figure 1D). Unlike other models of more extreme liver pathology leading to mortalities in *S. mansoni*-infected mice, there was no difference in survival rates between A+ and A- mice. The characteristic expansion of IL-4^+ T_H2 cells associated with egg deposition in the liver and the intestine was significantly reduced in A- mice (Figure 1E). Concomitantly, the numbers of IFNγ^+ and TNFα^+ $CD4^+$ T cells (Figure S1, A and B) as well as of $Foxp3^+$

Figure 1. Vitamin A deficiency impairs *S. mansoni*-elicited T_H2 responses.

(A) Quantification of *S. mansoni* eggs deposited per gram of liver.

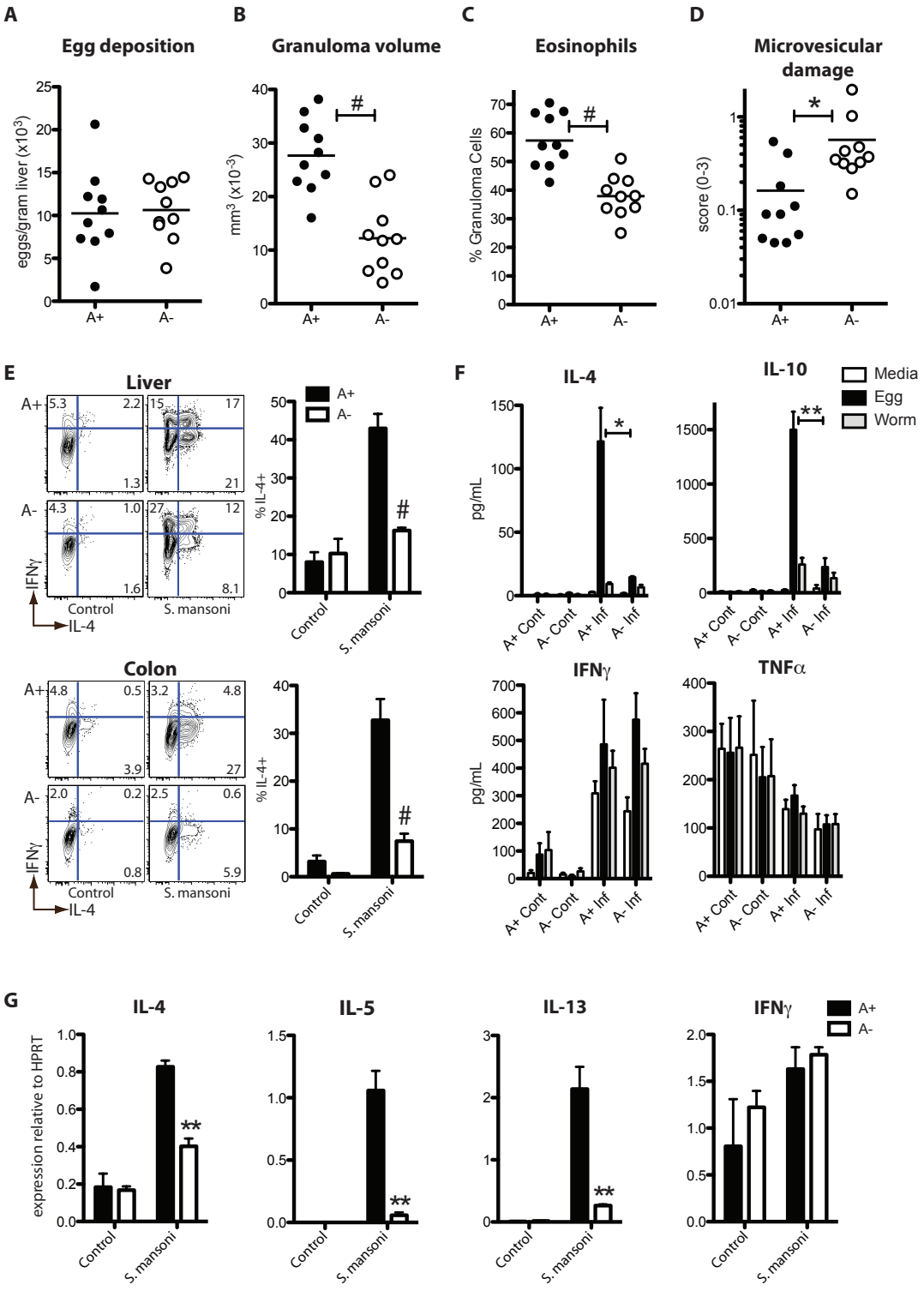
(B-D) Histopathology of liver tissue sections stained with hemotoxylin and eosin and evaluated for granuloma volume (B), eosinophil infiltration (C), and microvesicular liver damage (D). Liver damage was scored from 0 (no damaged hepatocytes) to 3 (every hepatocyte demonstrating microvesicular damage).

(E) Flow cytometric analysis of intracellular cytokines in cells from liver or colon following a 5-hour stimulation with PMA and ionomycin in the presence of brefeldin A. Representative contour plots are gated on live $CD4^+$ T cells. n = 3-5 mice per group.

(F) Cytometric bead array analysis of cytokine concentrations in culture supernatants. $3x10^5$ hepatic leukocytes harvested from control (Cont) and *S. mansoni*-infected (Inf) mice were cultured for 72 hours in the presence of egg homogenate (50 μg/mL), adult worm homogenate (50 μg/mL), or media alone. n = 3-4 mice per group.

(G) RT-PCR analysis of cytokine expression in hepatic leukocytes. Expression is normalized to HPRT. The bar graphs to the right represent composite results from the indicated groups (n = 3-5 mice per group). Error bars illustrate SEM; *$p<0.05$, **$p<0.01$, #$p<0.001$. Results are representative of two (A-D) or three (E, G) independent experiments.

FIGURE 1


A
Egg deposition
eggs/gram liver (x10^3)
A+
A-
B
Granuloma volume
mm^3 (x10^-3)
#
C
Eosinophils
% Granuloma Cells
D
Microvesicular damage
score (0-3)
*
E
Liver
Colon
IFNγ
IL-4
Control
S. mansoni
% IL-4+
F
IL-4
IL-10
IFNγ
TNFα
pg/mL
Media
Egg
Worm
A+ Cont
A- Cont
A+ Inf
A- Inf
G
IL-4
IL-5
IL-13
IFNγ
expression relative to HPRT
**

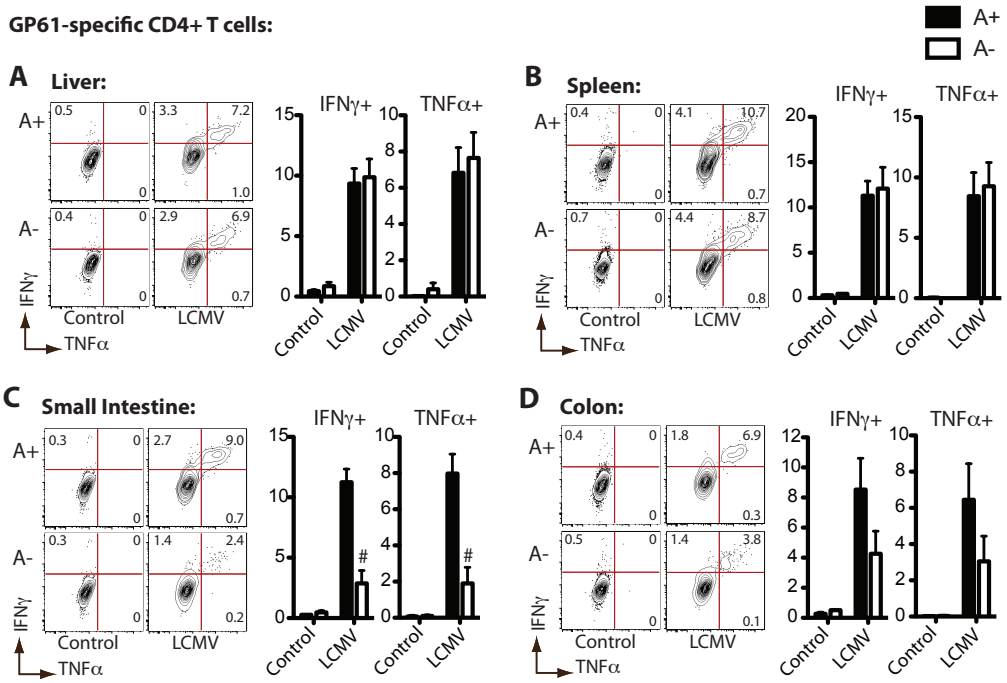
T_{reg} cells (Figure S2) were not impaired in A- mice, indicating a selective defect in the T_H2 response induced by vitamin A deficiency. When hepatic leukocytes were co-cultured with schistosome egg antigen (SEA) for 72 hours, we also found that SEA-specific IL-4 and IL-10 responses were dramatically reduced in A- mice. By contrast, IFNγ and TNFα production were indistinguishable between samples from A+ and A- mice (Figure 1F). By quantitative real-time PCR analysis (qRT-PCR) of isolated liver lymphocytes, we found that vitamin A deficiency significantly reduced the expression of IL-4, IL-5, and IL-13 but not IFNγ (Figure 1G).

The effects of vitamin A deficiency were less pronounced in the draining mesenteric lymph nodes than in the liver or intestine. Both the number of IL-4^+ T cells analyzed e*x vivo* and the SEA-specific IL-4 and IL-10 responses were either unaffected or only slightly reduced by vitamin A deficiency (Figure S1, C and D). However, the expression of IL-5 and IL-13 was vitamin A-dependent (Figure S1 E). The majority of IL-4-producing T cells in lymph nodes responding to helminth infection are follicular helper-T cells (T_{FH}), which are functionally distinct from T_H2 cells (King and Mohrs, 2009). Thus, these results suggest that RA signaling is critical for the expression of type-2 cytokines by T_H2 cells recruited to sites of tissue inflammation, but is not essential for IL-4 expression by T_{FH} cells.

