

**INDUCTION OF THE INTESTINAL TH2 IMMUNE RESPONSE TO THE PARASITIC  
NEMATODE *TRICHINELLA SPIRALIS*: RAPID EXPULSION, DISTINCT REGIONAL  
IMMUNITY, AND THE ROLE OF INNATE MEDIATORS.**

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By Lisa K. Blum

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**INDUCTION OF THE INTESTINAL TH2 IMMUNE RESPONSE TO THE PARASITIC NEMATODE *TRICHINELLA SPIRALIS*: RAPID EXPULSION, DISTINCT REGIONAL IMMUNITY, AND THE ROLE OF INNATE MEDIATORS.**

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Cornell University, 2012

Intestinal helminths infect over 2 billion people worldwide, and the mechanisms by which immunity to these pathogens is induced are not well-understood. *Trichinella spiralis* exhibits a broad geographic and host range, and is a natural pathogen of humans and rodents. Infection of mice and rats with *T. spiralis* provides a useful model of Th2 immunity in the mucosa. Rats exhibit a dramatic protective immune response, called rapid expulsion, in which 90% of parasites are cleared from the intestine within one hour of challenge. We observed that intestinal priming and mucosal mast cell degranulation are not required for rapid expulsion to occur. Thus, mast cells may play a role during the induction of Th2 immunity in rats, rather than during the effector phase.

The specific intestinal habitat of the parasite may influence immunity. Although many parasite species are restricted in their distribution in the gastrointestinal tract, *T. spiralis* colonizes both the small and large intestines (SI and LI). The results demonstrate that mucosal mastocytosis and the cytokines that influence it are not prominent in the LI during worm expulsion, and that immunopathology is more tightly regulated in the LI than the SI. Thus, mechanisms of immunity and enteropathy vary in different regions of the intestine during helminth infection.

The influence of innate mediators on the adaptive immune response to *T. spiralis* was also investigated. Evidence was collected suggesting that although MyD88 is influential in

promoting Th2 responses, and there was a modest influence of the microbiota, MyD88 operates independently of TLRs. Mice deficient in ST2, the receptor for IL-33, recapitulated the results seen in MyD88<sup>-/-</sup> mice, supporting the role of IL-33 as a mediator of Th2 responses. IL-33 was observed in intestinal leukocytes and in epithelial cells, where it concentrated in the nucleus within two days of infection. Array analysis revealed that at two days after infection, targets of NF- $\kappa$ B were downregulated in the epithelium, while the TGF- $\beta$  pathway was increased. IL-33 may carry out dual functions as part of a complex mechanism of innate induction of the Th2 response.

## **BIOGRAPHICAL SKETCH**

Lisa Kay Blum was born in Northern Oregon. After only two weeks of life in Oregon, she moved to San Luis Obispo, California, where she remained until graduating from San Luis Obispo High School. She earned her bachelors degree in Food Science from Cornell University in Ithaca, New York. In 2006, Lisa entered the Graduate Field of Immunology at Cornell for study culminating in a Doctor of Philosophy.

## **DEDICATION**

Dedicated to my wonderful family: Hank, Helen, Robert, Kay, Karen, Mike, Marlene, Nick, Ken, Jeannie, Sandy, Mike, Matt, Jen, Ben, Anne, Mikey, Brendan, Maria, Sheryl, Seiji, Marchella, Simon, Judy, Ken, Katie, Cody, Kate, Kysa, and Zaya.

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# Table of Contents

<b>CHAPTER 1: Introduction .....</b>	<b>1</b>
Significance .....	2
<i>Trichinella</i> .....	2
Intestinal Immunity to <i>T. spiralis</i> .....	3
<i>Primary Immunity</i> .....	3
<i>Protection against reinfection</i> .....	4
<i>Immunopathology</i> .....	5
Intestinal Habitat of the Parasite.....	6
PAMPs and DAMPs .....	10
Induction and Amplification of Th2 Immunity.....	12
Interleukin-33 .....	15
Synthesis.....	17
References .....	18
<b>CHAPTER 2: Expulsion of secondary <i>Trichinella spiralis</i> infection in rats occurs independently of mucosal mast cell release of mast cell protease II .....</b>	<b>39</b>
Abstract.....	40
Introduction .....	40
Materials and Methods .....	42
<i>Rats</i> .....	42
<i>Parasite</i> .....	42
<i>Histology</i> .....	43
<i>Sera and antibodies</i> .....	43
<i>ELISA</i> .....	44
<i>Passive immunization of rat pups</i> .....	44
<i>Statistical analyses</i> .....	45
Results .....	45
<i>Immunity induced by intravenous infection is indistinguishable from rapid expulsion.</i> .....	45
<i>Oral challenge of parenterally infected rats causes mast cell degranulation in the absence of mastocytosis.</i> .....	47
<i>Parenteral immunization generates a T cell-dependent mucosal response.</i> .....	49
<i>Mast cell degranulation is coincident with rapid expulsion in rat pups fostered by infected dams.</i> .....	49

<i>RMCP II release does not correlate with rapid expulsion in passively immunized rat pups.</i> .....	52
<i>RMCP II release is not sufficient to cause rapid expulsion in passively immunized adult rats</i> .....	52
Discussion.....	55
Acknowledgements .....	59
Disclosures.....	59
References .....	59
<b>CHAPTER 3: Intestinal infection with <i>Trichinella spiralis</i> induces distinct, regional immune responses</b> .....	<b>66</b>
Abstract.....	67
Introduction .....	67
Materials and Methods .....	67
<i>Animals</i> .....	67
<i>Parasite and infections</i> .....	68
<i>Tissue collection, preparation, and evaluation</i> .....	68
<i>Antibody treatment of rat pups</i> .....	68
<i>Cytokine Measurement</i> .....	69
<i>Statistical analysis</i> .....	69
Results .....	69
<i>Distribution of T. spiralis in the intestinal tract</i> .....	69
<i>Cytokine production and mast cell response</i> .....	71
<i>Enteropathy and the influence of IL-10</i> .....	71
<i>Protective immunity in the SI and LI</i> .....	74
Discussion.....	74
Acknowledgements .....	78
References .....	78
<b>CHAPTER 4: Epithelial disruption impacts intestinal immunity to <i>Trichinella spiralis</i>.</b> ..	<b>82</b>
Abstract.....	83
Introduction .....	83
Materials and Methods .....	85
<i>Mice and Rats</i> .....	85
<i>Parasite and infections</i> .....	86
<i>Culture of leukocytes and cytokine ELISA</i> .....	86
<i>Histology</i> .....	86

	<i>Flow cytometry</i> .....	87
	<i>TLR Transactivation Assay</i> .....	87
	<i>Antibiotic Treatment</i> .....	88
	<i>Quantitative PCR</i> .....	88
	<i>Invasion Assay</i> .....	88
	<i>Western Blotting</i> .....	89
	<i>Intestinal Epithelial Cell (IEC) Isolation</i> .....	89
	<i>Microarray</i> .....	90
	<i>Data Analysis</i> .....	90
	<i>Statistical Analysis</i> .....	91
Results	.....	91
	<i>MyD88 is required for efficient amplification of the Th2 cytokine response to infection.</i> .....	91
	<i>T. spiralis does not directly activate TLRs in vitro or in vivo</i> ....	93
	<i>ST2<sup>-/-</sup> mice have impaired Th2 immunity</i> .....	96
	<i>IL-33 localization is altered during infection</i> .....	96
	<i>IL-33 translocation does not occur in vitro</i> .....	99
	<i>Association of IL-33 with NF-<math>\kappa</math>B</i> .....	99
	<i>Gene expression microarray analysis of IECs</i> .....	102
Discussion	.....	104
Acknowledgements	.....	107
<b>CHAPTER 5: Summary and Conclusions</b> .....		<b>117</b>
References	.....	123
<b>APPENDIX A: Most highly altered genes in the murine epithelium during <i>T. spiralis</i> infection</b> .....		<b>128</b>
Upregulated Genes	.....	129
Downregulated Genes	.....	131

# List of Figures

<b>CHAPTER 1</b> .....	<b>1</b>
Figure 1.1: Intestinal immune response to <i>T. spiralis</i> .....	7
Figure 1.2: Regional differences in the rodent gastrointestinal tract.....	11
<b>CHAPTER 2</b> .....	<b>38</b>
FIGURE 2.1. Intestinal immunity induced by muscle infection. ....	45
FIGURE 2.2. Cellular and antibody responses to muscle infection. ....	47
FIGURE 2.3. Evaluation of intestinal immunity induced by muscle infection in nude rats. ....	49
FIGURE 2.4. Mucosal mast cell degranulation in rat pups born to infected dams.....	50
FIGURE 2.5. RMCPII release in rat pups passively immunized with parasite-specific monoclonal antibodies.....	52
FIGURE 2.6. RMCPII release in rat pups treated with fractions of serum from infected rats.....	53
FIGURE 2.7. RMCPII release in passively immunized adult rats. ....	55
<b>CHAPTER 3</b> .....	<b>65</b>
Figure 3.1: Colonization and expulsion of intestinal <i>T. spiralis</i> .....	70
Figure 3.2: Cytokine and mast cell responses in the SI and LI. ....	71
Figure 3.3: Enteropathy in C57Bl/6 and IL-10 <sup>-/-</sup> infected with <i>T. spiralis</i> .....	73
Figure 3.4: Cytokine response in the SI and LI of IL-10 <sup>-/-</sup> mice. ....	74
Figure 3.5: Protective immunity in the LI of suckling rats. ....	75
<b>CHAPTER 4</b> .....	<b>81</b>
Figure 4.1: Role of MyD88 in immunity to <i>T. spiralis</i> .....	92
Figure 4.2: Effect of the microbiota on Th2-mediated immunity.....	94
Figure 4.3: TLR response to <i>T. spiralis</i> <i>in vitro</i> and <i>in vivo</i> .....	95
Figure 4.4: Infection of ST2 <sup>-/-</sup> mice. ....	97
Figure 4.5: IL-33 mRNA and protein expression during <i>T. spiralis</i> infection. .	98
Figure 4.6: IL-33 localization in DLD-1 human colon carcinoma cells. ....	100
Figure 4.7: Location of NF-κB in IECs during infection. ....	101
Figure 4.8: Changes revealed by array analysis of NF-κB and TGF-β interaction networks.....	103
<b>CHAPTER 5</b> .....	<b>119</b>

# Chapter 1

## Introduction<sup>1</sup>

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<sup>1</sup> Sections of this chapter were taken from: Appleton JA, Blum LK, Gebreselassie NG Nematoda: *Trichinella*. In Lamb, TJ (Ed.), *Immunity to Parasitic Infections*. (In press) Oxford: Wiley-Blackwell.

## **Significance**

Intestinal helminths infect over 2 billion people worldwide, and immunity to these pathogens remains poorly understood (165). Secondary immunity to *Trichinella spiralis* involves a novel immune mechanism, in which the systemic antibody response is crucial to the expression of intestinal immunity (4). Understanding this mechanism may aid in the development of vaccines, which often aim to deliver antigen systemically in order to protect a mucosal surface. Furthermore, it has recently become clear that the absence of parasitic infection in many developed countries has secondary consequences, specifically by contributing to high rates of allergic and autoimmune diseases in these populations. This dissertation describes the induction and expression of intestinal immunity to different life stages of *T. spiralis*, in different regions of the host intestine, as well as the contributions of the microbiota, innate receptors, and the intestinal epithelium.

## **Trichinella**

*Trichinella* is a parasitic nematode in the class Adenophorea, or Clade I by the molecular classification (24). It is estimated that trichinellosis has a global incidence of 10,000 human cases per year, and it has been listed among ‘Europe’s neglected infections of poverty’ (35, 70). Geographically, *Trichinella* is widely distributed throughout the world, with different species occurring with higher prevalence in different host species and climates (48). The most widely studied species of *Trichinella* is *T. spiralis*, which is most commonly observed in cosmopolitan areas of Europe and Asia (48). *T. spiralis* infection occurs by oral ingestion of L<sub>1</sub> parasites that are present in the muscle tissue (meat) of the previous host. The L<sub>1</sub> are released by digestion in the stomach and activated by host gut contents (such as bile) before establishing their initial niche in the small intestine (SI). The larvae travel rapidly within the epithelium, occupying a ‘multi-intracellular’ habitat near the crypt-villus junction (168, 169). They then molt four times to mature into adult male and female worms, which mate within the intestine.

Four to five days after oral infection, adult females release newborn larvae (NBL), which exit the intestine into the bloodstream via the mesenteric vasculature. NBL migrate throughout the body, causing much of the pathology that is associated with *T. spiralis* infection, including liver, kidney, and CNS damage (48). Larvae that migrate to the skeletal muscle invade myotubes and establish a chronic infection.

Ultimately, an intestinal immune response expels the adult worms. L<sub>1</sub> initially invade the duodenum and move distally over time (132). The length of time that *T. spiralis* adults remain in the intestine varies greatly among host species. Worms are cleared from the intestines of otherwise healthy inbred mice within a period of two to three weeks, while they can persist in humans and swine for more than seven weeks (10, 48, 119).

### **Intestinal Immunity to *T. spiralis***

#### *Primary Immunity*

Clearance of adult worms from the small intestine is mediated by a potent Th2 response characterized by dramatic increases in the numbers of lymphocytes, eosinophils, goblet cells, and mucosal mast cells (10, 60, 76, 94). Blood and tissue eosinophilia are induced by interleukin (IL)-5, and local T cells and monocytes recruit eosinophils to the intestine by production of the chemokines CCL11 (eotaxin-1) and CCL24 (eotaxin-2) (46, 157). Despite the prominence of eosinophils in the intestinal immune response, they are not required for worm clearance (50).

Goblet cell hyperplasia during *T. spiralis* infection is dependent on IL-13 (44, 84, 106). Goblet cells synthesize and secrete mucins, and *T. spiralis* worms become entrapped in mucus during clearance of a challenge infection, although it is unclear whether this effect is causal (30). The production of several goblet cell-derived molecules is increased during infection, including mucin 2, mucin 3, mucin 5ac, trefoil factor 3 (TFF3), sialyl transferase 4c (Siat4c), intelectin-2, and resistin-like molecule- $\beta$  (RELM- $\beta$ ) (85). Infection of gene knockout mice has shown that mucin 5ac is necessary for efficient expulsion of *T. spiralis*, while RELM- $\beta$  is not required (65, 69).

Intestinal mastocytosis during infection is dependent on TNF- $\alpha$ , IL-4, IL-9, and IL-10 (51, 67, 72, 84, 138). Infection of mast cell-deficient rats and mice has revealed a role for mast cells in worm expulsion (64, 67, 149). Furthermore, mouse mast cell protease-1 (mMCP-1) and mast cell-derived IL-4 and TNF- $\alpha$  promote worm clearance (73, 86). The impact of these molecules is somewhat mild compared with the profound delay in expulsion observed in T cell-deficient mice, suggesting that mucosal mast cells and their mediators are part of a more complex mechanism (134). Evidence in support of this notion derives from the observation that mast cells contribute to the inductive phase of the immune response by promoting Th2 cytokine responses in the mesenteric lymph node (MLN) (73).

Clearance of adult worms is severely compromised in T cell-deficient mice, and adoptive transfer of lymphocytes reverses this phenotype (61, 134). The Th2 cytokines IL-4 and IL-13, which signal through a common receptor subunit (IL-4R $\alpha$ ), are required for efficient expulsion of adult worms (138). IL-4R $\alpha$  is present on many cell types, and expulsion is delayed in mice in which IL-4R $\alpha$  is absent from either bone marrow-derived or non-bone marrow-derived cells (154). Specifically, IL-4R $\alpha$  expression is not required on mast cells, CD4<sup>+</sup> T cells, or LysM<sup>+</sup> cells (macrophages and neutrophils); however, when IL-4R $\alpha$  ablation was targeted to intestinal epithelial cells (IECs), expulsion was delayed, supporting the conclusion that IL-4/IL-13 stimulate epithelial cells to promote worm rejection (69, 112). As IL-4R $\alpha$  signaling promotes goblet cell hyperplasia and RELM- $\beta$  production, it is likely that stimulation of goblet cells via IL-4R $\alpha$  also increases Muc5ac production, thereby promoting worm clearance (69).