In contrast to *S. mansoni* infection, we found that the numbers of GP61 and GP33 peptide-specific IFNγ- or TNFα-positive $CD4^+$ or $CD8^+$ T cells in the livers and spleens of LCMV-infected mice were unaffected by vitamin A deficiency (Figure 2, A and B; Figure S3). However, LCMV-specific (Figure 2, C and D) as well as polyclonal (Figure

Figure 2. LCMV-specific T_H1 responses in the intestinal mucosa are dependent on vitamin A metabolites. Flow cytometric analysis of intracellular cytokines expressed by cells harvested from the liver **(A)**, spleen **(B)**, small intestine **(C)**, or colon **(D)** of LCMV-infected mice following a 5-hour stimulation with GP61 peptides (10 μg/mL) in the presence of brefeldin A. Representative contour plots are gated on live $CD4^+$ T cells. The bar graphs to the right represent composite results from the indicated groups (n = 3-5 mice per group). Error bars illustrate SEM; #p<0.001. Results are representative of three independent experiments.

FIGURE 2



S4) T_H1 responses in the intestine were significantly diminished by vitamin A deficiency, consistent with a defect in intestinal homing (Iwata et al., 2004).

These results demonstrate that vitamin A deficiency does not impair all T cell responses to pathogens. Rather, our findings show that basal RA signaling required for efficient T cell activation (Hall et al., 2011) can be achieved in vitamin A-deficient individuals, but that higher levels of RA signaling are required to maintain intestinal homing of effector T cells and to support helminth-elicited T_H2 responses.

S. mansoni infection induces systemic RA signaling in T cells

The vitamin A-dependency of *S. mansoni*-elicited T_H2 responses suggested a critical role for RA during this infection. To determine whether RA signaling was directly targeted to $CD4^+$ T cells during infection, we measured CCR9 expression by T cells as a surrogate marker of RA activity (Iwata et al., 2004; Benson et al., 2007).

Baseline CCR9 expression on $CD4^+$ T cells in naïve, uninfected mice was reduced as a result of vitamin A deficiency in the MLN and intestinal mucosa but not in the spleen, confirming previous reports that homeostatic RA synthesis is a selective function of APCs in the GALT (Sun et al., 2007; Iwata et al., 2004; Coombes et al., 2007) (Figure S5). As expected, all mucosal $CCR9^+$ T cells were $CD62L^{neg}$ (effector/memory subset), consistent with the possibility that these cells homed to the intestinal mucosa following antigen presentation.

During LCMV infection, CCR9 induction was restricted to the intestinal tissues. During *S. mansoni* infection, by contrast, CCR9 expression was also induced in secondary lymphoid organs (e.g., the spleen) (Figure S5). In each case, the increase in

CCR9 expression was diminished in A- mice, indicating a dependency on vitamin A metabolites. These results reveal that *S. mansoni* infection drives RA signaling in T cells beyond the intestinal tissues.

Type-2 inflammatory cells express RA-synthesizing enzymes

We next sought to determine which cells produce RA in *S. mansoni*-infected mice. Using RT-PCR, we measured the three major Raldh isoforms that facilitate local RA synthesis in liver leukocytes isolated from *S. mansoni*- and LCMV-infected mice. Raldh2 and Raldh3 were expressed >50-fold higher in type-2 relative to type-1 inflammatory cells (Figure 3A), despite a similar increase in the number of inflammatory cells in the liver during both infections. Notably, Raldh2 is the isoform constitutively expressed by GALT APCs, while a role for Raldh3 in immunity has not been described.

S. mansoni egg-elicited granulomas are comprised of macrophages, eosinophils, and T cells (Davies and McKerrow, 2001). To determine if myeloid cells are the source of Raldh expression, liver sections from *S. mansoni*-infected mice were co-stained with antibodies reactive for CD11b and Raldh. The Raldh antibody recognizes Raldh1 as well as Raldh2. Hepatocytes stained brightly for Raldh (Figure 3B), most likely reflecting expression of Raldh1, a low efficiency isoform highly expressed in the liver. Raldh staining was also detectable within granuloma cells that co-stained for CD11b. To distinguish between expression of different Raldh isoforms in macrophages and eosinophils, which both express CD11b, liver leukocytes from *S. mansoni*-infected mice were sort-purified by fluorescence activated cell sorting (FACS) for RT-PCR analysis (Fig. 3 C). While expression of all three Raldh isoforms was detected in macrophages,

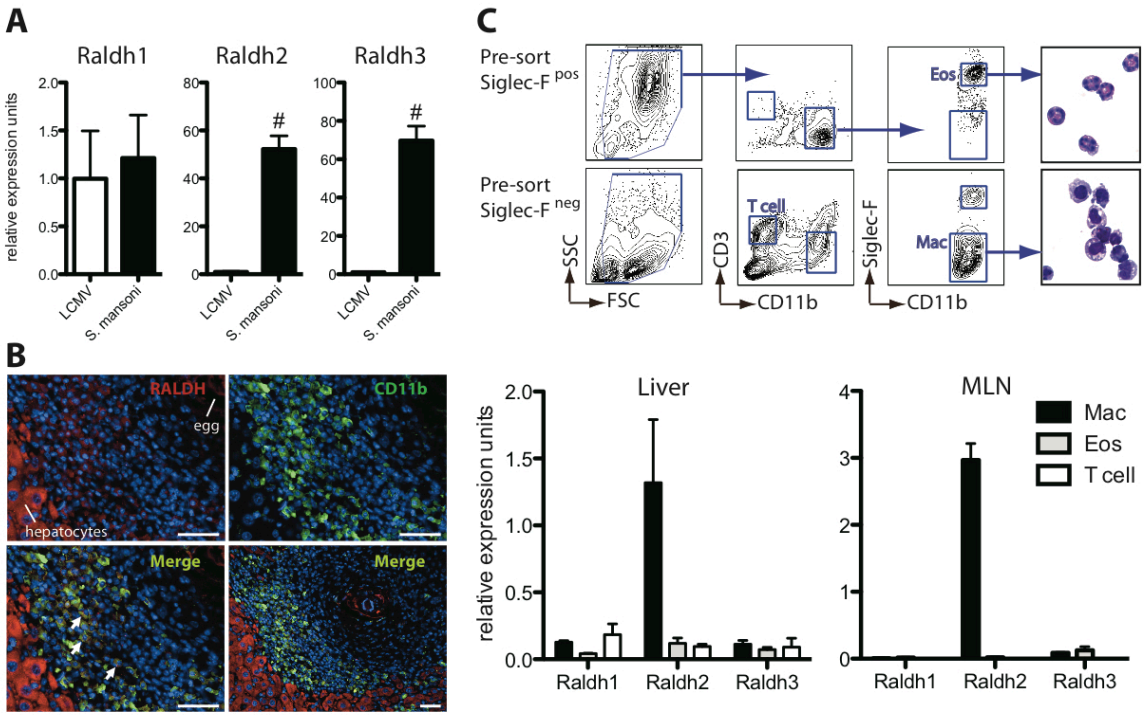
Figure 3. Type-2 inflammatory cells express Raldh2 and Raldh3, with Raldh2 most highly expressed in macrophages.

(A) qRT-PCR analysis of retinal dehydrogenase (Raldh) expression (isoforms 1-3) in hepatic inflammatory infiltrates. Expression is normalized to HPRT and presented as fold-change above the average expression in LCMV samples. n = 3-5 mice per group.

(B) Fluorescence microscopy of a hepatic granuloma co-stained with antibodies recognizing CD11b (green) and Raldh (red). Arrows point to cells within granulomas that co-stain for both CD11b and Raldh. Scale bar = 50μm.

(C) qRT-PCR analysis of Raldh expression in sorted macrophages (Mac), eosinophils (Eos), and T cells. Hepatic leukocytes from *S. mansoni*-infected mice were enriched by microbead selection of Siglec-F^+ cells. Cell fractions were then sorted to >90% purity by FACS, according to the gating strategy shown at the top. A modified Giemsa stain demonstrated the expected morphology of the sorted cells. The bar graphs at the bottom represent composite results from the indicated groups (n = 3 mice per group). Error bars illustrate SEM; #$p<0.001$. Results are representative of two (C) or three (A, B) independent experiments.

FIGURE 3


A
Raldh1
Raldh2
Raldh3
relative expression units
LCMV
S. mansoni
#
B
RALDH
egg
hepatocytes
CD11b
Merge
C
Pre-sort Siglec-F pos
Pre-sort Siglec-F neg
SSC
FSC
CD3
CD11b
T cell
Siglec-F
Eos
Mac
Liver
MLN
Mac
Eos
T cell

eosinophils, and T cells, Raldh2 in macrophages was the most abundant source of Raldh expression. Similar results were obtained from sorted MLN cells (Figure 3C).

AAMϕ macrophages recruited to liver granulomas highly express Raldh2

Macrophages sorted from the whole livers of *S. mansoni*-infected mice may include inflammatory AAMϕ recruited to granulomas as well as resident Kupffer cells. Recently, AAMϕ have been reported to originate from the proliferation of tissue resident macrophages (Jenkins et al., 2011), which may also be occurring in the liver granulomas. The CX_3CR1-GFP reporter mouse has been used to track monocyte-derived dendritic cells and macrophages in several different organs, including the liver and the intestinal tract (Geissmann et al., 2003). During steady state conditions, the only GFP^+ cells in the livers of these mice were "round" monocytes (white arrows) in the sinusoidal vessels (Figure 4A) (Geissmann et al., 2003). Kupffer cells did not express GFP, making this a convenient model to distinguish between them and inflammatory macrophages.

At eight weeks after infection with *S. mansoni*, almost all of the GFP^+ cells found in the tissues had a morphology (with multiple cellular processes; Fig. 4, B and C) and a localization (on the outer fringe of granulomas; Figure 4, B and C) consistent with that of AAMϕ. To better define these cells, liver leukocytes were sort purified into $CD11b^+$ subpopulations that were either positive or negative for GFP (Figure 4 D). RNA was extracted from these fractions and the expression of arginase 1 as well as of Raldh2 was measured by real-time PCR analysis within them (Figure 4 E). Compared to $CD11b^+CX_3CR1$-GFP^- cells, $CD11b^+$ CX_3CR1-GFP^+ cells expressed high levels of

Figure 4. $CX_3CR1\text{-}GFP^+$ AAMϕ in the liver granulomas of *S. mansoni* infected mice express Raldh2

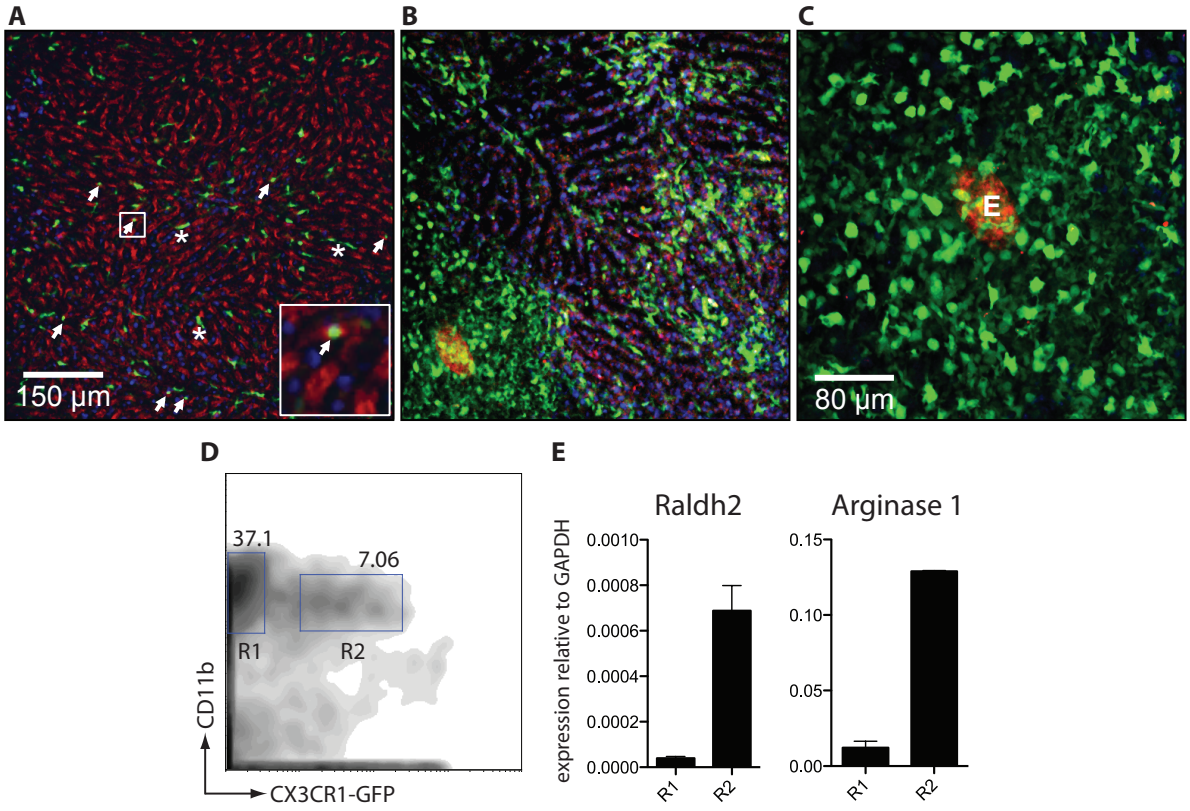
(A) Intravital confocal image analysis of the liver of an uninfected CX_3CR1-GFP/+ mouse injected i.v. with BSA-Alexa 647 and Hoechst 33342 to label sinusoidal vessels (red) and nuclei (blue), respectively. Only round monocytes in the sinusoid vessels (red), but not Kupffer cells, are GFP^+ (white arrows, inset). Large GFP^+ cells present in the capsule are also visible (white asterisks), but are not in the sinusoidal vessels.

(B) The liver of an *S. mansoni*-infected mouse at eight weeks post infection, showing that GFP^+ cells predominate in the parenchymal tissue and not in the sinusoids, and are incorporated into a hepatic granuloma that formed around an auto-fluorescent egg (red, labeled "E"). Auto-fluorescence of the tissue allows visualization of the sinusoids in black. **(C)** High resolution confocal image of the granuloma shown in (B), demonstrating macrophage-like morphology of the GFP^+ cells in the granuloma.

(D) Flow cytometry sorting analysis of GFP^- (R1) and GFP^+ (R2) $CD11b^+$ cells isolated from the liver of an infected CX_3CR1-GFP/+ mouse.

(E) qRT-PCR analysis for arginase 1 and Raldh2 message performed on cDNA of FACS sorted cells shown in (E). Expression is normalized to GAPDH. The bar graphs represent replicates of pooled samples (3 mice per group). Error bars illustrate SEM. Data are representative of three or more independent experiments.

FIGURE 4


A
B
C
150 µm
80 µm
E
D
37.1
7.06
R1
R2
CD11b
CX3CR1-GFP
E
Raldh2
Arginase 1
expression relative to GAPDH
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
0.15
0.10
0.05
0.00
R1
R2
R1
R2

arginase 1 and of Raldh2, indicating that $CX_3CR1\text{-}GFP^+$ cells are AAMϕ and an important source of RA synthesis during *S. mansoni* infection.

IL-4 activation induces Raldh2 expression in macrophages

To further explore the regulation of Raldh expression by AAMϕ, bone marrow-derived macrophages were treated with IL-4 or IFNγ *in vitro* and then assayed for expression of Raldh2 transcript using qRT-PCR. $Stat6^{-/-}$ macrophages were activated in parallel to confirm the specificity of IL-4 signaling. As expected, IL-4-induced arginase 1 expression was strictly Stat6-dependent while IFNγ-induced iNOS expression was unaffected in $Stat6^{-/-}$ macrophages (Figure 5). Raldh2 showed Stat6-dependent induction by IL-4. By contrast, IFNγ inhibited and the regulatory cytokines, IL-10 and TGFβ, had no effect on Raldh2 expression. We did not detect Raldh1 or Raldh3 expression in bone marrow-derived macrophages under any of these culture conditions. These results show that IL-4 is sufficient to induce Raldh2 activation in macrophages via Stat6 signaling, and support the conclusion that Raldh2 expression is a selective characteristic of AAMϕ and not of classically-activated macrophages.

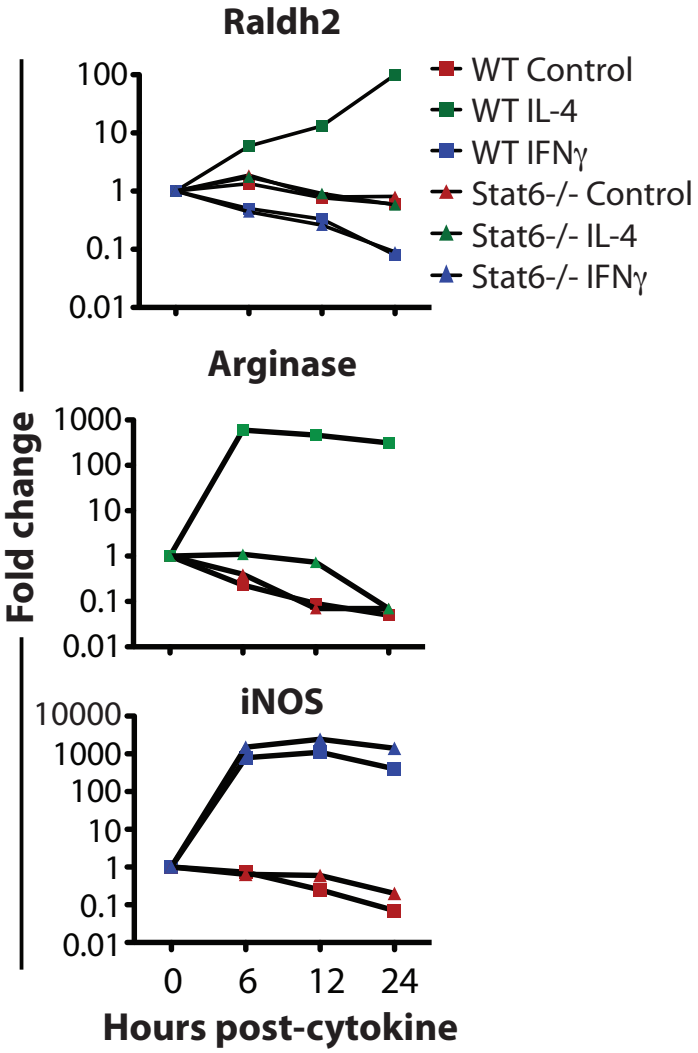
DISCUSSION

The increase in infectious disease morbidity and mortality associated with vitamin A deficiency can be reduced by vitamin A supplementation, suggesting that vitamin A metabolites are important in reducing the pathogenic effects of infection (Stephensen et al., 2001; Sommer et al., 2008). Retinoic acid mediates the effects of vitamin A in T cells (Hall et al., 2011; Iwata et al., 2004; Iwata et al., 2003); however, the regulation of

Figure 5. IL-4 induces arginase and Raldh2 expression in macrophages *in vitro*

qRT-PCR analysis of cytokine-treated bone marrow-derived macrophages from WT and Stat6$^{-/-}$ mice. Expression is normalized to HPRT and presented as a fold-change above untreated cells. Results are representative of three independent experiments.