### **Protection against reinfection**

The secondary immune response to *T. spiralis* varies among host species. Immune rats display a dramatic protective immunity in which 90% of intestinal larvae are cleared from the intestine within 30-60 minutes and 99% of larvae are cleared within 24 hours, a phenomenon that has been called rapid expulsion or rapid rejection (22, 33, 99). Secondary immunity in mice is much less robust; immune mice exhibit expulsion of adult worms that is accelerated by 2-7 days

(8, 9, 18). This immunity has not been thoroughly investigated. Secondary intestinal immunity in swine resembles that of mice, although swine also manifest strong immunity to NBL (102, 127).

In rats, tyvelose-specific antibodies play a key role in rapid expulsion and convey as much as 88% protection upon suckling rats (14). In adult rats, antibody alone is not sufficient, and either immune T cells or infection with a heterologous intestinal parasite can enable anti-tyvelose antibodies to effect expulsion (4, 20). Intestinal priming is not a strict requirement, as rats immunized with infections limited to the muscle phase produce anti-tyvelose IgG and display rapid expulsion (25).

Mucosal mast cells degranulate at the time of rapid expulsion in rats immunized with either oral or muscle infections, prompting speculation that mast cells are central to expulsion (25, 114, 166); however, recent evidence has shown that mucosal mast cell activation is neither sufficient nor required for rapid expulsion (Chapter 2, (25)). The mechanism that enables antibody-mediated protection in adult rats remains elusive.

The action of anti-tyvelose antibodies has been investigated in neonatal and adult rats, as well as in an *in vitro* model of epithelial cell infection. In the presence of monoclonal anti-tyvelose IgG, larvae are excluded from or encumbered in the epithelium (31, 108). *In vivo*, luminal larvae become entrapped in mucus, although entrapment is reversible (19, 31). All of these effects are directed solely at first-stage larvae (21). Following the first molt, larvae are no longer susceptible to the effects of anti-tyvelose IgG (13).

### **Immunopathology**

Many studies have differentiated protective from pathologic responses in the intestine during *T. spiralis* infection. *T. spiralis* enteropathy includes villous atrophy, crypt hyperplasia, edema, mastocytosis, and an increase in myeloperoxidase activity, which is associated with neutrophil infiltration (94-96). Furthermore, pathologic changes in smooth muscle function are induced by *Trichinella* infection, and these changes persist after worm clearance, in a syndrome that is referred to as post-infectious gut dysfunction (17, 34, 103).

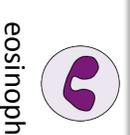
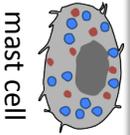
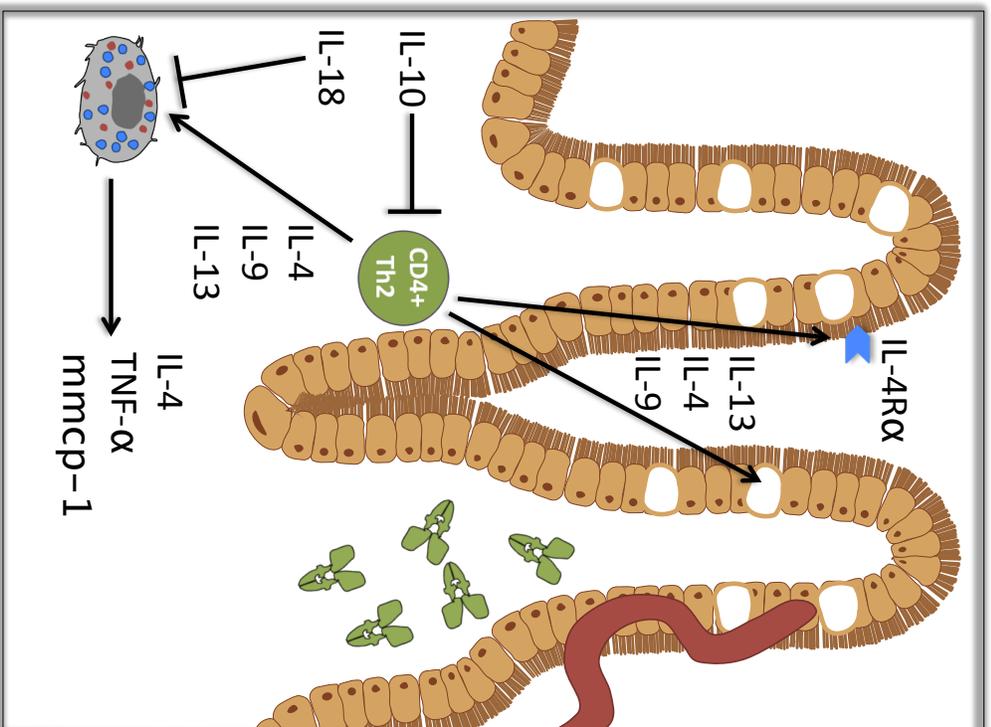
TNF- $\alpha$  drives much of the enteropathy caused by *Trichinella* infection, promoting villous atrophy, crypt hyperplasia and intestinal mastocytosis (72, 73). NO also plays a role, as infected iNOS-deficient mice display no significant pathologic changes (95). Furthermore, Th2 cytokines are essential for pathogenesis: IL-4 production is required for crypt hyperplasia while IL-13 mediates villous atrophy (as well as edema) (137). The cellular targets of IL-4 and IL-13 include epithelial cells, but not T lymphocytes, macrophages, or neutrophils, as specific deletion of IL-4R $\alpha$  on the latter cells has no effect on enteropathy (112). Innate cells are important sources of Th2 cytokines during *T. spiralis* infection. In fact, mast cell production of IL-4 (as well as TNF- $\alpha$  and mMCP-1) is required for enteropathy, whereas intraepithelial NK cells are believed to provide a potent, innate source of IL-13 (73, 106). Indeed, non-T cell derived IL-13 is sufficient to induce villous atrophy, crypt hyperplasia, and an increase in the number of goblet cells in immunodeficient SCID or nude (T cell deficient) mice (106); however, the recent discovery of type 2 innate cell populations, such as nuocytes, will prompt a reevaluation of the IL-13-producing cells in *T. spiralis*-infected immunodeficient mice.

Figure 1.1 provides a comparison of protective and pathologic immune mechanisms during *T. spiralis* infection.

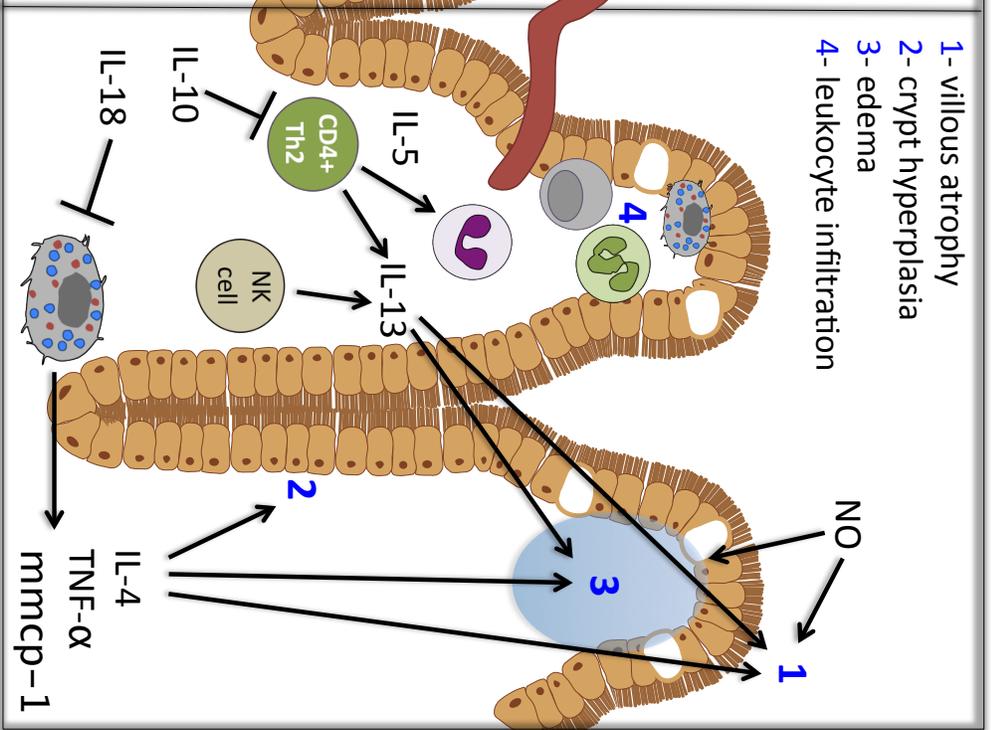
### **Intestinal Habitat of the Parasite**

During intestinal colonization and expulsion, *T. spiralis* ultimately passes through every region of the rodent intestine, and these sites vary greatly with regard to morphology, physiology, and immunology. The intestinal epithelium represents the largest surface in the body of the animal, with the length alone reaching three times the height of the animal, and with a surface area as great as 45,000 mm<sup>2</sup> for a rat (400 m<sup>2</sup> for a human) (43, 83). The gastrointestinal tract of rats and mice is composed of the stomach, small intestine (SI), cecum, and large intestine (LI). Unlike humans, they do not possess an appendix. The SI is divided into three sections, the duodenum, jejunum, and ileum (listed in proximal to distal order). The duodenum can be visibly distinguished from the other two sections because it is proximal to the Ligament of Treitz.

(A) Protective Response



(B) Pathologic Response



**Figure 1.1: Intestinal immune response to *T. spiralis*. (A) Mechanisms required for primary clearance of adult worms.** Parasites can survive for more than 80 days in the absence of Th2 cells. Expulsion is subtly delayed in the absence of IL-4R $\alpha$  on both epithelial cells and bone-marrow derived cells. Efficient expulsion also requires the induction of mast cells via Th2 cell-derived cytokines and mast cell production of IL-4, TNF- $\alpha$ , and mMCP-1. Goblet cells and parasite-specific antibodies also contribute to clearance. **(B) Mechanisms of enteropathy during *T. spiralis* infection.** (1) Villous atrophy is dependent on IL-13, NO, and mast cell production of IL-4, TNF- $\alpha$ , and mmcp-1. (2) Crypt hyperplasia is also dependent on mast cell products. (3) Edema is dependent on IL-13, NO, and mast cell products. (4) Leukocyte infiltration is induced by chemokines and Th2 cytokines. Tissue eosinophilia, driven by Th2 cell-derived IL-5, is shown. Th2 and NK cells are the primary sources of IL-13.

Differentiation between the jejunum and ileum is best done microscopically. The villi of the jejunum are taller than those of the ileum, and the ileum has a greater number of paneth cells (41). The crypt-to-villus ratio in the proximal duodenum is 14, while the ratio in the ileum is 6 (170). The large intestine does not contain villi, but rather a hexagonal-shaped cuff of surface epithelial cells that surrounds the opening of each crypt (gland) (139). The ileum also has a greater number of goblet cells than the duodenum or jejunum (41). The greatest proportion of goblet cells is found in the LI, although this site does not contain paneth cells under normal conditions (41).

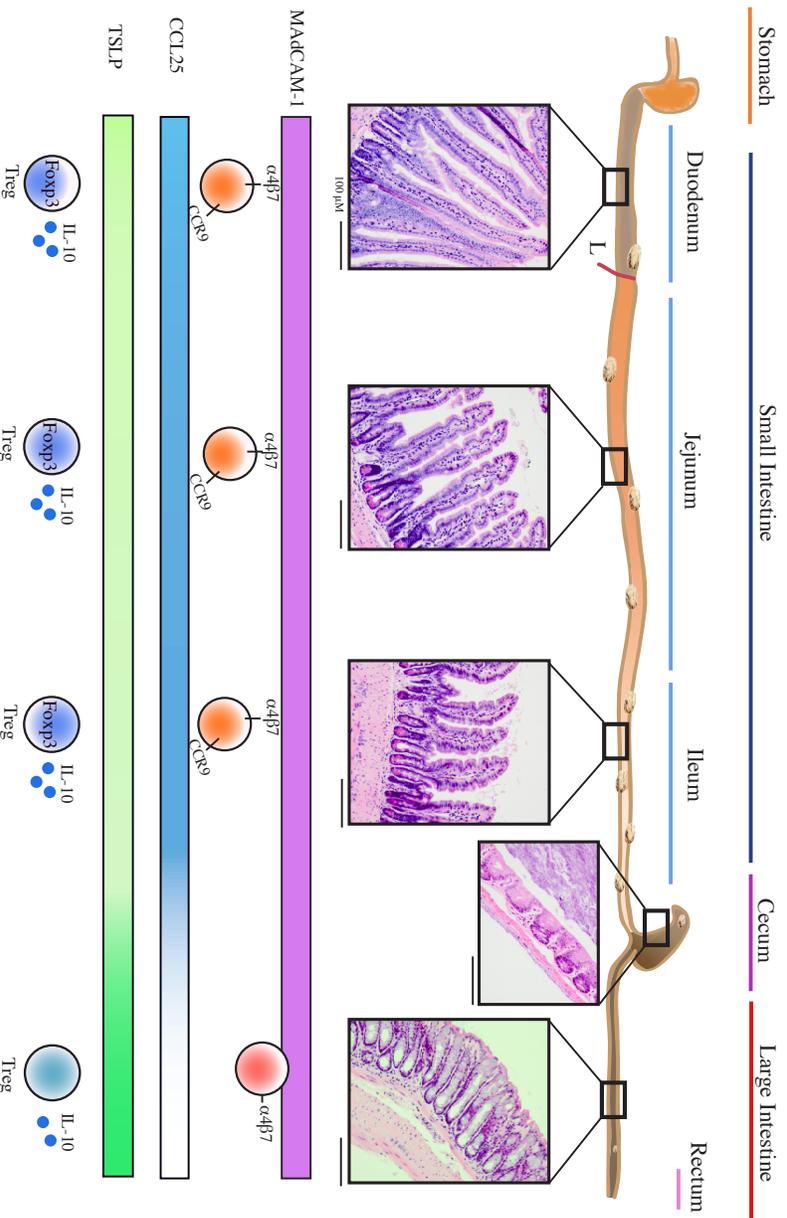
Regional differences in gene regulation exist between the jejunum and ileum. The transcription factors *Gata4* and *Hnf1 $\alpha$*  are highly expressed in the jejunum but not the ileum (26, 27). Genes that define the phenotype of the duodenum are regulated by *Pdx-1* (40, 63). The results of differential expression of these transcription factors along the cranial-caudal axis of the intestine are that enterocyte genes that are responsible for digestion and absorption of nutrients are most highly expressed in the duodenum and jejunum, while those that mediate absorption of bile salts and intrinsic factor-bound  $B_{12}$  are expressed in the ileum (26, 27, 40).

In addition to physiological and architectural differences among different regions of the intestine, the structural features of associated lymphoid tissues and the regulation of immunity varies by site. Peyer's patches are present in the SI, with the greatest number occurring in the ileum, while smaller cryptopatches (also called colonic lymphoid follicles) are present in the LI (123). Lymphatic drainage is regional, and the chain of MLN drains all sections of the intestine. However, lymphocytes are differentially recruited to the SI and LI, based on addressin and chemokine expression in each site. While MAdCAM-1 is displayed by endothelia throughout the intestine and recruits lymphocytes to all compartments, CCL25/TECK is mainly produced in the SI where it attracts CCR9+ lymphocytes (59). Priming in the presence of retinoic acid (RA) directs activated T cells to traffic to the SI (by inducing  $\alpha 4\beta 7$  and CCR9 expression) (77, 107). Although migration to the large intestine also depends on  $\alpha 4\beta 7$ , RA is neither necessary nor sufficient to promote homing to the LI (115).