FIGURE 5



RA synthesis and signaling during infection remains poorly understood. In this study, we identified two critical roles for RA in regulating T cell responses during infection. First, RA signaling in the gut, likely attributable to the constitutive synthesis of RA by GALT APCs, induced CCR9 expression in gut mucosal effector T cells during both helminth and viral infection, and was necessary for T_H2 and T_H1 responses at this site. Second, systemic RA signaling during helminth infection, likely driven by inducible Raldh expression in type-2 inflammatory cells, corresponded to the retinoid-dependency of T_H2 but not T_H1 responses in the liver. These findings offer new insights into the specific contributions of RA in supporting T cell responses during infection, and reveal the inducible expression of Raldh enzymes, especially in AAMϕ, as a regulated component of immune responses.

Homeostatic Raldh expression is considered to be a specialized function of gut-associated APCs (Sun et al., 2007; Denning et al., 2007; Iwata et al., 2004; Coombes et al., 2007). In particular, MLN and lamina propria DCs as well as lamina propria macrophages express Raldh2 at a steady state. The regulation of Raldh enzymes in the context of infection has not been characterized. We found that Raldh2 and Raldh3 were predominantly expressed in type-2 but not type-1 inflammatory infiltrates. We also found that IL-4 directly induced Raldh2 expression in macrophages *in vitro*, consistent with our finding that AAMϕ recruited to granulomas are the Raldh2-expressing macrophage population during *S. mansoni* infection. Raldh2 in macrophages appeared to be the dominant source of Raldh expression in type-2 inflammatory cells. However, the catalytic efficiency (V_{max}/K_m) of Raldh3 is ~10-fold higher than Raldh2 (Sima et al., 2009), thus it is possible that both of these enzymes are relevant sources of RA synthesis

within *S. mansoni* granulomas. Raldh3 is a recently characterized Raldh isoform, and its regulation and role in immunity has not been described. Raldh3 expression was nearly undetectable in the liver of uninfected and LCMV-infected mice, revealing a striking specificity of this enzyme for type-2 inflammation.

While RA promotes Foxp3$^+$ T_{reg} induction *in vitro* (Benson et al., 2007; Sun et al., 2007; Mucida et al., 2007; Kang et al., 2007; Coombes et al., 2007), recent studies have shown a higher frequency of lamina propria Tregs in vitamin A deficient mice and mice lacking RA receptor (RAR)-α (Hall et al., 2011; Hill et al., 2008). Here, we extend these findings to show that peripherally-induced Foxp3$^+$ T_{regs} increase systemically during vitamin A deficiency. Hall et al. noted that the higher frequency of lamina propria T_{regs} observed in vitamin A deficient and RAR-$\alpha^{-/-}$ mice was attributable to a loss of effector CD4$^+$ T cells in this tissue, rather than an increase in the number of T_{regs}. However, a relative loss of effector T cells cannot fully explain our observation that vitamin A deficiency induced higher Treg frequencies in the MLN and spleen, where total numbers of T cells were increased or unchanged during deficiency. Hall et al. found that T_{reg} ablation did not restore retinoid-dependent effector T cell responses in the lamina propria, demonstrating that T_{regs} were not responsible for the loss of these responses. Similarly, LCMV-elicited T cell responses were fully sustained in the spleen of vitamin A deficient hosts, despite a 2-fold increase in Treg frequency. Thus, further studies are needed to determine the mechanism of expansion and suppressive function of Foxp3$^+$ T_{regs} induced in the absence of RA signaling.

AAMϕ are present in type-2 inflammatory responses associated with helminth infection, atopy, and wound healing (Kreider et al., 2007; Loke et al., 2007). While innate

sources of IL-4 can drive alternative activation, T_H2-derived IL-4 is thought to be critical for maintaining AAMϕ during helminth infection (Anthony et al., 2006). Thus, IL-4-inducible RA synthesis by AAMϕ suggests a positive feedback mechanism that may contribute to the amplification of type-2 responses. RA is neither necessary nor sufficient to induce T_H2 polarization *in vitro*; instead, it has been shown to augment IL-4-mediated T_H2 induction (Hoag et al., 2002; Iwata et al., 2003). Accordingly, vitamin A deficiency curtailed the expansion of T_H2 responses at sites of egg deposition, resulting in partial granuloma formation. In MLN cells, the induction of IL-13 and IL-5 but not IL-4 was impaired by vitamin A deficiency. These findings may reflect differences in the regulation of IL-4 expression by T_H2 cells recruited to inflamed tissues and T_{FH} cells residing in the lymph nodes. The generation of mice with cell-specific defects in RA synthesis will be critical in confirming the contribution of macrophage-derived RA to the amplification of T_H2 responses *in vivo*.

While this study focused on the role of RA signaling in T cell responses, the induction of RA synthesis during helminth infection has important implications for other cell types involved in type-2 inflammation. For example, RA promotes eosinophil survival by inhibiting caspase-3 expression and function (Ueki et al., 2008). RA also inhibits IL-12 expression in DCs (Wada et al., 2009) and macrophages (Wang et al., 2007), reducing the T_H1-priming capacity of these cells. Interestingly, IL-3 activation has been shown to induce Raldh2 expression in human basophils *in vitro*, leading to both autocrine and paracrine RA signaling (Spiegl et al., 2008). Further investigation into these RA-mediated effects *in vivo* may better define the role of vitamin A in protective immunity.

Vitamin A deficiency affects ~200 million preschool age children and ~19 million pregnant woman globally (WHO 2009), the populations at the greatest risk for severe infections. The geographic distribution of vitamin A deficiency overlaps significantly with that of endemic helminth infections. Our results support a particularly important role for RA in the generation of protective T_H2 responses during helminth infection and reveal that RA-synthesizing enzymes are an inducible component of the immune response to helminths. It follows that the efficacy of vaccines aimed at eliciting protective T_H2 responses against helminth parasites (Hotez et al., 2010) will depend on both the vitamin A status of the host as well as on the ability to prime APCs such as AAMϕ that are competent for RA synthesis.

MATERIALS AND METHODS

Mice. Wild-type and Stat6$^{-/-}$ C57BL/6 mice were purchased from Jackson Laboratories. CX_3CR1-GFP mice were kindly provided by Dr. Dan Littman (Skirball Institute, NYU) and were used as heterozygotes from crosses of CX3-CR1-GFP/GFP with wildtype C57BL/6 mice. For vitamin A deficiency experiments, timed-pregnant C57BL/6 dams were purchased from Charles River. Mice were maintained in a specific pathogen free UCSF Laboratory Animal Resource Center facility. Pregnant dams were fed a vitamin A deficient (0 IU/g, TD.86143 Harlan Teklad) or control (20,000 IU/g, TD.93160) diet starting at day 10 of gestation and continuing through weaning. After weaning, mice were maintained on the same diet for the duration of the experiment. Animal protocols were approved by the UCSF Institutional Animal Care and Use Committee.

Infections. Mice were infected subcutaneously with 150 Puerto Rican *S. mansoni* cerceriae harvested from laboratory-maintained *Biomphalaria glabrata* snails. This number was titrated to result in a consistent chronic non-lethal infection in C57BL/6 mice. The intensity of infection was determined by counting adult worms recovered by perfusion of the portal system at euthanasia. To determine hepatic egg burden, liver samples were weighed, homogenized, and digested with trypsin; eggs were then sedimented and counted under a dissecting microscope. $2x10^5$ p.f.u. of LCMV-Armstrong was administered intraperitoneally.

Tissue preparation and histopathology. To obtain single-cell suspensions, livers were minced and digested with 100 U/ml type 8 collagenase (Sigma) and 150 μg/ml DNase I (Sigma) for 1 hour at 37°C followed by dispersal over 70 μm filters. Hepatic leukocytes were enriched by density centrifugation over a 40/80 Percoll (GE Healthcare) gradient. Spleens and MLN were dispersed over 70 μm filters, followed by lysis of splenic red blood cells with ACK lysis buffer (Invitrogen). Small intestine and colon tissue were first cleaned of mesentery, fat, and fecal contents, and then cut into ~2 cm pieces. Tissue pieces were incubated with 1 mM DTT followed by two consecutive incubations with 30 mM EDTA and 10 mM HEPES to remove epithelial cells. The remaining intestinal tissue was then digested as described above, and leukocytes were enriched by density centrifugation over a 40/80 percoll gradient. For histopathology, liver tissue was fixed in 10% formalin and paraffin-imbedded. Tissue sections were stained with hematoxylin and eosin for egg granuloma diameter measurements, eosinophil quantification, and scoring

of microvesicular damage, as described (Fallon and Dunne 1999), by two individuals blinded to treatment.

***Ex vivo* stimulation.** $5x10^5$ cells were stimulated for 5 hours at 37°C in the presence of 10 μg/ml brefeldin A (GolgiPlug, BD Pharmingen). Phorbol 12-myristate 13-acetate (PMA, 10 ng/ml) and ionomycin (1 μg/ml) were used for polyclonal T cell stimulations. LCMV peptides GP61 and GP33 (10 μg/ml) were used for antigen-specific $CD4^+$ and $CD8^+$ T cell stimulations, respectively. For detection of cytokines in culture supernatants, $5x10^5$ cells were cultured for 72 hours in the presence of adult schistosome worm homogenate or schistosome egg homogenate at a protein concentration of 50 μg/ml. Cytokines were quantified using a multiplex bead-based assay (Th1/Th2/Th17 Cytometric Bead Array, BD Biosciences), according to the manufacturer's instructions. Samples were acquired on an LSRII with FACSDiVa software (BD Biosciences) and data were analyzed with FCAP Array software.

Flow cytometry

T cell phenotyping. Cells were incubated for 30 minutes at 4°C with fluorochrome-conjugated antibodies against CD3 (500A2, BD Biosciences), CD4 (RM4-5, Invitrogen), CD8 (5H10, Invitrogen), CCR9 (CW-1.2, eBioscience), CD62L (MEL-14, eBioscience), and CD44 (IM7, eBioscience).

Intracellular cytokine staining. Following surface staining with antibodies against CD3, CD4, and CD8, cells were fixed with 2% paraformaldehyde and permeabilized with 0.5%

saponin. Cells were then incubated with anti-mouse CD16/32 (eBioscience) to block Fc receptors, followed by a 30 minute incubation at 4°C with fluorochrome-conjugated antibodies against IL-4 (11B11, eBioscience), IFNγ (XMG1.2, BD Biosciences), and TNFα (MP6-XT22, eBioscience).

Treg staining. Following surface staining with antibodies against CD3, CD4, CD8, and CD25 (PC61, BD Biosciences), cells were washed with PhosFlow permeabilization buffer (BD Biosciences), blocked with anti-mouse CD16/32, and stained with Foxp3 antibody (FJK-16s, eBioscience) for 1 hour at 4°C. For all experiments, dead cells were excluded with LIVE/DEAD Fixable Aqua Dead Cell Stain kit (Invitrogen). Samples were fixed in 2% paraformaldehyde and acquired on an LSRII with FACSDiVa software (BD Biosciences). Data were analyzed with FlowJo software (TreeStar).

Cell sorting. Cells were stained with PE-conjugated anti-Siglec-F antibody (E50-2440, BD Biosciences) for 20 minutes at 4°C and then incubated with anti-PE magnetic beads (Miltenyi Biotec). Siglec-F^+ cells were positively selected on MS columns (Miltenyi Biotec) according to the manufacturer's instructions; Siglec-F^- cells were collected in the flow-through. Both fractions were stained with antibodies against CD3, CD11b, and Siglec-F, and sorted directly into TRIzol (Invitrogen) using a BD FACSAria cell sorter.

RT-PCR. Tissue samples were homogenized in TRIzol. RNA was collected in the aqueous extraction phase and column purified using an RNeasy kit (Qiagen). cDNA was generated using an Omniscript Reverse Transcription kit (Qiagen) with oligo-dT primers

in the presence of RNasin Plus RNase inhibitor (Promega). PCR reactions were carried out with Taqman primer/probe sets (Applied Biosystems) in a StepOne Plus machine (Applied Biosystems).

Immunofluorescence. Sections of formalin-fixed, paraffin-imbedded tissue were deparaffinized and rehydrated according to standard protocols. Slides were immersed in citrate buffer (pH 6.0) and heated in a pressure cooker for antigen retrieval. After blocking, tissue sections were stained for 1 hour at room temperature with antibodies against CD11b (M1/70, Abcam) and Raldh (Abcam) followed by a 1-hour incubation with Alexafluor 488- and Alexafluor 555-conjugated secondary antibodies (Invitrogen). Images were acquired on a Leica DM6000B microscope with a QImaging Retiga EXi Fast 1394 camera using QCapture Pro software.

Intravital imaging. CX3CR1-GFP/+ mice were anesthetized with a combination of ketamine, xylazine and acepromazine injected intraperitoneally and were kept warm on a heating pad or pre-warmed stage. Livers of anesthetized mice were exposed by carefully cutting through the skin and peritoneum just below the rib cage and gently coaxing out a lobe of the liver. Mice were then inverted onto a pre-warmed aluminum stage insert with a 2.5 cm hole fitted with a glass coverslip secured with vacuum grease and tape. The liver was stabilized with gauze soaked in PBS to limit movement during imaging and to keep the liver moist. Mice were injected retro-orbitally with 250 μg of Hoechst 33342 to visualize nuclei and 250 μg BSA conjugated to Alexa 647 to detect blood vessels. Mice were then transferred to a heated chamber that was used to keep the microscope,

objectives, mice and stage at 37°C during imaging. Images were acquired on a Leica SP2 inverted confocal microscope with light generated from UV, 488 nm, and 633 nm laser lines and detected using tunable filters.

Derivation and activation of bone marrow-derived macrophages. Macrophages were derived from bone marrow cells harvested from the femurs and tibias of C57BL/6 mice. Cells were differentiated for six days in the presence of fetal bovine serum (FBS) and 3T3 fibroblast supernatant containing M-CSF and cryopreserved. Thawed macrophages were rested for 12 hours, followed by activation with IL-4 (20 ng/ml) or IFNγ (50 ng/ml; all cytokines were purchased from Peprotech). Cells were lysed in TRIzol (Invitrogen) at the indicated time points for RNA extraction.

Statistical analysis. Statistical significance was determined with the unpaired Students's t test using Prism software (GraphPad).

Figure S1. T_H1 responses during *S. mansoni* infection are not retinoid-dependent

(A - C) Flow cytometric analysis of intracellular cytokines expressed by liver (A), colon (B), and mesenteric lymph node (MLN, C) leukocytes following a 5-hour stimulation with PMA and ionomycin in the presence of brefeldin A. n = 3-5 mice per group. **(D)** qRT-PCR analysis of cytokine expression in whole MLN. Expression is normalized to HPRT. n = 3-5 mice per group. **(E)** Cytometric bead array analysis of cytokine concentrations in culture supernatants. MLN cells harvested from *S. mansoni*-infected (Inf) and control (Cont) mice were cultured as described in Figure 2. Error bars illustrate SEM. Results are representative of two (E) or three (A-D) independent experiments.

SUPPLEMENTARY FIGURE 1

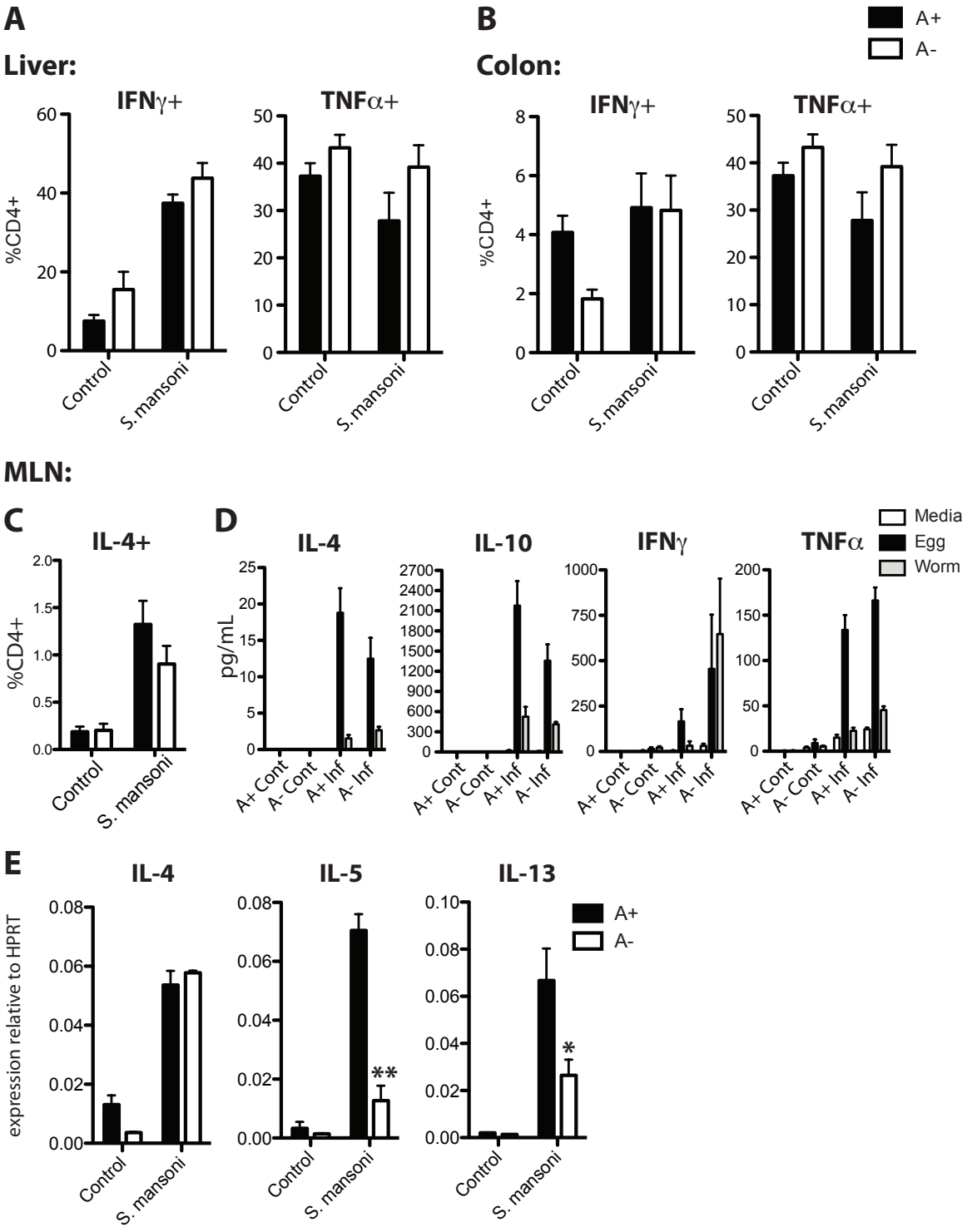


Figure S2. Foxp3$^+$ T_{reg} cells are sustained during vitamin A deficiency

Flow cytometric analysis of intranuclear Foxp3. Representative contour plots are gated on live CD4$^+$ T cells from mesenteric lymph nodes (MLN), small intestine, thymus, spleen, or colon. The bar graphs to the right of each plot represent composite results from the indicated groups (n = 3-5 mice per group). Error bars illustrate SEM; *p<0.05, #p<0.001. Results are representative of three independent experiments.