Evidence derived from a variety of disease conditions confirms that the SI and LI constitute distinct immune environments. Reports describe variation in regulatory T cell phenotypes and in the production of IL-10 and TGF- $\beta$  (15, 105). Specifically, Foxp3+IL-10+ T cells play a predominant role in controlling inflammation in the SI, while Foxp3-IL-10+ T cells are of primary importance in regulating LI inflammation (105). NADPH oxidase 1 (NOX1), which catalyzes the production of reactive oxygen species, is preferentially expressed in the LI where it is modulated by IL-10 (80, 131). Furthermore, the epithelial-derived Th2 cytokine TSLP is more highly expressed in the proximal LI than in the cecum or distal SI (150). The expression of individual toll-like receptors (TLRs) also differs between the SI and LI (reviewed in (1)). For example, TLR5 is expressed on the basolateral surface of IECs in the ileum and colon, but on the apical surface in the follicle-associated epithelium of the SI (37, 55, 130). Contrasts in immune function are evident in two different inflammatory bowel diseases (IBD) in humans. Ulcerative colitis is a Th2-mediated disease of the LI (75). Conversely, a mixed Th1/Th17 response has been implicated in the etiology of Crohn's disease, which mainly affects the distal ileum of the SI (54, 125). It is unclear how differences in the affected intestinal sites contribute to differences in the induction and manifestation of each disease. Figure 1.2 illustrates relevant differences among the regions of the rodent intestinal tract.

### **PAMPs and DAMPs**

One of the major tenets of immunology is the differentiation between 'self' and 'non-self', which can be modernized as differentiation between 'non-danger' and 'danger'. Charles Janeway proposed that conserved molecular patterns alert the immune system to the presence of a non-self pathogen (78). These molecules were termed pathogen-associated molecular patterns (PAMPs), and the Nobel Prize was recently awarded to Bruce Buetler and Jules Hoffman for their elucidation of the identity and interaction between the bacterial PAMP lipopolysaccharide (LPS) and its immune receptor, TLR4. Further studies have identified 12 mouse and 10 human TLRs, which sense ligands ranging from viral RNA to profilin in



**Figure 1.2: Regional differences in the rodent gastrointestinal tract.** Colored bars denote the segments of the intestine (Stomach, SI, Cecum, and LI), the subsections of the SI (duodenum, jejunum, and ileum), and the rectum. L (red line) indicates the separation of the duodenum by the Ligament of Treitz. Micrographs illustrate the morphology of the intestinal architecture in each region. Colored rectangles show the relative expression of MACdCAM-1, CCL25, and TSLP along the length of the intestine. Round cells indicate the presence of CCR9+ $\alpha$ 4 $\beta$ 7+ lymphocytes in the SI and CCR9- $\alpha$ 4 $\beta$ 7+ in the LI, as well as Foxp3+IL-10+ Tregs in the SI and Foxp3-IL-10+ Tregs in the LI.

*Toxoplasma gondii* (reviewed in (81)). Intracellular pathogens are also sensed by nod-like receptors (NLRs) and rig-like receptors (RLRs) (reviewed in (98, 141)). In the intestine, dendritic cells (DCs) with high and low TLR expression are both present, but neither population responds strongly to activation by the microbiota under normal conditions (36, 113). The mechanisms that induce and maintain this tolerogenic phenotype are not well understood. IECs in the villous epithelium of the SI do not highly express TLR5, and IECs in the LI express TLR5 only on the basolateral surface of the cell, limiting activation to situations where the epithelium has been disrupted, or the microbiota have translocated across the epithelium (55). TLR3, 7, 8, and 9 are intracellular, and IECs do not express high levels of TLR2 or TLR4 under homeostatic conditions (2, 109, 110).

In addition to sensing exogenous threats (pathogens), dangerous states within the host are sensed by the detection of so-called danger associated molecular patterns (DAMPs). DAMPs are much less well understood than PAMPs, but this is an area of active research. At this time, the DAMP superfamily includes debris produced from the extracellular matrix, such as hyaluronan fragments, heat shock proteins, S100 family proteins, uric acid, IL-1 $\alpha$ , IL-33, and the canonical DAMP, high mobility group box protein 1 (HMGB1) (49, 52, 89, 118, 124, 151, 155, 156, 164). HMGB1 is a nuclear-associated protein that is released (bound to DNA or nucleosomes) by necrotic cells and activates Th1 inflammation via the receptor for advanced glycation end products (RAGE), TLR2, and TLR4 (124, 171). DAMPs, which are also referred to as ‘alarmins’ or ‘endokines’, are involved in the pathology associated with sterile inflammatory conditions such as myocardial infarction and gout, but their role in infectious diseases where a pathogen causes tissue necrosis is not well known (reviewed in (144)).

### **Induction and Amplification of Th2 Immunity**

Despite the prodigious increase in the past 20 years in our understanding of how Th1 responses are induced, the innate mediators responsible for Th2 responses remain poorly defined. Although there appear to be context-dependent differences in the mechanisms

of protective immunity between *T. spiralis* and *S. mansoni* or *T. muris*, observations from these related helminths may provide insight into the potential pathways of activation during *T. spiralis* infection. DCs play a critical role in driving the direction of an immune response; when DCs are highly activated by bacterial or viral stimuli, they drive a strong Th1 cell response. Th2-inducing DCs are considered to be in a lower state of activation (100). Currently, the major innate pathways that are known to promote a Th2 response are TLRs, C-type lectin receptors (CLRs), notch signaling, and protease activation of basophils. Newly identified innate cell populations also initiate Th2 immunity.

Among the helminths, several molecules have been identified that influence DC activation. Soluble egg antigen (SEA) from *S. mansoni* contains lacto-N-fucopentaose III that activates MAPK signaling via TLR4, while schistosome lysophosphatidylserine (lyso-PS) induces a Th2 phenotype via TLR2 (152, 158). The protein ES-62 from the filarial nematode *Acanthocheilonema vitae* can bind to TLR4 in a manner that leads to the suppression, rather than induction, of IL-12 production (57, 58). However, TLR activation suppresses the Th2 response during *T. muris* infection, as Th2 immunity is diminished in MyD88<sup>-/-</sup> and TLR4 mutant C3H/HeJ mice (68). The role of TLRs and MyD88 signaling during *T. spiralis* infection has not been reported previously.

Helminths are known to produce and secrete large quantities of immunogenic glycoproteins (reviewed in (159)). These motifs can be recognized by the calcium-dependent carbohydrate binding protein family of receptors called C-type lectin receptors (CLRs). This family includes DC-SIGN (human CD209) and its murine homologs, as well as intelectins and the mannose receptor family. These receptors bind to carbohydrate moieties and contribute to Th2 immunity, although their mechanism of action requires further study. *S. mansoni* products bind to the mannose receptor on macrophages (97); glycan microarray profiling in humans showed that macrophage galactose-type lectin (MGL, CD301) on DCs bound to specific glycan moieties found in *S. mansoni* eggs, and human DC-SIGN binds to whole *S. mansoni* cercariae (111, 160, 161). Once bound, glycan-lectin complexes are internalized, and activation of TLRs

is suppressed (160). The impact of these binding events in the context of infection is less clear. For example, although *S. mansoni* products activated the murine DC-SIGN homolog SIGNR1 *in vitro*, SIGNR1-deficient mice did not have an altered immune response (136). In addition, *T. muris* ES products bound to the mannose receptor (MR, CD206) on macrophages, and binding induced cytokine production, but MR<sup>-/-</sup> mice had normal clearance of the parasite (45). It has been proposed that carbohydrate binding to host CLRs is a mechanism of immune suppression by helminths, rather than activation (159). Mannan-binding lectin (MBL) does bind to *T. spiralis* muscle larvae *in vivo*, although the downstream effects of MBL binding are not clear (62).

Notch signaling is an evolutionarily conserved pathway for cell-to-cell communication that is important in many types of cell fate determination. Notch receptors are present on many cells of the immune system, including DCs, lymphocytes, and macrophages (3, 101, 121). Vertebrates have four notch receptors (Notch1-4), which can be bound by five different ligands (Delta-1, -3, and -4 and Jagged-1 and -2)(28). Stimulation of T cells or antigen presenting cells (APCs) in the presence of the delta ligands induces a Th1 phenotype, while stimulation in the presence of the jagged ligands induces a Th2 phenotype (12, 101). LPS induces the expression of delta family ligands in a MyD88-dependent manner, while Th2 stimuli such as PGE<sub>2</sub> induce a rapid increase in the expression of the jagged family (12). Mice in which notch signaling was conditionally inactivated in T cells developed a non-protective Th1 response during *T. muris* infection (153). It is believed that notch signaling contributes to Th2 differentiation in response to SEA, although contradictory results have been reported regarding the requirement for individual jagged ligands (56, 90, 167). It is likely that jagged1 and jagged2 play redundant roles during *S. mansoni* infection, and that knockout of both molecules is required for a deficiency in Th2 induction. Interestingly, IL-33 decreases the expression of both delta and jagged ligands during experimentally induced dextran sodium sulfate (DSS) colitis (74).

It was recently reported that microRNAs play a role in the induction of intestinal Th2 responses. During *T. muris* infection, IL-13 induces miR-375, which regulates the expression of TSLP, an epithelial-derived cytokine that amplifies Th2 responses (23). TSLP can potentiate the

response of basophils, a cell type known to promote induction of Th2 responses (143). Basophils respond to protease allergens, such as papain, by upregulating MHC II expression and migrating to the draining lymph node, where they provide an early source of IL-4 (146, 147). Exposure to schistosome eggs elicits a robust recruitment of MHC II<sup>+</sup> basophils to the draining lymph node, and depletion of basophils impairs Th2 cytokine-mediated expulsion of *T. muris* (126). Basophils are also increased by up to 10-fold in the MLN and spleens of mice infected with *T. spiralis* (Gebreselassie and Appleton, in preparation).

Previously unrecognized innate cell populations have recently been described as crucial to the initiation of Th2 responses. It is unclear whether natural helper cells, nuocytes, multi-potent progenitor type 2 (MPP<sup>type2</sup>) cells, and innate helper 2 (Ih2) cells, which were independently reported by four research groups, are the same or distinct populations (117, 120, 128, 135). These cells are induced by the tissue-derived cytokines IL-25 and IL-33 to potentiate the Th2 cytokine response, particularly IL-5 and IL-13 (117, 120, 128, 135). In addition, they share a surface phenotype that is negative for lineage markers (T, B, NK, macrophage, DC, neutrophil, and LTI markers), negative for FcεRI, CCR3, and CD49b, yet positive for c-kit (117, 120, 128, 135). MPP<sup>type2</sup> cells are preferentially activated by IL-25 and do not express ST2, the receptor for IL-33, while the other three populations can respond to both IL-25 and IL-33 (117, 120, 128, 135). MPP<sup>type2</sup> cells, nuocytes, and Ih2 cells have all been documented to be active in Th2 immunity during helminth infections (117, 120, 128, 135).

### **Interleukin-33**

IL-33 (IL-1F11) is an IL-1 family cytokine that contains the classic β trefoil structure found in this family (140). It was identified as the ligand for the orphan-receptor ST2, and was originally called nuclear factor of high endothelial venules (NF-HEV) (16, 140). IL-33 is predominantly expressed in cells that are exposed to the external environment, such as the skin, intestine, and lung, but it is also present at crucial sites in the body such as the brain, heart, and airway smooth muscle (118). In addition, IL-33 mRNA is expressed by activated mast

cells, DCs, and macrophages (122). Although it was originally described as being processed by caspase-1 into a cleaved active form, subsequent studies have shown that full-length (30 kDa) IL-33 is biologically active (7). Furthermore, it is cleaved by caspase-3 and -7 during apoptosis, which leads to inactivation of the ST2-dependent functions of IL-33 (7). It is believed that IL-33 is released in its active form by necrotic cells and is inactivated during apoptosis.

The paracrine effects of IL-33 are mediated via its receptor, ST2, which is expressed on a wide variety of leukocytes, including mast cells, basophils, eosinophils, neutrophils, Th2 cells, NK cells, alternatively activated macrophages, and nuocytes/natural helper cells (42, 93, 116, 128, 129, 140, 145). The receptor complex includes ST2 and the IL-1 receptor accessory protein, IL-1RAcP (38). Binding of IL-33 to ST2 leads to activation of NF- $\kappa$ B via MyD88, IRAK-1, and TRAF6 (5, 53, 91, 140). In mast cells, IL-33 receptor signaling also synergizes with activation of c-kit, and to a lesser extent Fc $\epsilon$ RI to amplify cytokine production and degranulation, respectively (47, 142). The ST2-IL-33 axis is regulated by a soluble alternative splice variant of ST2, sST2, which blocks activation of the functional receptor (66). Intracellularly, single Ig IL-1R-related molecule (SIGIRR) acts as a negative regulator of ST2 signaling (29).

IL-33 demonstrates biological functions in the mucosa that include an increase in Th2 cytokine expression, goblet cell hyperplasia, airway hyper-responsiveness, neutrophil and Th2 cell migration, and activation of eosinophils (11, 71, 82, 87, 88, 140, 148, 163). IL-33 is highly expressed in the intestines of patients with UC, and it was found to be crucial in the Samp1/YitFc mouse model of IBD. Expression of IL-33 was increased at a single early time point (day 3) of *T. muris* infection, and injection of recombinant IL-33 promoted intestinal parasite clearance (71). In addition to its role in Th2 immunity, IL-33 is also influential in *Toxoplasma* encephalitis, inflammatory hypernociception, and *Influenza* virus infection (39, 79, 162). More work is needed to determine the precise mechanisms by which ST2 induces the amplification of immune responses under different conditions, such as Th1 or Th2 inflammation, as well as whether IL-33 influences Th17-mediated immunity.

In addition to its role as a paracrine cytokine, IL-33 also carries out an

intracellular signaling mechanism, which is not fully understood (32). In the endothelia of certain tissues, IL-33 is constitutively located in the nucleus, where it binds to the histone H2A/H2B acidic pocket, and is released in response to inflammatory stimuli (92, 133). Although the function of this shift has not been extensively studied, nuclear translocation was associated with reduced expression of a Gal4 reporter gene, and was proposed as a mechanism for controlling proliferation in pancreatic stellate cells (32, 104). Many of the studies regarding IL-33 nuclear translocation have been carried out in transfected cell lines, which may not be an accurate model of the natural functions of nuclear IL-33. It was recently reported that nuclear IL-33 binds to NF- $\kappa$ B and prevents it from acting as a transcriptional activator, but the significance of NF- $\kappa$ B inhibition by IL-33 has not yet been investigated *in vivo* (6). Further work is needed to dissect the pleiotropic functions of IL-33 during intestinal helminth infection.

### Synthesis

The results presented in this dissertation describe the role of mast cells in protective secondary immunity, differences in protection and pathology among different regions of the intestine, and the contribution of innate mediators in initiating the Th2 response to infection. Primary infection with *T. spiralis* induces many cellular and physical changes in the rodent intestine. Some of these changes contribute to protective immunity, while others are merely pathologic. The study reported in Chapter 2 shows that although mucosal mast cells dramatically increase in number during natural infection of rats, mastocytosis and mucosal mast cell degranulation are not required for protective immunity. We also report that the mechanisms of primary immunity may differ among different regions of the intestine, and that pathology induced by infection is regulated differently in the different sites (Chapter 3). These results fit well with known differences in the immune milieus between the SI and LI; in particular, cytokine production during infection was parallel between the jejunum and ileum (but not the LI), which fits with the specific re-circulation of CCR9<sup>+</sup> leukocytes within the SI. The mechanisms by which Th2 immune responses are induced in the intestine are not fully understood. However, it

is known that *T. spiralis* causes physical disruption of the intestinal epithelium during infection. The results presented in Chapter 4 illustrate dramatic changes that occur in the intestinal epithelium during infection, and the mechanisms by which those changes promote a robust Th2 response.