SUPPLEMENTARY FIGURE 2

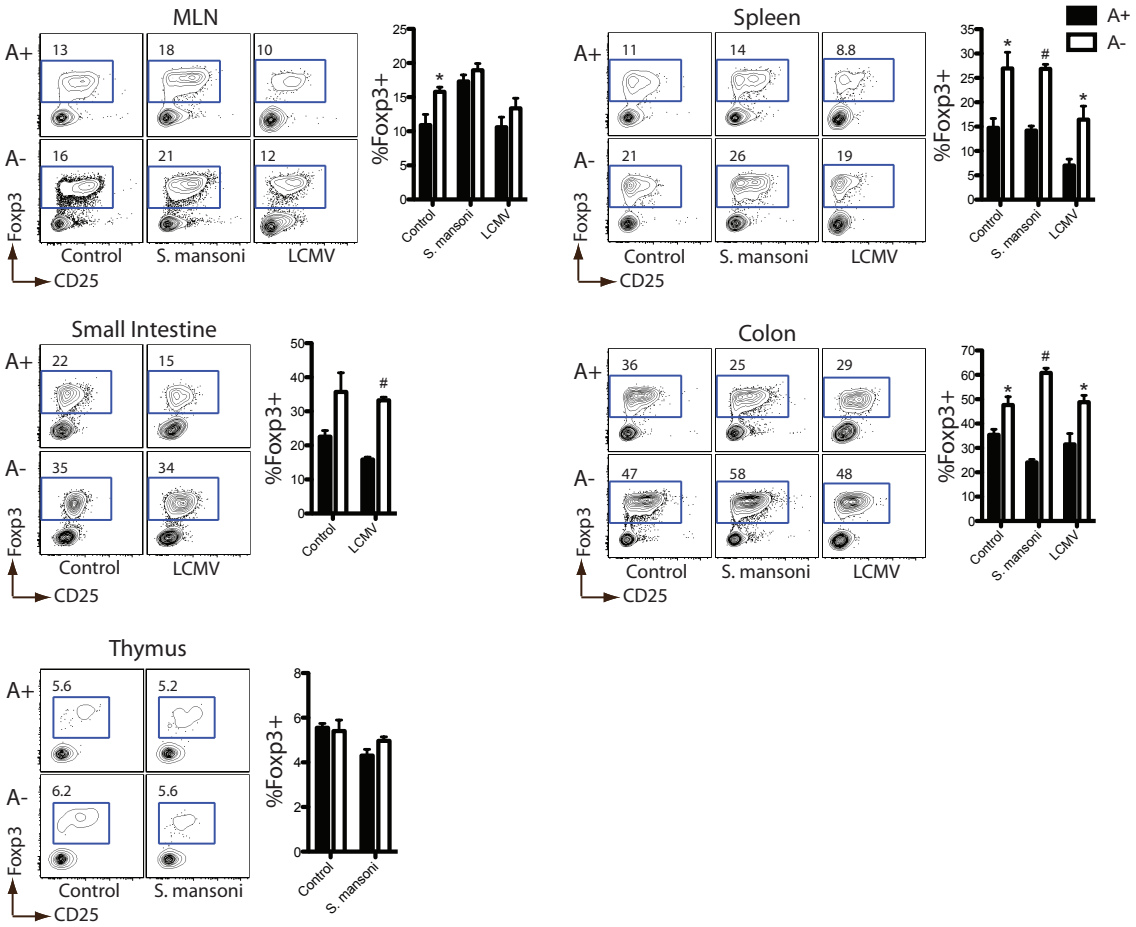


Figure S3. LCMV-specific CD8$^+$ T cell responses in the intestinal mucosa are partially retinoid-dependent

Flow cytometric analysis of intracellular cytokines expressed by cells harvested from the liver, spleen, small intestine, or colon following a 5-hour stimulation with GP33 peptides (10 μg/mL) in the presence of brefeldin A. Representative contour plots are gated on live CD8$^+$ T cells. The bar graphs to the right of each plot represent composite results from the indicated groups (n = 3-5 mice per group). Error bars illustrate SEM. Results are representative of three independent experiments.

SUPPLEMENTARY FIGURE 3

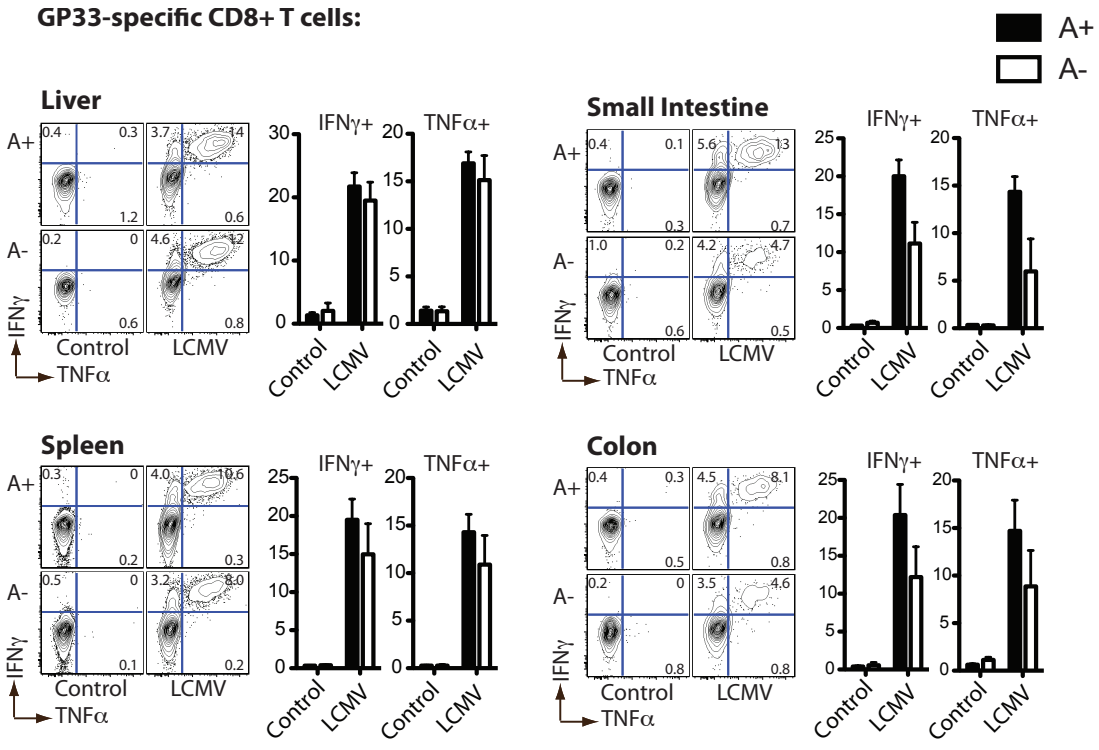


Figure S4. Polyclonal T_H1 responses in the intestinal mucosa are retinoid-dependent

Flow cytometric analysis of intracellular cytokines following a 5-hour stimulation with PMA and ionomycin in the presence of brefeldin A. Bars represent average frequencies of IFNγ^+ cells within the live CD4$^+$ T cell gate. n = 3-5 mice per group. Error bars illustrate SEM; *p<0.05, **p<0.01. Results are representative of three independent experiments.

SUPPLEMENTARY FIGURE 4

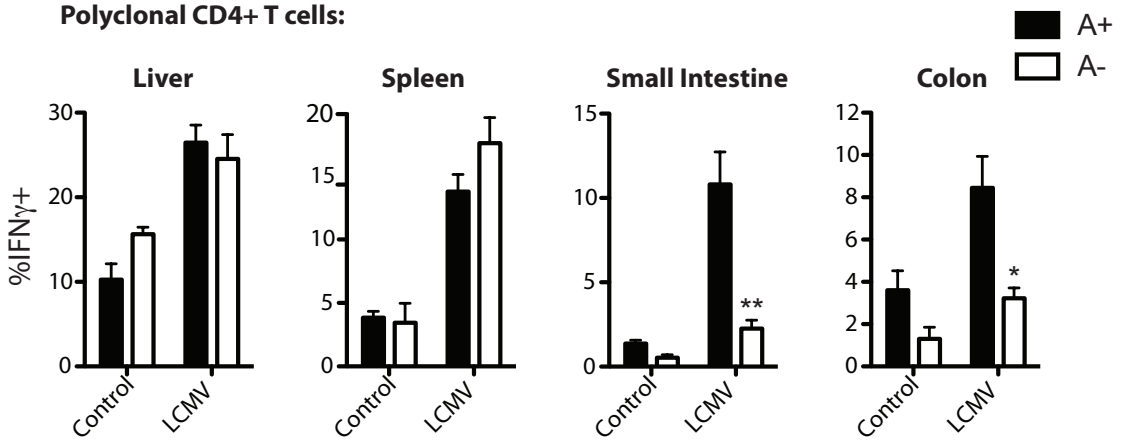
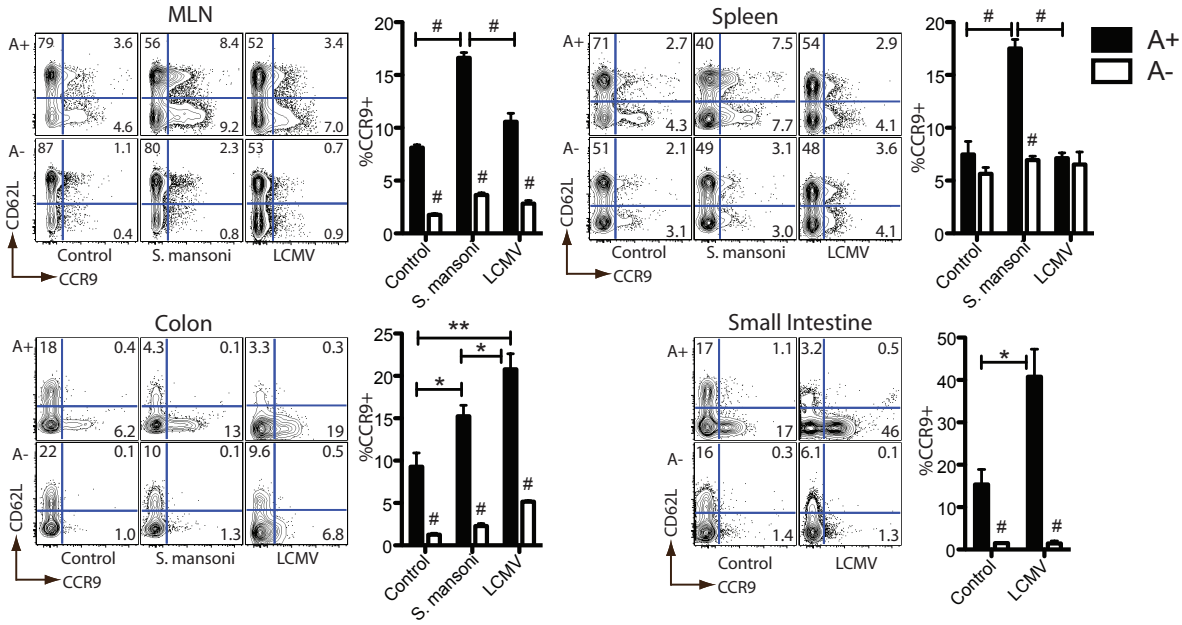


Figure S5. Retinoid-dependent CCR9 expression is variably induced during infection

Flow cytometric analysis of cells harvested from mesenteric lymph nodes (MLN), colon, spleen, or small intestine at 7 weeks (*S. mansoni*) or 7 days (LCMV) post-infection (p.i.) from A+ or A- mice. Representative contour plots are gated on live $CD4^+$ T cells. The bar graphs to the right of each plot represent composite results from the indicated groups (n = 3-5 mice per group). Error bars illustrate SEM; *p<0.05, **p<0.01, #p<0.001. Results are representative of three independent experiments. MLN = mesenteric lymph node.

SUPPLEMENTARY FIGURE 5



CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Exploring the immunology of helminthic therapy: an ecological perspective.

T_{regs} play an important, but not exclusive, role in helminth-mediated immune modulation

Upon the first epidemiological observations in the 1960's that autoimmune diseases rarely occur in regions of endemic helminth infection, it was suggested that immunological changes induced by these parasites protect against immune-mediated pathology (Greenwood, 1968). Given the propensity of helminth infections to subvert T_H1-, T_H2- and T_H17-driven diseases, a strong focus has been placed on the role of regulatory networks that broadly dampen effector T cell responses (Maizels, 2003). In particular, a role for helminth-induced T_{regs} has garnered much interest. Convincing evidence has accumulated that T_{regs} dampen parasite-specific T cell responses, contributing to parasite survival and limiting inflammatory pathology during chronic infection (Baumgart et al., 2006; Taylor et al., 2006; McSorley et al., 2008; Taylor et al., 2009; Blankenhaus et al., 2011). As a bystander effect, these cells also dampen T cell responses to unrelated antigens, which has both harmful (e.g. reduced vaccine efficacy; Elias et al., 2006; Urban et al., 2007) and beneficial (suppression of allergy and autoimmunity; Fallon and Mangan, 2007; Wilson and Maizels, 2004) consequences. However, there is also evidence for T_{reg}-independent mechanisms driving helminth-mediated amelioration of inflammatory disease, particularly in the setting of colitis (Smith et al., 2007). Furthermore, T_{reg}-targeted therapies have shown limited clinical success for IBD despite promising studies in murine models (Plevy and Targan, 2011). It is therefore useful to consider all facets of the mucosal type-2 immune response when deriving a mechanistic understanding of helminthic therapy for IBD.

Tolerance: a common goal for host and parasite

In immunological terms, tolerance is defined by the deletion or suppression of antigen-specific immune responses, such as the well-known examples of central tolerance to auto-antigens via negative selection and $Foxp3^+$ T_{reg} induction in the thymus. In the ecological study of host-parasite relationships, tolerance is defined by the collection of host mechanisms that maximize host fitness despite a persistent pathogen burden (Schneider and Ayres, 2008). Tolerance is thus distinct from resistance, the ability to reduce pathogen burden. As host-parasite relationships evolve, parasites will adapt to neutralize host resistance mechanisms. Ecological tolerance, in contrast, will in most cases be favored by both host and parasite selection.

Ecological tolerance is a critical component of the host response to helminth infection, as resistance mechanisms are largely ineffectual and chronic infection is typically established (Allen and Maizels, 2011). The induction of immunological tolerance can represent an element of ecological tolerance, as the host benefits from the mitigation of inflammatory pathology. Thus, as discussed above, T_{reg} induction is a common feature of chronic helminth infection that benefits both host and parasite. Due to the migratory and invasive nature of these large parasites, the ability of the host to tolerate helminth infection also requires mechanisms for tissue repair and wound healing. As with T_{reg} induction, it would be predicted that both host and parasite positively select these mechanisms. Thus, a consideration of mechanisms underlying helminthic therapy should encompass the full complement of host tolerance mechanisms that comprise the type-2 immune response.

A variety of helminth parasites have demonstrated efficacy for a range of inflammatory diseases, including conditions where the inflamed tissue and site of infection are widely separated (e.g. intestinal hookworm infection in a multiple sclerosis patient). Thus, it is unlikely that a single mechanism can account for the therapeutic effect of helminth exposure in all cases. The studies presented here have explored therapeutic *T. trichiura* infection in the setting of colitis, where the worms reside in close proximity to the inflamed tissue and localized immune responses in the colonic mucosa are expected to be most relevant. *T. trichiura* infection elicits resistance and tolerance mechanisms tailored to the intestinal mucosa, including the enhancement of mucosal barrier functions that have evolved to promote expulsion and to cope with mucosal injury caused by adult worms as they burrow into the intestinal epithelial layer. We propose that these specialized functions of the mucosal type-2 response contribute to the amelioration of colitis by limiting epithelial exposure to luminal bacteria.

Lessons from worms: targeting mucosal barrier function in IBD treatment.

The data presented in Chapters 2 and 3 showed that symptomatic improvement following *T. trichiura* infection in an UC patient and rhesus macaques suffering from CIC was associated with a localized T_H2 response (and, in the UC patient, an IL-22 response) in the colonic mucosa. The T_H2 cytokines IL-4, IL-9, and IL-13 (Finkelman et al., 2004; Steenwinckel et al., 2009) as well as IL-22 (Sonnenberg et al., 2011) have profound effects on colonic epithelial cell function, including the stimulation of goblet cell and Paneth cell differentiation with their attendant mucus production and anti-microbial peptide expression, and the activation of anti-apoptotic pathways. Furthermore,

accessory cells recruited and activated by type-2 cytokines, most notably AAMϕ, can promote mucosal healing (Seno et al., 2009). Taken together, these functions enhance the epithelial barrier against luminal antigens, and they have demonstrated protective effects in murine models of colitis (Seno et al., 2009; Sugimoto et al., 2008; Zenewicz et al., 2008). In our studies, gene expression analyses revealed the activation of these signaling pathways, and functional read-outs (restoration of colonic mucus and reduced bacterial attachment) confirmed a positive effect on mucosal barrier function.

While T_{reg} induction likely contributes to the beneficial effects of helminth exposure in other settings, we did not find evidence to support a role for T_{regs} in the amelioration of colitis following *T. trichiura* infection. $Foxp3^+$ T_{regs} were most abundant in the colonic mucosa during severe inflammation in both studies and decreased in parallel with the resolution of inflammation, consistent with other clinical studies of chronic mucosal inflammation (Loke et al., 2010). However, we did not directly measure IL-10 expression, and thus cannot rule out a role for IL-10-producing Tr1 cells.

Mucus: keeper of the peace?

Intestinal mucus is a carbohydrate-rich gel, approximately 1 millimeter thick, charged with the formidable task of separating the intestinal epithelium from ~10^{13} commensal bacteria. The scaffolding of the mucus gel is primarily composed of mucins, high molecular weight glycoproteins bearing O-linked oligosaccharides that are commonly decorated with chemical moieties such as sulphate and acetyl groups. Of the nineteen mucins identified in humans, Muc2 is the most important mucin secreted in the intestine (Johansson et al., 2011). Muc2 forms two distinct layers following secretion by

goblet cells. The loosely packed outer layer is the main bulk of the mucus gel, and harbors a large number of bacteria. Conversely, the thin inner layer is composed of tightly packed lamellar sheets that are normally impermeable to bacteria (Johansson et al., 2008). Below the Muc2 layers, transmembrane mucins (e.g. Muc3) cover the apical surface of enterocytes. A lipid fraction largely composed of amphipathic phospholipids contributes to the viscosity and hydrophobicity of the mucus gel (Gibson and Muir, 2005). Phosphatidylcholine (PC) and lyso-PC are the most abundant phospholipids in colonic mucus (Ehehalt et al., 2004).