### References

1. **Abreu, M. T. 2010.** Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nature reviews. Immunology* 10:131-144.
2. **Abreu, M. T., P. Vora, E. Faure, L. S. Thomas, E. T. Arnold, and M. Arditi. 2001.** Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. *Journal of immunology* 167:1609-1616.
3. **Adler, S. H., E. Chiffoleau, L. Xu, N. M. Dalton, J. M. Burg, A. D. Wells, M. S. Wolfe, L. A. Turka, and W. S. Pear. 2003.** Notch signaling augments T cell responsiveness by enhancing CD25 expression. *Journal of immunology* 171:2896-2903.
4. **Ahmad, A., C. H. Wang, M. Korenaga, R. G. Bell, and L. S. Adams. 1990.** Synergistic interaction between immune serum and thoracic duct cells in the adoptive transfer of rapid expulsion of *Trichinella spiralis* in adult rats. *Exp Parasitol* 71:90-99.
5. **Ali, S., M. Huber, C. Kollwe, S. C. Bischoff, W. Falk, and M. U. Martin. 2007.** IL-1 receptor accessory protein is essential for IL-33-induced activation of T lymphocytes and mast cells. *Proceedings of the National Academy of Sciences of the United States of America* 104:18660-18665.
6. **Ali, S., A. Mohs, M. Thomas, J. Klare, R. Ross, M. L. Schmitz, and M. U. Martin. 2011.** The dual function cytokine IL-33 interacts with the transcription factor NF-kappaB to dampen NF-kappaB-stimulated gene transcription. *Journal of immunology* 187:1609-1616.
7. **Ali, S., D. Q. Nguyen, W. Falk, and M. U. Martin. 2010.** Caspase 3 inactivates

- biologically active full length interleukin-33 as a classical cytokine but does not prohibit nuclear translocation. *Biochem Biophys Res Commun* 391:1512-1516.
8. **Alizadeh, H., and K. D. Murrell.** 1984. The intestinal mast cell response to *Trichinella spiralis* infection in mast cell-deficient w/wv mice. *The Journal of Parasitology* 70:767-773.
  9. **Alizadeh, H., and D. Wakelin.** 1982. Comparison of rapid expulsion of *Trichinella spiralis* in mice and rats. *International journal for parasitology* 12:65-73.
  10. **Alizadeh, H., and D. Wakelin.** 1982. Comparison of rapid expulsion of *Trichinella spiralis* in mice and rats. *Int J Parasitol* 12:65-73.
  11. **Alves-Filho, J. C., F. Sonogo, F. O. Souto, A. Freitas, W. A. Verri, Jr., M. Auxiliadora-Martins, A. Basile-Filho, A. N. McKenzie, D. Xu, F. Q. Cunha, and F. Y. Liew.** 2010. Interleukin-33 attenuates sepsis by enhancing neutrophil influx to the site of infection. *Nat Med* 16:708-712.
  12. **Amsen, D., J. M. Blander, G. R. Lee, K. Tanigaki, T. Honjo, and R. A. Flavell.** 2004. Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. *Cell* 117:515-526.
  13. **Appleton, J. A., and D. D. McGregor.** 1985. Life-phase specific induction and expression of rapid expulsion in rats suckling *Trichinella spiralis*-infected dams. *Immunology* 55:225-232.
  14. **Appleton, J. A., L. R. Schain, and D. D. McGregor.** 1988. Rapid expulsion of *Trichinella spiralis* in suckling rats: mediation by monoclonal antibodies. *Immunology* 65:487-492.
  15. **Autenrieth, I. B., N. Bucheler, E. Bohn, G. Heinze, and I. Horak.** 1997. Cytokine mRNA expression in intestinal tissue of interleukin-2 deficient mice with bowel inflammation. *Gut* 41:793-800.
  16. **Baekkevold, E. S., M. Roussigne, T. Yamanaka, F. E. Johansen, F. L. Jahnsen, F. Amalric, P. Brandtzaeg, M. Erard, G. Haraldsen, and J. P. Girard.** 2003. Molecular

- characterization of NF-HEV, a nuclear factor preferentially expressed in human high endothelial venules. *The American journal of pathology* 163:69-79.
17. **Barbara, G., B. A. Vallance, and S. M. Collins.** 1997. Persistent intestinal neuromuscular dysfunction after acute nematode infection in mice. *Gastroenterology* 113:1224-1232.
  18. **Bell, R. G.** 1992. *Trichinella spiralis*: evidence that mice do not express rapid expulsion. *Exp Parasitol* 74:417-430.
  19. **Bell, R. G., L. S. Adams, and R. W. Ogden.** 1984. Intestinal mucus trapping in the rapid expulsion of *Trichinella spiralis* by rats: induction and expression analyzed by quantitative worm recovery. *Infect Immun* 45:267-272.
  20. **Bell, R. G., J. A. Appleton, D. A. Negrao-Correa, and L. S. Adams.** 1992. Rapid expulsion of *Trichinella spiralis* in adult rats mediated by monoclonal antibodies of distinct IgG isotypes. *Immunology* 75:520-527.
  21. **Bell, R. G., and D. D. McGregor.** 1980. Requirement for two discrete stimuli for induction of the intestinal rapid expulsion response against *Trichinella spiralis* in rats. *Infect Immun* 29:186-193.
  22. **Bell, R. G., and D. D. McGregor.** 1979. *Trichinella spiralis*: expression of rapid expulsion in rats exposed to an abbreviated enteral infection. *Exp Parasitol* 48:42-50.
  23. **Biton, M., A. Levin, M. Slyper, I. Alkalay, E. Horwitz, H. Mor, S. Kredo-Russo, T. Avnit-Sagi, G. Cojocar, F. Zreik, Z. Bentwich, M. N. Poy, D. Artis, M. D. Walker, E. Hornstein, E. Pikarsky, and Y. Ben-Neriah.** 2011. Epithelial microRNAs regulate gut mucosal immunity via epithelium-T cell crosstalk. *Nature immunology* 12:239-246.
  24. **Blaxter, M. L., P. De Ley, J. R. Garey, L. X. Liu, P. Scheldeman, A. Vierstraete, J. R. Vanfleteren, L. Y. Mackey, M. Dorris, L. M. Frisse, J. T. Vida, and W. K. Thomas.** 1998. A molecular evolutionary framework for the phylum Nematoda. *Nature* 392:71-75.
  25. **Blum, L. K., S. M. Thrasher, L. F. Gagliardo, V. Fabre, and J. A. Appleton.** 2009. Expulsion of secondary *Trichinella spiralis* infection in rats occurs independently of

- mucosal mast cell release of mast cell protease II. *J Immunol* 183:5816-5822.
26. **Bosse, T., J. J. Fialkovich, C. M. Piaseckyj, E. Beuling, H. Broekman, R. J. Grand, R. K. Montgomery, and S. D. Krasinski.** 2007. Gata4 and Hnf1 alpha are partially required for the expression of specific intestinal genes during development. *American journal of physiology. Gastrointestinal and liver physiology* 292:G1302-1314.
  27. **Bosse, T., C. M. Piaseckyj, E. Burghard, J. J. Fialkovich, S. Rajagopal, W. T. Pu, and S. D. Krasinski.** 2006. Gata4 is essential for the maintenance of jejunal-ileal identities in the adult mouse small intestine. *Mol Cell Biol* 26:9060-9070.
  28. **Bray, S. J.** 2006. Notch signalling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol* 7:678-689.
  29. **Bulek, K., S. Swaidani, J. Qin, Y. Lu, M. F. Gulen, T. Herjan, B. Min, R. A. Kastelein, M. Aronica, M. Kosz-Vnenchak, and X. Li.** 2009. The essential role of single Ig IL-1 receptor-related molecule/Toll IL-1R8 in regulation of Th2 immune response. *Journal of immunology* 182:2601-2609.
  30. **Carlisle, M. S., D. D. McGregor, and J. A. Appleton.** 1991. Intestinal mucus entrapment of *Trichinella spiralis* larvae induced by specific antibodies. *Immunology* 74:546-551.
  31. **Carlisle, M. S., D. D. McGregor, and J. A. Appleton.** 1990. The role of mucus in antibody-mediated rapid expulsion of *Trichinella spiralis* in suckling rats. *Immunology* 70:126-132.
  32. **Carriere, V., L. Roussel, N. Ortega, D. A. Lacorre, L. Americh, L. Aguilar, G. Bouche, and J. P. Girard.** 2007. IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. *Proceedings of the National Academy of Sciences of the United States of America* 104:282-287.
  33. **Castro, G. A., F. Badial-Aceves, P. R. Adams, E. M. Copeland, and S. J. Dudrick.** 1976. Response to immunized, parenterally nourished rats to challenge infection with the nematode, *Trichinella spiralis*. *J Nutr* 106:1484-1491.

34. **Castro, G. A., F. Badial-Aceves, J. W. Smith, S. J. Dudrick, and N. W. Weisbrodt.** 1976. Altered small bowel propulsion associated with parasitism. *Gastroenterology* 71:620-625.
35. **Centers for Disease Control and Prevention, C.** 2010, posting date. Parasites - Trichinellosis (also known as Trichinosis). [Online.]
36. **Cerovic, V., C. D. Jenkins, A. G. Barnes, S. W. Milling, G. G. MacPherson, and L. S. Klavinskis.** 2009. Hyporesponsiveness of intestinal dendritic cells to TLR stimulation is limited to TLR4. *Journal of immunology* 182:2405-2415.
37. **Chabot, S., J. S. Wagner, S. Farrant, and M. R. Neutra.** 2006. TLRs regulate the gatekeeping functions of the intestinal follicle-associated epithelium. *Journal of immunology* 176:4275-4283.
38. **Chackerian, A. A., E. R. Oldham, E. E. Murphy, J. Schmitz, S. Pflanz, and R. A. Kastelein.** 2007. IL-1 receptor accessory protein and ST2 comprise the IL-33 receptor complex. *Journal of immunology* 179:2551-2555.
39. **Chang, Y. J., H. Y. Kim, L. A. Albacker, N. Baumgarth, A. N. McKenzie, D. E. Smith, R. H. Dekruyff, and D. T. Umetsu.** 2011. Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. *Nature immunology* 12:631-638.
40. **Chen, C., R. Fang, C. Davis, C. Maravelias, and E. Sibley.** 2009. Pdx1 inactivation restricted to the intestinal epithelium in mice alters duodenal gene expression in enterocytes and enteroendocrine cells. *American journal of physiology. Gastrointestinal and liver physiology* 297:G1126-1137.
41. **Cheng, H., and M. Bjerknes.** 1982. Whole population cell kinetics of mouse duodenal, jejunal, ileal, and colonic epithelia as determined by radioautography and flow cytometry. *Anat Rec* 203:251-264.
42. **Cherry, W. B., J. Yoon, K. R. Bartemes, K. Iijima, and H. Kita.** 2008. A novel IL-1 family cytokine, IL-33, potently activates human eosinophils. *The Journal of allergy and*

- clinical immunology 121:1484-1490.
43. **Childers, N. K., M. G. Bruce, and J. R. McGhee.** 1989. Molecular mechanisms of immunoglobulin A defense. *Annu Rev Microbiol* 43:503-536.
  44. **Dehlawi, M. S., Y. R. Mahida, K. Hughes, and D. Wakelin.** 2006. Effects of *Trichinella spiralis* infection on intestinal pathology in mice lacking interleukin-4 (IL-4) or intestinal trefoil factor (ITF/TFF3). *Parasitology international* 55:207-211.
  45. **deSchoolmeester, M. L., L. Martinez-Pomares, S. Gordon, and K. J. Else.** 2009. The mannose receptor binds *Trichuris muris* excretory/secretory proteins but is not essential for protective immunity. *Immunology* 126:246-255.
  46. **Dixon, H., C. Blanchard, M. L. Deschoolmeester, N. C. Yuill, J. W. Christie, M. E. Rothenberg, and K. J. Else.** 2006. The role of Th2 cytokines, chemokines and parasite products in eosinophil recruitment to the gastrointestinal mucosa during helminth infection. *European journal of immunology* 36:1753-1763.
  47. **Drube, S., S. Heink, S. Walter, T. Lohn, M. Grusser, A. Gerbault, L. Berod, J. Schons, A. Dudeck, J. Freitag, S. Grotha, D. Reich, O. Rudeschko, J. Norgauer, K. Hartmann, A. Roers, and T. Kamradt.** 2010. The receptor tyrosine kinase c-Kit controls IL-33 receptor signaling in mast cells. *Blood* 115:3899-3906.
  48. **Dupouy-Camet, J., K. D. Murrell, E.** International Office of Food, N. Agriculture Organization of the United, and O. World Health. 2007. *FAO/WHO/OIE guidelines for the surveillance, management, prevention and control of trichinellosis.* Food and Agriculture Organization of the United Nations : World Health Organization : World Organization for Animal Health, Paris.
  49. **Eigenbrod, T., J. H. Park, J. Harder, Y. Iwakura, and G. Nunez.** 2008. Cutting edge: critical role for mesothelial cells in necrosis-induced inflammation through the recognition of IL-1 alpha released from dying cells. *Journal of immunology* 181:8194-8198.
  50. **Fabre, V., D. P. Beiting, S. K. Bliss, N. G. Gebreselassie, L. F. Gagliardo, N. A. Lee, J.**

- J. Lee, and J. A. Appleton.** 2009. Eosinophil deficiency compromises parasite survival in chronic nematode infection. *J Immunol* 182:1577-1583.
51. **Faulkner, H., N. Humphreys, J. C. Renauld, J. Van Snick, and R. Grecis.** 1997. Interleukin-9 is involved in host protective immunity to intestinal nematode infection. *European journal of immunology* 27:2536-2540.
52. **Foell, D., H. Wittkowski, Z. Ren, J. Turton, G. Pang, J. Daebritz, J. Ehrchen, J. Heidemann, T. Borody, J. Roth, and R. Clancy.** 2008. Phagocyte-specific S100 proteins are released from affected mucosa and promote immune responses during inflammatory bowel disease. *J Pathol* 216:183-192.
53. **Funakoshi-Tago, M., K. Tago, M. Hayakawa, S. Tominaga, T. Ohshio, Y. Sonoda, and T. Kasahara.** 2008. TRAF6 is a critical signal transducer in IL-33 signaling pathway. *Cell Signal* 20:1679-1686.
54. **Fuss, I. J., M. Neurath, M. Boirivant, J. S. Klein, C. de la Motte, S. A. Strong, C. Focchi, and W. Strober.** 1996. Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *Journal of immunology* 157:1261-1270.
55. **Gewirtz, A. T., T. A. Navas, S. Lyons, P. J. Godowski, and J. L. Madara.** 2001. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *Journal of immunology* 167:1882-1885.
56. **Goh, F., K. M. Irvine, E. Lovelace, S. Donnelly, M. K. Jones, K. Brion, D. A. Hume, A. C. Kotze, J. P. Dalton, A. Ingham, and M. J. Sweet.** 2009. Selective induction of the Notch ligand Jagged-1 in macrophages by soluble egg antigen from *Schistosoma mansoni* involves ERK signalling. *Immunology* 127:326-337.
57. **Goodridge, H. S., F. A. Marshall, K. J. Else, K. M. Houston, C. Egan, L. Al-Riyami, F. Y. Liew, W. Harnett, and M. M. Harnett.** 2005. Immunomodulation via novel use of TLR4 by the filarial nematode phosphorylcholine-containing secreted product, ES-62.