Histochemical studies have demonstrated that the mucus gel is abnormal in both quantity and quality in a large fraction of UC patients (Pullan et al., 1994). Muc2 abundance is lower in rectal mucus samples from UC patients (Tytgat et al., 1996) and furthermore displays altered glycosylation (Larsson et al., 2011) and reduced sulphation (Corfield et al., 1996). A causal role for altered expression and post-translational processing of mucins in the pathogenesis of colitis is supported in several mouse models. Genetic deficiency (van der Sluis et al., 2006; Burger-van Plaass et al., 2011) or terminal misfolding (Heazlewood et al., 2008) of Muc2 precipitates severe, spontaneous colitis in mice. Impaired glycolylation of mucins due to specific glycosyltransferase deficiencies also increases susceptibility to colitis (Fu et al., 2011; An et al., 2007). More recently, abnormalities in phospholipid species have also been described in UC patients, with a significant decrease in PC (Ehehalt et al., 2004; Stremmel et al., 2005; Stremmel et al., 2010). Intriguingly, clinical trials in which the phospholipid content of mucus in UC patients was restored to that of healthy individuals by oral intake of delayed-release PC

have shown promising results (Stremmel et al., 2005; Stremmel et al., 2007; Stremmel et al., 2010).

Our studies of *T. trichiura* infection support the development of IBD therapies that target mucosal healing, particularly for patients with an underlying defect in barrier function. It is interesting to note that helminth infection is associated with qualitative changes in mucus composition, including increased sulphation of mucins (Koninkx et al., 1988; Soga et al., 2008), in addition to stimulating bulk mucus production via goblet cell hyperplasia and increased mucin expression (Shekels et al., 2001; Karlsson et al., 2000; Olson et al., 2002; Yamauchi et al., 2006). The further study of host tolerance mechanisms activated during intestinal helminth infection may identify novel pathways that bolster mucosal barrier functions and thereby reduce inflammation driven by luminal bacteria without the risks of immunosuppression associated with current treatments for severe IBD.

Retinoic acid: a central player in type-2 immunity?

Using a murine model of schistosomiasis, we found a selective dependency of type-2 immunity on vitamin A metabolites (Chapter 4). Importantly, AAMϕ highly expressed RA-synthesizing enzymes in this setting, demonstrating that RA synthesis is an inducible component of the type-2 response. Given the diverse roles of RA in cellular physiology, the ability of AAMϕ to synthesize RA may contribute to their pleiotropic function during helminth infection. In their capacity as APCs, AAMϕ could influence helper T cell polarization via RA-dependent mechanisms, for example, by promoting T_H2 and T_{reg} subsets while inhibiting T_H1 and T_H17 differentiation. AAMϕ-derived RA could

also impact other innate type-2 effector cells, as RA signaling promotes eosinophil survival (Ueki et al., 2008) and basophil activation (Spiegl et al., 2008). Finally, RA plays a critical role in the maintenance and repair of mucosal epithelia (reviewed in Stephensen, 2001), raising the interesting possibility that RA synthesis contributes to the wound healing function of AAMϕ. In summary, the induction of RA synthesis during helminth infection has important implications for both resistance and tolerance mechanisms of type-2 immunity, and elucidating the functions of AAMϕ-derived RA merits further investigation.

Future directions

Larger mechanistic studies of Trichuris-based therapy for UC and CIC

To extend the findings of the case study, a clinical trial titled Mucosal Immunity of Ulcerative Colitis Patients Undergoing Therapy with *Trichuris Suis* Ova (MUCUS) has been initiated at New York University Medical Center. This is a randomized, double-blind, crossover trial in which patients with established and active UC are treated with either *T. suis* ova for 12 weeks followed by placebo for 12 weeks, or placebo followed by *T. suis* ova. Colon biopsies and peripheral blood will be collected at three timepoints: baseline, 12 weeks, and 24 weeks. The primary outcome measures will include changes in 1) effector and regulatory $CD4^+$ T cell subsets determined by flow cytometry, 2) mucus production determined by immunohistochemistry, and 3) gene expression determined by microarray and RT-PCR. As secondary outcome measures, disease activity will be assessed using the Mayo score and Simple Clinical Colitis Activity Index.

An extended study is also planned to confirm our findings in rhesus macaques suffering from CIC. 16 CIC subjects housed at the CNPRC will be enrolled in the study, and randomly assigned to treatment with *T. trichiura* eggs (n = 10) or a standard 8-week course of methylprednisone that is used to induce remission in IBD patients (n = 6). Adult Trichuris worms will be collected at necropsy from naturally infected macaques, and eggs will be harvested and germinated *in vitro* for 5-6 weeks. Colon biopsies and peripheral blood will be collected at baseline and at 8 weeks. Similar to the primary outcome measures of the MUCUS trial, T cell subsets, mucus production, and gene expression will be analyzed. Weight and fecal consistency scoring will be used to monitor clinical status.

For both studies described above, changes in the abundance and composition of bacteria residing in the colon will be evaluated. The intestinal microbiota is strongly implicated in the pathogenesis of chronic colitis (Nell et al., 2010) and compositional changes in luminal bacteria have been associated with colitis in macaques (McKenna et al., 2008). Intestinal helminth infection in mice can alter the composition of the microbiota, including a higher representation of the family *Lactobacillaceae* that can reduce intestinal inflammation (Walk et al, 2010). However, helminth-induced changes in the composition and mucosal adherence of intestinal bacteria have not been investigated in the context of colitis. The results of our pilot study suggest that bacteria closely associated with the mucosal epithelium are particularly relevant for driving inflammation during CIC. Thus, colonic brushings will be collected in addition to stool to evaluate shifts in both adherent and luminal bacteria. It will also be intriguing to explore functional shifts in the microbiome, such as the expression of mucolytic enzymes that

alter glycoprotein and phosopholipid components of mucus and could therefore mediate effects on mucosal barrier function following helminth infection.

We expect that not all UC patients and CIC subjects will respond to helminthic therapy due to the heterogeneous pathoetiology of these diseases. An important goal of future studies will be to identify factors that predict a beneficial (or detrimental) response to helminth exposure. For example, individuals with an underlying defect in mucosal barrier functions such as mucus production may respond more favorably to helminthic therapy than those individuals with disrupted immune regulation. Indeed, helminth infection can exacerbate colitis when regulatory networks are dysfunctional (Schopf et al., 2002) or when the underlying inflammation is strongly T_H2-polarized (Hunter et al., 2007), highlighting the need for predictive biomarkers.

Defining the role of RA in murine models of helminthic therapy

Retinoid signaling can suppress Th1- and Th17-driven inflammation in murine models of autoimmunity (Kinoshita et al., 2003; Escribese et al., 2007; Klemann et al., 2009). Our studies of RA synthesis during *S. mansoni* infection suggest that AAMϕ-derived RA may contribute to the suppression of autoimmunity mediated by this parasite. To investigate this hypothesis, we are generating a genetically engineered mouse strain (called ATIC) in which Raldh2-expressing cells can be tracked by a fluorescent reporter and inducibly deleted. Our reporter strategy is to replace the first exon of the Raldh2 gene with the tdTomato (red) fluorescent protein, stabilized with a bovine growth hormone polyA tail. The reporter function will enable us to monitor cellular behavior and trafficking, as well as to isolate live cells for *in vitro* functional assays. An additional

cassette following the reporter gene contains an internal ribosomal entry site and Cre gene, such that Raldh2-expressing cells will also express Cre recombinase. Crossing the ATIC mice to mice in which lysozyme expression is linked with Cre-inducible expression of diphtheria toxin receptor (DTR) will generate a strain in which Raldh2-expressing macrophages are susceptible to inducible deletion by DT injection. These tools will allow us to specifically interrogate the contribution of Raldh2-expressing macrophages to *S. mansoni*-mediated suppression of inflammation in disease models such as chemically-induced colitis.

Can a pill replace worms?

A prevailing question in the field of helminth immunology remains: can the beneficial effects of helminth exposure be recapitulated with a biologic therapy that avoids active infection? Helminth-derived molecules that can drive certain aspects of a tolerogenic type-2 response have been identified, and offer important stepping-stones towards this goal. Two components of schistosome eggs, lacto-*N*-fucopentoase III (Thomas et al., 2003) and the omega-1 glycoprotein (Steinfelder et al., 2009; Everts et al., 2009) condition DCs to prime T_H2 polarization. Omega-1 also acts directly on T cells to promote FoxP3 expression (Zaccone et al., 2011). Another major schistosome egg-derived glycoprotein, IL-4-inducing principle of *S. mansoni* eggs (IPSE/alpha-1) activates IL-4 expression by basophils (Schramm et al., 2007), contributing to T_H2 responses *in vivo*. Toll-like receptor stimulation by schistosomal lysophosphatidylserine (van der Kleij et al., 2002) and the ES-62 glycoprotein secreted by *Acanthocheilonema vitae* (Goodridge et al., 2005) promotes tolerogenic DCs with reduced pro-inflammatory

cytokine expression. Finally, a TGFβ-mimic secreted by *H. polyrus* augments FoxP3^{+} T_{reg} reponses (Grainger et al., 2010). The elucidation of signals that mediate cross-talk between type-2 immune cells and resident tissue cells during helminth infection, as well as a better understanding of indirect immune modulation driven by changes in the microbiome, may also lead to novel therapeutic targets. However, significant challenges will remain, including the ability to localize and sustain the targeted signaling pathways. This active field of research is sure to yield promising new classes of immunomodulatory and probiotic therapeutic agents in the coming years.

The study of helminthic therapy is an exciting field at the intersection of molecular and translational immunology, evolutionary ecology, and the clinical management of inflammatory diseases. When fully unveiled, the mechanisms driving the benefits of helminth exposure will no doubt tell a fascinating story about our millennia-old relationship with these uninvited guests, and help us to cope with the unintended consequences of their absence.

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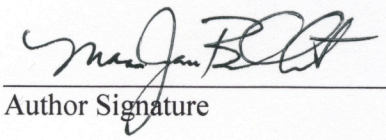

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