- Journal of immunology 174:284-293.
58. **Goodridge, H. S., E. H. Wilson, W. Harnett, C. C. Campbell, M. M. Harnett, and F. Y. Liew.** 2001. Modulation of macrophage cytokine production by ES-62, a secreted product of the filarial nematode *Acanthocheilonema viteae*. Journal of immunology 167:940-945.
  59. **Gorfu, G., J. Rivera-Nieves, and K. Ley.** 2009. Role of beta7 integrins in intestinal lymphocyte homing and retention. Curr Mol Med 9:836-850.
  60. **Grencis, R. K., L. Hultner, and K. J. Else.** 1991. Host protective immunity to *Trichinella spiralis* in mice: activation of Th cell subsets and lymphokine secretion in mice expressing different response phenotypes. Immunology 74:329-332.
  61. **Grencis, R. K., and D. Wakelin.** 1982. Short lived, dividing cells mediate adoptive transfer of immunity to *Trichinella spiralis* in mice. I. Availability of cells in primary and secondary infections in relation to cellular changes in the mesenteric lymph node. Immunology 46:443-450.
  62. **Gruden-Movsesijan, A., M. Petrovic, and L. Sofronic-Milosavljevic.** 2003. Interaction of mannan-binding lectin with *Trichinella spiralis* glycoproteins, a possible innate immune mechanism. Parasite immunology 25:545-552.
  63. **Guz, Y., M. R. Montminy, R. Stein, J. Leonard, L. W. Gamer, C. V. Wright, and G. Teitelman.** 1995. Expression of murine STF-1, a putative insulin gene transcription factor, in beta cells of pancreas, duodenal epithelium and pancreatic exocrine and endocrine progenitors during ontogeny. Development 121:11-18.
  64. **Ha, T. Y., N. D. Reed, and P. K. Crowle.** 1983. Delayed expulsion of adult *Trichinella spiralis* by mast cell-deficient W/W<sup>v</sup> mice. Infect Immun 41:445-447.
  65. **Hasnain, S. Z., C. M. Evans, M. Roy, A. L. Gallagher, K. N. Kindrachuk, L. Barron, B. F. Dickey, M. S. Wilson, T. A. Wynn, R. K. Grencis, and D. J. Thornton.** 2011. Muc5ac: a critical component mediating the rejection of enteric nematodes. The Journal of experimental medicine 208:893-900.

66. **Hayakawa, H., M. Hayakawa, A. Kume, and S. Tominaga.** 2007. Soluble ST2 blocks interleukin-33 signaling in allergic airway inflammation. *The Journal of biological chemistry* 282:26369-26380.
67. **Helmby, H., and R. K. Grencis.** 2003. Contrasting roles for IL-10 in protective immunity to different life cycle stages of intestinal nematode parasites. *Eur J Immunol* 33:2382-2390.
68. **Helmby, H., and R. K. Grencis.** 2003. Essential role for TLR4 and MyD88 in the development of chronic intestinal nematode infection. *European journal of immunology* 33:2974-2979.
69. **Herbert, D. R., J. Q. Yang, S. P. Hogan, K. Groschwitz, M. Khodoun, A. Munitz, T. Orekov, C. Perkins, Q. Wang, F. Brombacher, J. F. Urban, Jr., M. E. Rothenberg, and F. D. Finkelman.** 2009. Intestinal epithelial cell secretion of RELM-beta protects against gastrointestinal worm infection. *The Journal of experimental medicine* 206:2947-2957.
70. **Hotez, P. J., and M. Gurwith.** 2011. Europe's neglected infections of poverty. *Int J Infect Dis* 15:e611-619.
71. **Humphreys, N. E., D. Xu, M. R. Hepworth, F. Y. Liew, and R. K. Grencis.** 2008. IL-33, a potent inducer of adaptive immunity to intestinal nematodes. *Journal of immunology* 180:2443-2449.
72. **Ierna, M. X., H. E. Scales, C. Mueller, and C. E. Lawrence.** 2009. Transmembrane tumor necrosis factor alpha is required for enteropathy and is sufficient to promote parasite expulsion in gastrointestinal helminth infection. *Infection and immunity* 77:3879-3885.
73. **Ierna, M. X., H. E. Scales, K. L. Saunders, and C. E. Lawrence.** 2008. Mast cell production of IL-4 and TNF may be required for protective and pathological responses in gastrointestinal helminth infection. *Mucosal Immunol* 1:147-155.
74. **Imaeda, H., A. Andoh, T. Aomatsu, K. Uchiyama, S. Bamba, T. Tsujikawa, Y. Naito,**

- and Y. Fujiyama.** 2011. Interleukin-33 suppresses Notch ligand expression and prevents goblet cell depletion in dextran sulfate sodium-induced colitis. *Int J Mol Med* 28:573-578.
75. **Inoue, S., T. Matsumoto, M. Iida, M. Mizuno, F. Kuroki, K. Hoshika, and M. Shimizu.** 1999. Characterization of cytokine expression in the rectal mucosa of ulcerative colitis: correlation with disease activity. *Am J Gastroenterol* 94:2441-2446.
76. **Ismail, M. M., and C. E. Tanner.** 1972. *Trichinella spiralis*: peripheral blood, intestinal, and bone-marrow eosinophilia in rats and its relationship to the inoculating dose of larvae, antibody response and parasitism. *Experimental parasitology* 31:262-272.
77. **Iwata, M., A. Hirakiyama, Y. Eshima, H. Kagechika, C. Kato, and S. Y. Song.** 2004. Retinoic acid imprints gut-homing specificity on T cells. *Immunity* 21:527-538.
78. **Janeway, C. A., Jr.** 1989. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* 54 Pt 1:1-13.
79. **Jones, L. A., F. Roberts, M. B. Nickdel, F. Brombacher, A. N. McKenzie, F. L. Henriquez, J. Alexander, and C. W. Roberts.** 2010. IL-33 receptor (T1/ST2) signalling is necessary to prevent the development of encephalitis in mice infected with *Toxoplasma gondii*. *European journal of immunology* 40:426-436.
80. **Kamizato, M., K. Nishida, K. Masuda, K. Takeo, Y. Yamamoto, T. Kawai, S. Teshima-Kondo, T. Tanahashi, and K. Rokutan.** 2009. Interleukin 10 inhibits interferon gamma- and tumor necrosis factor alpha-stimulated activation of NADPH oxidase 1 in human colonic epithelial cells and the mouse colon. *J Gastroenterol* 44:1172-1184.
81. **Kawai, T., and S. Akira.** 2011. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* 34:637-650.
82. **Kearley, J., K. F. Buckland, S. A. Mathie, and C. M. Lloyd.** 2009. Resolution of allergic inflammation and airway hyperreactivity is dependent upon disruption of the T1/ST2-IL-33 pathway. *Am J Respir Crit Care Med* 179:772-781.

83. **Kenworthy, R.** 1967. Influence of bacteria on absorption from the small intestine. *Proc Nutr Soc* 26:18-23.
84. **Khan, W. I., M. Richard, H. Akiho, P. A. Blennerhasset, N. E. Humphreys, R. K. Grecis, J. Van Snick, and S. M. Collins.** 2003. Modulation of intestinal muscle contraction by interleukin-9 (IL-9) or IL-9 neutralization: correlation with worm expulsion in murine nematode infections. *Infection and immunity* 71:2430-2438.
85. **Knight, P. A., A. D. Pemberton, K. A. Robertson, D. J. Roy, S. H. Wright, and H. R. Miller.** 2004. Expression profiling reveals novel innate and inflammatory responses in the jejunal epithelial compartment during infection with *Trichinella spiralis*. *Infect Immun* 72:6076-6086.
86. **Knight, P. A., S. H. Wright, C. E. Lawrence, Y. Y. Paterson, and H. R. Miller.** 2000. Delayed expulsion of the nematode *Trichinella spiralis* in mice lacking the mucosal mast cell-specific granule chymase, mouse mast cell protease-1. *The Journal of experimental medicine* 192:1849-1856.
87. **Komai-Koma, M., D. Xu, Y. Li, A. N. McKenzie, I. B. McInnes, and F. Y. Liew.** 2007. IL-33 is a chemoattractant for human Th2 cells. *European journal of immunology* 37:2779-2786.
88. **Kondo, Y., T. Yoshimoto, K. Yasuda, S. Futatsugi-Yumikura, M. Morimoto, N. Hayashi, T. Hoshino, J. Fujimoto, and K. Nakanishi.** 2008. Administration of IL-33 induces airway hyperresponsiveness and goblet cell hyperplasia in the lungs in the absence of adaptive immune system. *International immunology* 20:791-800.
89. **Kono, H., C. J. Chen, F. Ontiveros, and K. L. Rock.** 2010. Uric acid promotes an acute inflammatory response to sterile cell death in mice. *The Journal of Clinical Investigation* 120:1939-1949.
90. **Krawczyk, C. M., J. Sun, and E. J. Pearce.** 2008. Th2 differentiation is unaffected by Jagged2 expression on dendritic cells. *Journal of immunology* 180:7931-7937.
91. **Kroeger, K. M., B. M. Sullivan, and R. M. Locksley.** 2009. IL-18 and IL-33 elicit Th2

- cytokines from basophils via a MyD88- and p38alpha-dependent pathway. *J Leukoc Biol* 86:769-778.
92. **Kuchler, A. M., J. Pollheimer, J. Balogh, J. Sponheim, L. Manley, D. R. Sorensen, P. M. De Angelis, H. Scott, and G. Haraldsen.** 2008. Nuclear interleukin-33 is generally expressed in resting endothelium but rapidly lost upon angiogenic or proinflammatory activation. *The American journal of pathology* 173:1229-1242.
93. **Kurowska-Stolarska, M., B. Stolarski, P. Kewin, G. Murphy, C. J. Corrigan, S. Ying, N. Pitman, A. Mirchandani, B. Rana, N. van Rooijen, M. Shepherd, C. McSharry, I. B. McInnes, D. Xu, and F. Y. Liew.** 2009. IL-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation. *Journal of immunology* 183:6469-6477.
94. **Lawrence, C. E., J. C. Paterson, L. M. Higgins, T. T. MacDonald, M. W. Kennedy, and P. Garside.** 1998. IL-4-regulated enteropathy in an intestinal nematode infection. *European journal of immunology* 28:2672-2684.
95. **Lawrence, C. E., J. C. Paterson, X. Q. Wei, F. Y. Liew, P. Garside, and M. W. Kennedy.** 2000. Nitric oxide mediates intestinal pathology but not immune expulsion during *Trichinella spiralis* infection in mice. *Journal of immunology* 164:4229-4234.
96. **Lawrence, C. E., Y. Y. Paterson, S. H. Wright, P. A. Knight, and H. R. Miller.** 2004. Mouse mast cell protease-1 is required for the enteropathy induced by gastrointestinal helminth infection in the mouse. *Gastroenterology* 127:155-165.
97. **Linehan, S. A., P. S. Coulson, R. A. Wilson, A. P. Mountford, F. Brombacher, L. Martinez-Pomares, and S. Gordon.** 2003. IL-4 receptor signaling is required for mannose receptor expression by macrophages recruited to granulomata but not resident cells in mice infected with *Schistosoma mansoni*. *Laboratory investigation; a journal of technical methods and pathology* 83:1223-1231.
98. **Loo, Y. M., and M. Gale, Jr.** 2011. Immune signaling by RIG-I-like receptors. *Immunity* 34:680-692.

99. **Love, R. J., B. M. Ogilvie, and D. J. McLaren.** 1976. The immune mechanism which expels the intestinal stage of *Trichinella spiralis* from rats. *Immunology* 30:7-15.
100. **MacDonald, A. S., and R. M. Maizels.** 2008. Alarming dendritic cells for Th2 induction. *The Journal of experimental medicine* 205:13-17.
101. **Maekawa, Y., S. Tsukumo, S. Chiba, H. Hirai, Y. Hayashi, H. Okada, K. Kishihara, and K. Yasutomo.** 2003. Delta1-Notch3 interactions bias the functional differentiation of activated CD4+ T cells. *Immunity* 19:549-559.
102. **Marti, H. P., and K. D. Murrell.** 1986. *Trichinella spiralis*: antifecundity and antinewborn larvae immunity in swine. *Experimental parasitology* 62:370-375.
103. **Marzio, L., P. Blennerhassett, S. Chiverton, D. L. Vermillion, J. Langer, and S. M. Collins.** 1990. Altered smooth muscle function in worm-free gut regions of *Trichinella*-infected rats. *The American journal of physiology* 259:G306-313.
104. **Masamune, A., T. Watanabe, K. Kikuta, K. Satoh, A. Kanno, and T. Shimosegawa.** 2010. Nuclear expression of interleukin-33 in pancreatic stellate cells. *American journal of physiology. Gastrointestinal and liver physiology* 299:G821-832.
105. **Maynard, C. L., L. E. Harrington, K. M. Janowski, J. R. Oliver, C. L. Zindl, A. Y. Rudensky, and C. T. Weaver.** 2007. Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10. *Nat Immunol* 8:931-941.
106. **McDermott, J. R., N. E. Humphreys, S. P. Forman, D. D. Donaldson, and R. K. Grencis.** 2005. Intraepithelial NK cell-derived IL-13 induces intestinal pathology associated with nematode infection. *J Immunol* 175:3207-3213.
107. **McDermott, M. R., D. A. Mark, A. D. Befus, B. S. Baliga, R. M. Suskind, and J. Bienenstock.** 1982. Impaired intestinal localization of mesenteric lymphoblasts associated with vitamin A deficiency and protein-calorie malnutrition. *Immunology* 45:1-5.
108. **McVay, C. S., P. Bracken, L. F. Gagliardo, and J. Appleton.** 2000. Antibodies to

- tyvelose exhibit multiple modes of interference with the epithelial niche of *Trichinella spiralis*. *Infect Immun* 68:1912-1918.
109. **Medzhitov, R.** 2007. Recognition of microorganisms and activation of the immune response. *Nature* 449:819-826.
110. **Melmed, G., L. S. Thomas, N. Lee, S. Y. Tesfay, K. Lukasek, K. S. Michelsen, Y. Zhou, B. Hu, M. Arditi, and M. T. Abreu.** 2003. Human intestinal epithelial cells are broadly unresponsive to Toll-like receptor 2-dependent bacterial ligands: implications for host-microbial interactions in the gut. *Journal of immunology* 170:1406-1415.
111. **Meyer, S., E. van Liempt, A. Imberty, Y. van Kooyk, H. Geyer, R. Geyer, and I. van Die.** 2005. DC-SIGN mediates binding of dendritic cells to authentic pseudo-LewisY glycolipids of *Schistosoma mansoni* cercariae, the first parasite-specific ligand of DC-SIGN. *The Journal of biological chemistry* 280:37349-37359.
112. **Michels, C. E., H. E. Scales, K. A. Saunders, S. McGowan, F. Brombracher, J. Alexander, and C. E. Lawrence.** 2009. Neither interleukin-4 receptor alpha expression on CD4+ T cells, or macrophages and neutrophils is required for protective immunity to *Trichinella spiralis*. *Immunology* 128:e385-394.
113. **Monteleone, I., A. M. Platt, E. Jaensson, W. W. Agace, and A. M. Mowat.** 2008. IL-10-dependent partial refractoriness to Toll-like receptor stimulation modulates gut mucosal dendritic cell function. *European journal of immunology* 38:1533-1547.
114. **Moqbel, R., D. Wakelin, A. J. MacDonald, S. J. King, R. K. Grencis, and A. B. Kay.** 1987. Release of leukotrienes during rapid expulsion of *Trichinella spiralis* from immune rats. *Immunology* 60:425-430.
115. **Mora, J. R., M. R. Bono, N. Manjunath, W. Weninger, L. L. Cavanagh, M. Roseblatt, and U. H. Von Andrian.** 2003. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature* 424:88-93.
116. **Moritz, D. R., H. R. Rodewald, J. Gheyselinck, and R. Klemenz.** 1998. The IL-1 receptor-related T1 antigen is expressed on immature and mature mast cells and on fetal

- blood mast cell progenitors. *Journal of immunology* 161:4866-4874.
117. **Moro, K., T. Yamada, M. Tanabe, T. Takeuchi, T. Ikawa, H. Kawamoto, J. Furusawa, M. Ohtani, H. Fujii, and S. Koyasu.** 2010. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature* 463:540-544.
118. **Moussion, C., N. Ortega, and J. P. Girard.** 2008. The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? *PLoS One* 3:e3331.
119. **Murrell, K. D.** 1985. *Trichinella spiralis*: acquired immunity in swine. *Experimental parasitology* 59:347-354.
120. **Neill, D. R., S. H. Wong, A. Bellosi, R. J. Flynn, M. Daly, T. K. Langford, C. Bucks, C. M. Kane, P. G. Fallon, R. Pannell, H. E. Jolin, and A. N. McKenzie.** 2010. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* 464:1367-1370.
121. **Ohishi, K., B. Varnum-Finney, R. E. Serda, C. Anasetti, and I. D. Bernstein.** 2001. The Notch ligand, Delta-1, inhibits the differentiation of monocytes into macrophages but permits their differentiation into dendritic cells. *Blood* 98:1402-1407.
122. **Ohno, T., K. Oboki, N. Kajiwara, E. Morii, K. Aozasa, R. A. Flavell, K. Okumura, H. Saito, and S. Nakae.** 2009. Caspase-1, caspase-8, and calpain are dispensable for IL-33 release by macrophages. *Journal of immunology* 183:7890-7897.
123. **Owen, R. L., A. J. Piazza, and T. H. Ermak.** 1991. Ultrastructural and cytoarchitectural features of lymphoreticular organs in the colon and rectum of adult BALB/c mice. *Am J Anat* 190:10-18.
124. **Park, J. S., D. Svetkauskaite, Q. He, J. Y. Kim, D. Strassheim, A. Ishizaka, and E. Abraham.** 2004. Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. *The Journal of biological chemistry* 279:7370-7377.
125. **Pene, J., S. Chevalier, L. Preisser, E. Venereau, M. H. Guilleux, S. Ghannam, J. P.**

- Moles, Y. Danger, E. Ravon, S. Lesaux, H. Yssel, and H. Gascan.** 2008. Chronically inflamed human tissues are infiltrated by highly differentiated Th17 lymphocytes. *Journal of immunology* 180:7423-7430.
126. **Perrigoue, J. G., S. A. Saenz, M. C. Siracusa, E. J. Allenspach, B. C. Taylor, P. R. Giacomini, M. G. Nair, Y. Du, C. Zaph, N. van Rooijen, M. R. Comeau, E. J. Pearce, T. M. Laufer, and D. Artis.** 2009. MHC class II-dependent basophil-CD4+ T cell interactions promote T(H)2 cytokine-dependent immunity. *Nature immunology* 10:697-705.
127. **Picherot, M., I. P. Oswald, M. Cote, K. Noeckler, F. Le Guerhier, P. Boireau, and I. Vallee.** 2007. Swine infection with *Trichinella spiralis*: Comparative analysis of the mucosal intestinal and systemic immune responses. *Veterinary parasitology* 143:122-130.
128. **Price, A. E., H. E. Liang, B. M. Sullivan, R. L. Reinhardt, C. J. Eisley, D. J. Erle, and R. M. Locksley.** 2010. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proceedings of the National Academy of Sciences of the United States of America* 107:11489-11494.
129. **Rank, M. A., T. Kobayashi, H. Kozaki, K. R. Bartemes, D. L. Squillace, and H. Kita.** 2009. IL-33-activated dendritic cells induce an atypical TH2-type response. *The Journal of allergy and clinical immunology* 123:1047-1054.
130. **Rhee, S. H., E. Im, M. Riegler, E. Kokkotou, M. O'Brien, and C. Pothoulakis.** 2005. Pathophysiological role of Toll-like receptor 5 engagement by bacterial flagellin in colonic inflammation. *Proceedings of the National Academy of Sciences of the United States of America* 102:13610-13615.
131. **Rokutan, K., T. Kawahara, Y. Kuwano, K. Tominaga, K. Nishida, and S. Teshima-Kondo.** 2008. Nox enzymes and oxidative stress in the immunopathology of the gastrointestinal tract. *Semin Immunopathol* 30:315-327.
132. **Roth, H.** 1938. On the localization of adult *Trichinella spiralis* in the intestinal tract. *J Parasitol* 24:225-231.

133. **Roussel, L., M. Erard, C. Cayrol, and J. P. Girard.** 2008. Molecular mimicry between IL-33 and KSHV for attachment to chromatin through the H2A-H2B acidic pocket. *EMBO Rep* 9:1006-1012.
134. **Ruitenbergh, E. J., and P. A. Steerenberg.** 1974. Intestinal phase of *Trichinella spiralis* in congenitally athymic (nude) mice. *The Journal of Parasitology* 60:1056-1057.
135. **Saenz, S. A., M. C. Siracusa, J. G. Perrigoue, S. P. Spencer, J. F. Urban, Jr., J. E. Tocker, A. L. Budelsky, M. A. Kleinschek, R. A. Kastelein, T. Kambayashi, A. Bhandoola, and D. Artis.** 2010. IL25 elicits a multipotent progenitor cell population that promotes T(H)2 cytokine responses. *Nature* 464:1362-1366.
136. **Saunders, S. P., C. M. Walsh, J. L. Barlow, N. E. Mangan, P. R. Taylor, A. N. McKenzie, P. Smith, and P. G. Fallon.** 2009. The C-type lectin SIGNR1 binds *Schistosoma mansoni* antigens in vitro, but SIGNR1-deficient mice have normal responses during schistosome infection. *Infection and immunity* 77:399-404.
137. **Scales, H. E., M. X. Ierna, and C. E. Lawrence.** 2007. The role of IL-4, IL-13 and IL-4Ralpha in the development of protective and pathological responses to *Trichinella spiralis*. *Parasite Immunol* 29:81-91.
138. **Scales, H. E., M. X. Ierna, and C. E. Lawrence.** 2007. The role of IL-4, IL-13 and IL-4Ralpha in the development of protective and pathological responses to *Trichinella spiralis*. *Parasite immunology* 29:81-91.
139. **Schmidt, G. H., M. M. Wilkinson, and B. A. Ponder.** 1985. Cell migration pathway in the intestinal epithelium: an in situ marker system using mouse aggregation chimeras. *Cell* 40:425-429.
140. **Schmitz, J., A. Owyang, E. Oldham, Y. Song, E. Murphy, T. K. McClanahan, G. Zurawski, M. Moshrefi, J. Qin, X. Li, D. M. Gorman, J. F. Bazan, and R. A. Kastelein.** 2005. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 23:479-490.

141. **Shaw, P. J., M. Lamkanfi, and T. D. Kanneganti.** 2010. NOD-like receptor (NLR) signaling beyond the inflammasome. *European journal of immunology* 40:624-627.
142. **Silver, M. R., A. Margulis, N. Wood, S. J. Goldman, M. Kasaian, and D. Chaudhary.** 2010. IL-33 synergizes with IgE-dependent and IgE-independent agents to promote mast cell and basophil activation. *Inflamm Res* 59:207-218.
143. **Siracusa, M. C., S. A. Saenz, D. A. Hill, B. S. Kim, M. B. Headley, T. A. Doering, E. J. Wherry, H. K. Jessup, L. A. Siegel, T. Kambayashi, E. C. Dudek, M. Kubo, A. Cianferoni, J. M. Spergel, S. F. Ziegler, M. R. Comeau, and D. Artis.** 2011. TSLP promotes interleukin-3-independent basophil haematopoiesis and type 2 inflammation. *Nature* 477:229-233.
144. **Sirisinha, S.** 2011. Insight into the mechanisms regulating immune homeostasis in health and disease. *Asian Pac J Allergy Immunol* 29:1-14.
145. **Smithgall, M. D., M. R. Comeau, B. R. Yoon, D. Kaufman, R. Armitage, and D. E. Smith.** 2008. IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. *International immunology* 20:1019-1030.
146. **Sokol, C. L., G. M. Barton, A. G. Farr, and R. Medzhitov.** 2008. A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nature immunology* 9:310-318.
147. **Sokol, C. L., N. Q. Chu, S. Yu, S. A. Nish, T. M. Laufer, and R. Medzhitov.** 2009. Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response. *Nature immunology* 10:713-720.
148. **Suzukawa, M., R. Koketsu, M. Iikura, S. Nakae, K. Matsumoto, H. Nagase, H. Saito, K. Matsushima, K. Ohta, K. Yamamoto, and M. Yamaguchi.** 2008. Interleukin-33 enhances adhesion, CD11b expression and survival in human eosinophils. *Laboratory investigation; a journal of technical methods and pathology* 88:1245-1253.
149. **Suzuki, T., T. Sasaki, H. Takagi, K. Sato, and K. Ueda.** 2008. The effectors responsible for gastrointestinal nematode parasites, *Trichinella spiralis*, expulsion in rats.

- Parasitology research 103:1289-1295.
150. **Taylor, B. C., C. Zaph, A. E. Troy, Y. Du, K. J. Guild, M. R. Comeau, and D. Artis.** 2009. TSLP regulates intestinal immunity and inflammation in mouse models of helminth infection and colitis. *The Journal of experimental medicine* 206:655-667.
  151. **Termeer, C., F. Benedix, J. Sleeman, C. Fieber, U. Voith, T. Ahrens, K. Miyake, M. Freudenberg, C. Galanos, and J. C. Simon.** 2002. Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. *The Journal of experimental medicine* 195:99-111.
  152. **Thomas, P. G., M. R. Carter, O. Atochina, A. A. Da'Dara, D. Piskorska, E. McGuire, and D. A. Harn.** 2003. Maturation of dendritic cell 2 phenotype by a helminth glycan uses a Toll-like receptor 4-dependent mechanism. *Journal of immunology* 171:5837-5841.
  153. **Tu, L., T. C. Fang, D. Artis, O. Shestova, S. E. Pross, I. Maillard, and W. S. Pear.** 2005. Notch signaling is an important regulator of type 2 immunity. *The Journal of experimental medicine* 202:1037-1042.
  154. **Urban, J. F., Jr., N. Noben-Trauth, L. Schopf, K. B. Madden, and F. D. Finkelman.** 2001. Cutting edge: IL-4 receptor expression by non-bone marrow-derived cells is required to expel gastrointestinal nematode parasites. *Journal of immunology* 167:6078-6081.
  155. **Vabulas, R. M., P. Ahmad-Nejad, S. Ghose, C. J. Kirschning, R. D. Issels, and H. Wagner.** 2002. HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. *The Journal of biological chemistry* 277:15107-15112.
  156. **Vabulas, R. M., S. Braedel, N. Hilf, H. Singh-Jasuja, S. Herter, P. Ahmad-Nejad, C. J. Kirschning, C. Da Costa, H. G. Rammensee, H. Wagner, and H. Schild.** 2002. The endoplasmic reticulum-resident heat shock protein Gp96 activates dendritic cells via the Toll-like receptor 2/4 pathway. *The Journal of biological chemistry* 277:20847-20853.
  157. **Vallance, B. A., K. I. Matthaei, S. Sanovic, I. G. Young, and S. M. Collins.** 2000.

- Interleukin-5 deficient mice exhibit impaired host defence against challenge *Trichinella spiralis* infections. *Parasite immunology* 22:487-492.
158. **van der Kleij, D., E. Latz, J. F. Brouwers, Y. C. Kruize, M. Schmitz, E. A. Kurt-Jones, T. Espevik, E. C. de Jong, M. L. Kapsenberg, D. T. Golenbock, A. G. Tielens, and M. Yazdanbakhsh.** 2002. A novel host-parasite lipid cross-talk. Schistosomal lysophosphatidylserine activates toll-like receptor 2 and affects immune polarization. *The Journal of biological chemistry* 277:48122-48129.
159. **van Die, I., and R. D. Cummings.** 2010. Glycan gimmickry by parasitic helminths: a strategy for modulating the host immune response? *Glycobiology* 20:2-12.
160. **van Liempt, E., S. J. van Vliet, A. Engering, J. J. Garcia Vallejo, C. M. Bank, M. Sanchez-Hernandez, Y. van Kooyk, and I. van Die.** 2007. *Schistosoma mansoni* soluble egg antigens are internalized by human dendritic cells through multiple C-type lectins and suppress TLR-induced dendritic cell activation. *Molecular immunology* 44:2605-2615.
161. **van Vliet, S. J., E. van Liempt, E. Saeland, C. A. Aarnoudse, B. Appelmelk, T. Irimura, T. B. Geijtenbeek, O. Blixt, R. Alvarez, I. van Die, and Y. van Kooyk.** 2005. Carbohydrate profiling reveals a distinctive role for the C-type lectin MGL in the recognition of helminth parasites and tumor antigens by dendritic cells. *International immunology* 17:661-669.
162. **Verri, W. A., Jr., A. T. Guerrero, S. Y. Fukada, D. A. Valerio, T. M. Cunha, D. Xu, S. H. Ferreira, F. Y. Liew, and F. Q. Cunha.** 2008. IL-33 mediates antigen-induced cutaneous and articular hypernociception in mice. *Proceedings of the National Academy of Sciences of the United States of America* 105:2723-2728.
163. **Verri, W. A., Jr., F. O. Souto, S. M. Vieira, S. C. Almeida, S. Y. Fukada, D. Xu, J. C. Alves-Filho, T. M. Cunha, A. T. Guerrero, R. B. Mattos-Guimaraes, F. R. Oliveira, M. M. Teixeira, J. S. Silva, I. B. McInnes, S. H. Ferreira, P. Louzada-Junior, F. Y. Liew, and F. Q. Cunha.** 2010. IL-33 induces neutrophil migration in rheumatoid arthritis

- and is a target of anti-TNF therapy. *Ann Rheum Dis* 69:1697-1703.
164. **Wheeler, D. S., M. A. Chase, A. P. Senft, S. E. Poynter, H. R. Wong, and K. Page.** 2009. Extracellular Hsp72, an endogenous DAMP, is released by virally infected airway epithelial cells and activates neutrophils via Toll-like receptor (TLR)-4. *Respir Res* 10:31.
  165. **WHO** 2010, posting date. PCT databank. [Online.]
  166. **Woodbury, R. G., H. R. Miller, J. F. Huntley, G. F. Newlands, A. C. Palliser, and D. Wakelin.** 1984. Mucosal mast cells are functionally active during spontaneous expulsion of intestinal nematode infections in rat. *Nature* 312:450-452.
  167. **Worsley, A. G., S. LeibundGut-Landmann, E. Slack, L. K. Phng, H. Gerhardt, C. Reis e Sousa, and A. S. MacDonald.** 2008. Dendritic cell expression of the Notch ligand jagged2 is not essential for Th2 response induction in vivo. *European journal of immunology* 38:1043-1049.
  168. **Wright, K. A.** 1979. *Trichinella spiralis*: an intracellular parasite in the intestinal phase. *J Parasitol* 65:441-445.
  169. **Wright, K. A., E. Weidman, and H. Hong.** 1987. The distribution of cells killed by *Trichinella spiralis* in the mucosal epithelium of two strains of mice. *J Parasitol* 73:935-939.
  170. **Wright, N. A., and M. Irwin.** 1982. The kinetics of villus cell populations in the mouse small intestine. I. Normal villi: the steady state requirement. *Cell Tissue Kinet* 15:595-609.
  171. **Yang, D., Q. Chen, H. Yang, K. J. Tracey, M. Bustin, and J. J. Oppenheim.** 2007. High mobility group box-1 protein induces the migration and activation of human dendritic cells and acts as an alarmin. *J Leukoc Biol* 81:59-66.

## CHAPTER 2

**Expulsion of secondary *Trichinella spiralis* infection in rats occurs independently of mucosal mast cell release of mast cell protease II<sup>1</sup>**

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1 Originally published in *The Journal of Immunology*.  
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## Abstract

Our aim was to elucidate the contribution of mucosal mast cells to the effector phase of a secondary immune response to *Trichinella spiralis*. During secondary infection, rats expel 90 to 99% of *T. spiralis* L<sub>1</sub> from the intestine in a matter of hours. This phenomenon appears to be unique to rats and has been called rapid expulsion. Primary intestinal infection by *T. spiralis* induces mastocytosis and mast cell degranulation occurs when challenged rats exhibit rapid expulsion. These observations have engendered the view that mast cells mediate rapid expulsion. Here we report that immunization of adult AO rats by an infection limited to the muscle phase did not induce intestinal mastocytosis, yet such rats exhibited rapid expulsion when challenged orally. Although mastocytosis was absent, the protease unique to mucosal mast cells, rat mast cell protease II (RMCPII), was detected in sera at the time of expulsion. We further evaluated mast cell activity in neonatal rats that display rapid expulsion. Pups born to infected dams displayed rapid expulsion and RMCPII was detected in their sera. By feeding pups parasite-specific monoclonal or polyclonal antibodies before challenge infection, it was possible to dissociate mast cell degranulation from parasite expulsion. These results indicate that rapid expulsion can occur in the absence of either intestinal mastocytosis or RMCPII release. Furthermore, release of RMCPII is not sufficient to cause expulsion. The data argue against a role for mast cells in the mechanism underlying the effector phase of protective immunity against *T. spiralis* in rats.

## Introduction

Rodents are natural hosts for *Trichinella spiralis*, a parasitic nematode that colonizes the epithelium of the small intestine. In both mice and rats, primary infection induces a potent Th2 response leading to intestinal mastocytosis (1). Mast cell degranulation can be detected at the time of adult worm expulsion (2-4). The temporal alignment of intestinal mastocytosis and mast cell degranulation with the rejection of adult worms provided evidence that mast cells may be key to intestinal defense against the parasite. Support for this notion has been provided by reports that rejection of both primary and secondary intestinal infections

with *T. spiralis* is delayed in mast cell deficient W/W<sup>v</sup> mice (5, 6) and that the capacity to reject adult worms can be reconstituted with bone marrow cells (7). Studies with wild type and IL-9 transgenic mice treated with anti-c-kit antibodies confirmed these results (8, 9). Furthermore, mice deficient in mast cell protease-1 (mMCP-1) show delayed rejection of *T. spiralis* (10). It has been hypothesized that mMCP-1 increases intestinal permeability, providing antibodies access to parasite antigens (11); however, the mechanism of adult worm rejection remains unproven.

Rats respond to a secondary *T. spiralis* infection with a dramatic and immediate immune response. This rapid expulsion of first-stage larvae (L<sub>1</sub>) has been likened to intestinal anaphylaxis (12-14). Mice do not exhibit rapid expulsion, although they do reject secondary intestinal infections in an accelerated fashion (15, 16). Mast cell degranulation is coincident with rapid expulsion in rats, as revealed by detection of rat mast cell protease II (RMCPII) in the serum (2); however, there is no direct evidence that mast cells contribute to expulsion. Mast cells have been reported to be the sole cellular source of RMCPII in intestinal tissue of naïve rats (17, 18). RMCPII-positive cells are present in the lung, liver and primary lymphoid tissue (19); however, intestinal mast cells are very likely to be the major source of blood RMCPII, when antigenic challenge is limited to the intestine.

The participation of antibodies in rapid expulsion of *T. spiralis* has been documented in passively immunized rat pups. Immune serum or parasite-specific monoclonal IgG1 or IgG2c confer an immunity on pups that is indistinguishable from rapid expulsion displayed by previously infected adult rats (20, 21). Protective antibodies are specific for tyvelose-bearing glycans that are unique to L<sub>1</sub> (22). In the presence of tyvelose-specific antibodies, L<sub>1</sub> are excluded from the epithelium, entrapped in mucus, or encumbered in epithelial cells (23, 24). Antibodies are protective when delivered to pups orally or by intra-peritoneal injection, or when passively transferred in milk from the mother (20, 21). Passive immunization is not effective in rats after weaning (25). The two rat isotypes known to activate mast cells, IgE and IgG2a, do not protect neonates (21, 26).

In contrast to rat pups, passive immunization of adult rats is effective only after intestinal

priming. Infection with either *Nippostrongylus brasiliensis* (27, 28) or *Heligmosomoides polygyrus* (27) enables passively transferred serum antibodies or monoclonal anti-tyvelose antibodies to confer immunity. Infection with either of these parasites induces mastocytosis as well as many other innate cellular changes in the small intestine. In the studies described here, we sought to investigate the requirement for intestinal priming using an immunization scheme that did not involve intestinal infection. In addition, we investigated the activity of mast cells in passively immunized rat pups, as pups do not require intestinal priming for immunity. Our findings indicate that rapid expulsion occurs in the absence of mastocytosis and that mast cell release of RMCPII is neither required nor sufficient for immunity.

## **Materials and Methods**

### **Rats**

Albino Oxford (AO) and PVG strain rats were produced and maintained under specific-pathogen-free conditions in the James A. Baker Institute vivarium according to the guidelines of the American Association for Accreditation of Laboratory Animal Care.

Outbred, male NIH-RNU (athymic) and heterozygous NIH-RNU/+ rats (02N01 Cr:NIH-rnu) were obtained from NCI-Frederick and were barrier maintained. Rats were euthanized by CO<sub>2</sub> inhalation and cervical dislocation. Blood samples were collected from the tails of rats under anesthesia with isoflurane, or from the heart post-mortem.

### **Parasite**

*Trichinella spiralis* (pig strain) was maintained in irradiated adult AO rats as described by Crum *et al.* (29). Infectious L<sub>1</sub> were harvested from muscle by digestion in a solution of 1% pepsin-1% HCl at 37°C. Newborn larvae (NBL) were recovered from cultures of adult worms as described by Beiting *et al.* (30). Adult rats, 8 to 12 weeks old, were immunized by oral infection with 1000 L<sub>1</sub> or by infection limited to muscle by injection of 80,000 NBL in the lateral tail vein. Adult rats were challenged 9 weeks after the immunizing infection. The challenge dose was

500 L<sub>1</sub> for adult rat experiments. Pups (12 to 16 days old) were challenged with 200 L<sub>1</sub>. Small intestines were collected for parasite enumeration 18 to 24 hours post-challenge for adult rats, and 3 hours post-challenge for pups.

To evaluate entrapment of intestinal L<sub>1</sub> in mucus, rats were euthanized 60 to 90 minutes post-challenge and the contents of stomach and small intestine (divided into the proximal 50% and distal 50% of the small intestine) were examined as previously described (24). Briefly, intestinal contents were flushed with 0.85% NaCl, collected into 50 ml polypropylene conical tubes, and packed on ice. L<sub>1</sub> trapped in mucus were observed by pressing mucus between glass slides. L<sub>1</sub> free in saline were considered to have been free in the lumen. Intestines were incubated at 37°C for 5 hours, and L<sub>1</sub> migrating out of the epithelium were counted.

### **Histology**

Two-centimeter sections of jejunum were collected, fixed in Carnoy's solution (60% ethanol, 30% chloroform, 10% glacial acetic acid) for 3 hours, transferred to 70% ethanol, and embedded in paraffin. Mast cells were counted in sections stained with Alcian blue (pH 0.4) and counterstained with Nuclear Fast Red. Goblet cells and eosinophils were counted in sections stained with hematoxylin and eosin (H & E). Each cell type was counted in twenty crypt-villus units (CVU) per rat, and values reported as the mean number per CVU for rats in each treatment group.

### **Sera and antibodies**

Tyvelose-specific monoclonal antibodies 9D4 (IgG1), 18H1(IgG2a), and 9E6 (IgG2c) have been described previously (21). Antibodies were concentrated by (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation of nude mouse ascites fluid, dialyzed against saline, and were adjusted to a concentration of 5 mg/ml (21). Immune serum was collected from adult AO rats 9 to 12 weeks following infection with 1000 L<sub>1</sub>. Normal rat serum was collected from naïve rats. Polyclonal IgG was produced by precipitation of immune or normal serum with 40% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> leaving polyclonal IgE in the supernatant (31). Both fractions were dialyzed against saline and concentrated to match the

original volume of serum using a 10,000 molecular weight cut-off centrifugal device (Millipore-Centricon, Billerica, MA).

## **ELISA**

Serum antibodies were measured using *T. spiralis* excretory-secretory antigen (32) or anti-IgE (clone A2) (33) for capture, and biotinylated mouse anti-isotype antibodies followed by horseradish peroxidase-conjugated streptavidin. Antibodies were detected with biotinylated monoclonal mouse anti-rat IgG1 (RB11/39), IgG2a (RG7/1), IgG2b (RG7/11), or IgE (MARE-1). Antibodies were biotinylated with N-hydroxysuccinimidobiotin (Sigma; St Louis, MO) in bicarbonate buffer (pH 9.5; 120 µg biotin per 1 mg of antibody) for 4 hours at room temperature and used at 1 µg/ml. Biotin-conjugated anti-rat IgG2c (BD Pharmingen; San Diego, CA) was used at 0.25 µg/ml. Standard curves for IgG1, IgG2a, IgG2b, and IgG2c utilized tyvelose-specific monoclonal antibodies 9D4, 18H1, 10G11, and 9E6, respectively. Sera from rats were diluted 1:6000 for measuring IgG1 and IgG2a, 1:100 or 1:500 for IgG2b, or 1:2500 for IgG2c. In order to measure total serum IgE, ELISA plates were coated with anti-rat IgE mAb A2 (5 µg/ml) rat myeloma IgE 162 (34) served as a standard, rat sera were diluted 1:100, and biotin-conjugated MARE-1 (5 µg/ml) was used as the detection reagent. RMCPII was detected in rat sera using the RMCPII ELISA kit purchased from Moredun Scientific Limited (Midlothian, Scotland).

## **Passive immunization of rat pups**

One hour prior to oral infection with  $L_1$ , groups of 6 to 8 rat pups were fed antibodies (5 mg/ml in 0.85% NaCl) at a dose of 2.5mg/20g of body weight. Antibody treatments included serum immunoglobulin from infected or uninfected rats, tyvelose-specific mAb, and precipitates or supernatants from immune serum. Pups treated with whole serum or serum fractions were fed two doses of 0.5 ml, two hours and one hour prior to challenge infection. Adult rats were passively immunized by intraperitoneal injection of 5 mg of tyvelose-specific mAb or normal serum immunoglobulin and challenged orally 16 hours later.

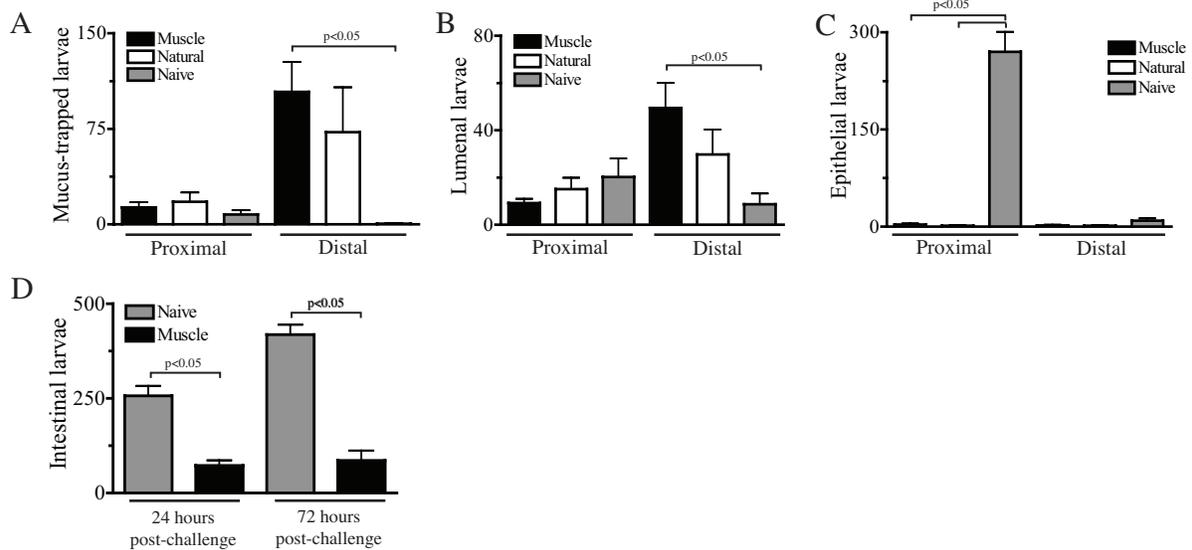
## Statistical analyses

Student's t test was used to compare means for treatment groups. Analysis of variance and Tukey's least significant difference test were used to detect differences among three or more means. Data are presented as mean  $\pm$  standard deviation (n = 5-8 rats). In experiments where pups of infected dams were challenged, means were calculated for each litter and those values assigned to the dam in order to calculate the mean for the treatment group. For passive immunization experiments, rat pups in each group were selected from a randomized pool from several litters. Differences were considered significant when the p-value was less than 0.05.

## Results

### **Immunity induced by intravenous infection is indistinguishable from rapid expulsion.**

Infection with *T. spiralis* occurs when a host ingests muscle tissue containing *T. spiralis* L<sub>1</sub>. Infection begins when L<sub>1</sub> invade epithelial cells lining the small intestine, where they molt four times to become adult worms (35). Adult worms in this epithelial syncytium mate, and the females release NBL, which enter the blood stream and subsequently establish a chronic muscle infection. We have shown previously that rats infected by intravenous injection of NBL develop muscle infections that engender immunity against re-infection of the intestine (36). In order to confirm that this immunity was equivalent to rapid expulsion induced by natural infection, we evaluated the distribution of larvae in the period immediately following challenge infection. Mucus entrapment of luminal larvae within the first hour of challenge is a hallmark of rapid expulsion (27, 37). Such entrapment occurred in both parenterally and orally infected rats after oral challenge (Fig 2.1A). In both groups, L<sub>1</sub> were excluded from the small intestinal epithelium, as evidenced by increased numbers of L<sub>1</sub> in the lumen of the distal intestine (Fig 2.1B) and dramatically fewer larvae recoverable from the epithelium (Fig 2.1C), compared to naïve control animals. Intestinal burdens were not further reduced between 24 and 72 hours post-challenge, indicating that the response was specific and restricted to larval, rather than adult, stages (Fig. 2.1D). The increased number of worms recovered from naïve rats at 72 hours post-challenge can



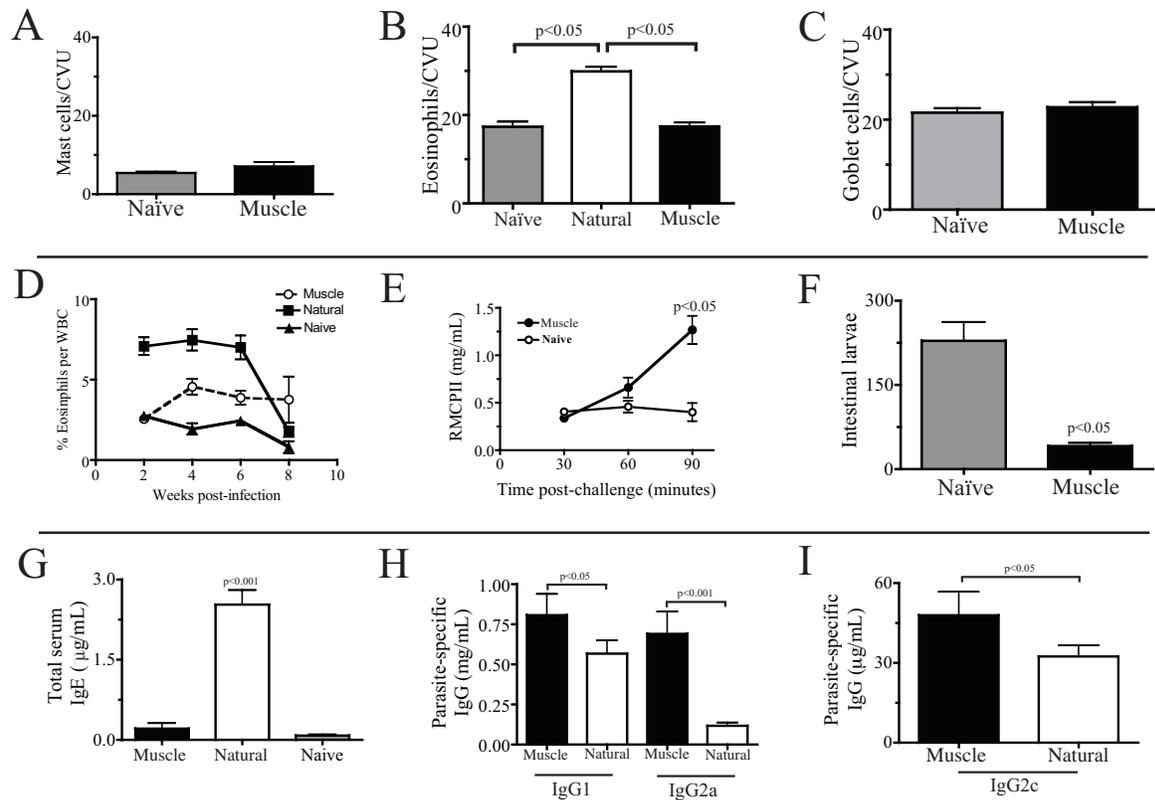
**FIGURE 2.1. Intestinal immunity induced by muscle infection.** (A-C) Distribution of larvae in previously infected and naïve AO rats sixty to ninety minutes post-challenge with 500  $L_1$ . Rats were infected intravenously with 80,000 NBL (muscle) or with 1000  $L_1$  (natural). Proximal and distal small intestine contents were collected separately, and larvae were counted that were (A) trapped in mucus, (B) free in the intestinal lumen or (C) able to migrate into saline from the epithelium. (D) Intestinal burdens in naïve rats and rats immunized by muscle infection. Burdens were stable after 24 hours, indicating that immunity was specific for larval stages.

be explained by the failure of molting larvae (present in the intestine 24 hours post-infection) to emerge from the intestinal epithelium. Overall, the kinetics of entrapment and exclusion following intestinal challenge of parenterally infected rats are entirely consistent with rapid expulsion.

### **Oral challenge of parenterally infected rats causes mast cell degranulation in the absence of mastocytosis.**

We sought to evaluate the cellular response in the intestine after parenteral infection, including evaluation of mast cell degranulation upon challenge infection. Jejunal mast cells, eosinophils, and goblet cells were not increased in parenterally infected rats (Fig. 2.2A, B, C), although there was a mild but prolonged blood eosinophilia (Fig. 2.2D). Despite the absence of intestinal mastocytosis, oral challenge of parenterally infected rats induced a significant increase in serum RMCPII concentration (Fig. 2.2E) that correlated with larval expulsion (Fig. 2.2F). The amount of RMCPII detected in parenterally immunized rats was consistent with what we have detected in orally immunized rats in several other experiments (1,000 - 2,000 ng/mL). These results reveal that degranulation of mucosal mast cells was coincident with expulsion, even in the absence of mastocytosis. Attempts to inhibit mast cell degranulation by treating rats with the mast cell stabilizer, doxantrazole, were unsuccessful. Therefore, we were unable to determine whether mast cell activation contributed to rapid expulsion.

Comparison of antibody responses between naturally and parenterally infected rats revealed a striking difference. Infection limited to the muscle did not cause a significant increase in the total serum concentration of IgE, but did induce a strong parasite-specific IgG2a response (Fig. 2.2G,H). Both IgE and IgG2a bind to the high affinity IgE receptor, FcεRI (38), so it is possible that mast cell activation was mediated by IgG2a in parenterally infected rats and by both isotypes in naturally infected rats. Either infection regime induced antigen-specific IgG1 and IgG2c (Fig. 2.2H, I), two isotypes known to mediate rapid expulsion in rat pups (21).



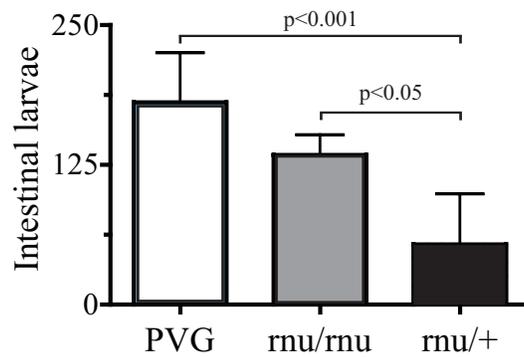
**FIGURE 2.2. Cellular and antibody responses to muscle infection.** Intestinal sections from rats immunized via muscle infection were evaluated for (A) mastocytosis, (B) eosinophilia, and (C) goblet cell hyperplasia. (D) Blood eosinophilia during infection. (E) RMCPII concentrations in sera collected 30 to 90 minutes post-challenge. (F) Intestinal burden 24 hours after challenge with 500  $L_1$  in naïve rats and rats immunized with muscle infection. (G-I) Serum antibodies 9 weeks following infection with 1,000  $L_1$  or 80,000 NBL.

### **Parenteral immunization generates a T cell-dependent mucosal response.**

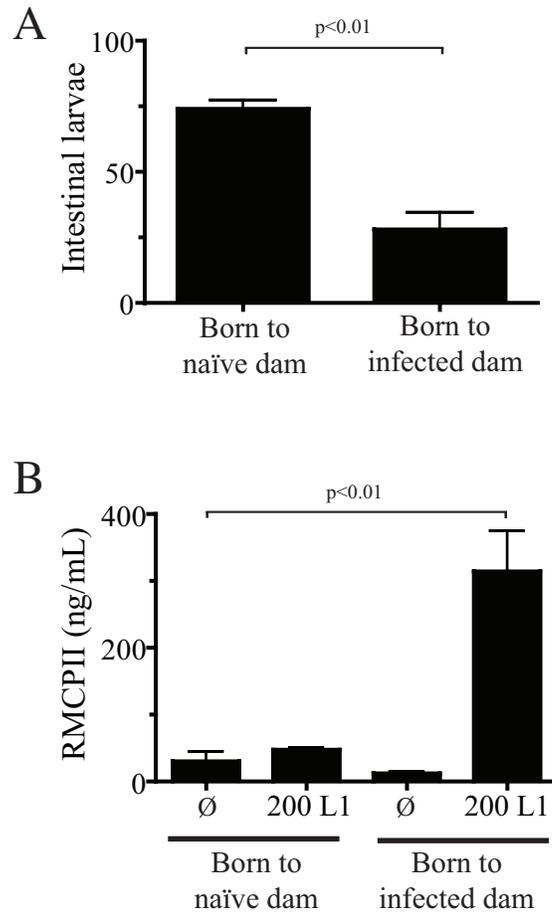
Mastocytosis in *T. spiralis* infection is T cell-dependent (39, 40). Because intestinal mastocytosis was not required for rapid expulsion, and knowing that IgG2c mediates rapid expulsion in rat pups and can be produced in a T-cell independent manner (41), we tested the requirement for T cells in rapid expulsion by challenging parenterally infected, T cell-deficient rats. Only euthymic rats demonstrated rapid expulsion, indicating that T cells were required for immunity (Fig. 2.3). Similar results were obtained in PVG rats made T lymphocyte-deficient by thymectomy, followed by irradiation and bone marrow reconstitution (data not shown). In another experiment, we passively immunized parenterally infected rnu/rnu rats with monoclonal, tyvelose-specific IgG1. These animals did not demonstrate rapid expulsion, a result that is consistent with a requirement for T cells in addition to antibody production (data not shown). Blood RMCPII was not elevated in nude rats following challenge, consistent with a failure of both T lymphocytes and T-dependent, antibody production (data not shown).

### **Mast cell degranulation is coincident with rapid expulsion in rat pups fostered by infected dams.**

With the aim of using suckling rat pups to study secondary expulsion of *T. spiralis*, we evaluated mast cell degranulation in this system. Rat pups fostered by a dam infected with *T. spiralis* demonstrate rapid expulsion (20, 42). Passive immunization with tyvelose-specific mAbs confers immunity upon pups born to naïve dams (21). Antibodies delivered immediately prior to infection are protective, indicating that cells that may interact with antibodies to effect expulsion are resident in the normal neonatal rat gut. We evaluated MMC degranulation in rat pups that displayed rapid expulsion (Fig. 2.4A). Prior to challenge, serum RMCPII concentrations were similar in pups born to infected dams compared with pups of naïve dams (Fig. 2.4B). When both groups were challenged with 200 L<sub>1</sub>, only pups born to infected dams showed significantly elevated serum RMCPII concentrations (Fig. 2.4B). Thus, maternal immunity confers upon pups the capacity to activate mast cells upon oral infection with *T.*



**FIGURE 2.3. Evaluation of intestinal immunity induced by muscle infection in nude rats.** Athymic nude (rnu/rnu) rats and euthymic (rnu/+) littermates were immunized by intravenous injection of 30,000 NBL. Uninfected PVG strain rats served as controls. Rats were challenged orally with 500 L<sub>1</sub> 9 weeks after muscle infection. Intestinal parasite burden was determined 24 hours after challenge.



**FIGURE 2.4. Mucosal mast cell degranulation in rat pups born to infected dams.<sup>2</sup> (A)**

Rat pups born to a naturally infected dam exhibit rapid expulsion upon challenge infection.

(B) RMCPII concentrations in sera of pups born to infected and uninfected dams. Pups were unchallenged (Ø) or challenged orally with 200 L<sub>1</sub>. Blood was collected 3 hours post-challenge. Mean RMCPII concentration in sera of challenged pups of immune dams is significantly higher than all other groups (p < 0.01).

<sup>2</sup>Experiments performed by L.K. Blum and others.

*spiralis*.

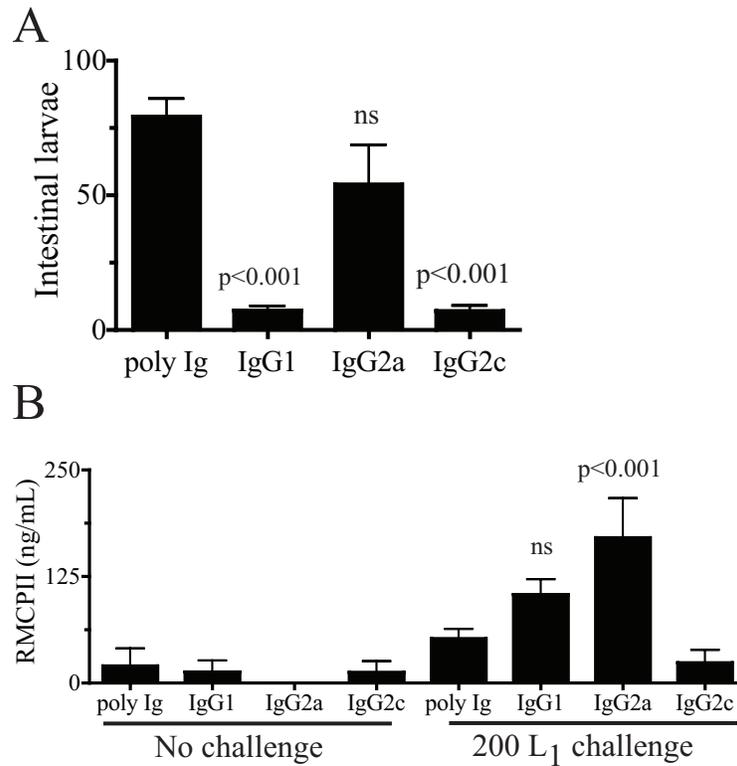
### **RMCP II release does not correlate with rapid expulsion in passively immunized rat pups.**

Since the antibody isotypes that provide immunity to rat pups are not the same isotypes known to cause mast cell degranulation in rats, we were able to manipulate this system to separate mast cell degranulation from protective immunity. Pups were fed tyvelose-specific mAbs of different isotypes one hour prior to oral challenge (Fig. 2.5). Treatment with IgG2c afforded protection but did not cause a significant increase in serum RMCP II concentration. Conversely, IgG2a caused a significant release of RMCP II, but did not provide protection. Pups treated with IgG1 were protected and modest increases in serum RMCP II were induced. These results indicate that MMC degranulation is not required for rapid expulsion. Furthermore, activation of mast cells by IgG2a was not sufficient to cause rapid expulsion.

To test whether the effects observed were unique to mAbs, pups were fed fractions of serum enriched for either IgE or IgG. IgG precipitates in 40% saturated  $(\text{NH}_4)_2\text{SO}_4$ , while IgE remains soluble (31). As shown in Figure 2.6, whole immune serum and precipitated IgG from immune serum caused rapid expulsion, while the supernatant was not protective, as we have shown previously (26) (Fig. 2.6A). Administration of serum or fractions of serum did not cause RMCP II release prior to challenge with parasites (Fig. 2.6B). Following challenge, pups treated with any fraction of immune serum had elevated concentrations of RMCP II in their sera (Fig. 2.6C). These results confirm that RMCP II release is not sufficient to cause rapid expulsion.

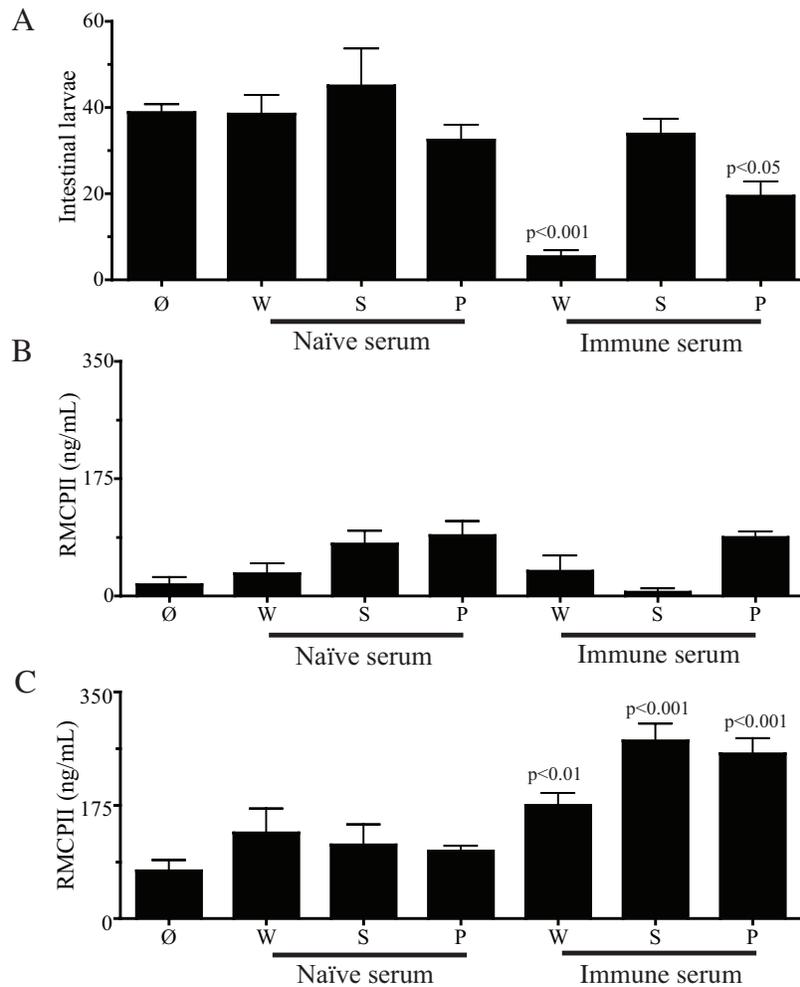
### **RMCP II release is not sufficient to cause rapid expulsion in passively immunized adult rats**

We continued these studies by investigating whether the dissociation of mast cell degranulation from rapid expulsion was possible in adult rats. Five- to six-week old rats (weaned at 3 weeks of age) were injected intraperitoneally with tyvelose-specific mAbs 16 hours prior to oral challenge with  $L_1$  (Figure 2.7). We have previously reported that although treatment with tyvelose-specific IgG2c is not protective in weaned rats, it causes  $L_1$  to become encumbered in the intestinal epithelium such that parasites are not released into saline during the standard



**FIGURE 2.5. RMCPII release in rat pups passively immunized with parasite-specific monoclonal antibodies.**<sup>3</sup> (A) Intestinal larvae in passively immunized pups 3 hours post-challenge. (B) RMCPII concentration was measured in serum collected prior to challenge or 3 hours post-challenge. Values in IgG1 treated rats were reproducibly elevated in replicate experiments, although not to the level of statistical significance.

<sup>3</sup>Experiments performed by L.K. Blum and others.



**FIGURE 2.6. RMCPII release in rat pups treated with fractions of serum from infected rats.**<sup>4</sup> Rat pups were left untreated ( $\emptyset$ ) or were treated orally with  $(\text{NH}_4)_2\text{SO}_4$  precipitate (P), supernatant (S) or whole serum (W) collected from naturally infected or naïve rats. (A) Intestinal burden in pups treated with serum fractions and challenged orally with 200  $L_1$ . (B) RMCPII concentrations in sera of passively immunized pups prior to challenge. (C) RMCPII concentrations in sera of passively immunized pups 3 hours post-challenge with 200  $L_1$ .

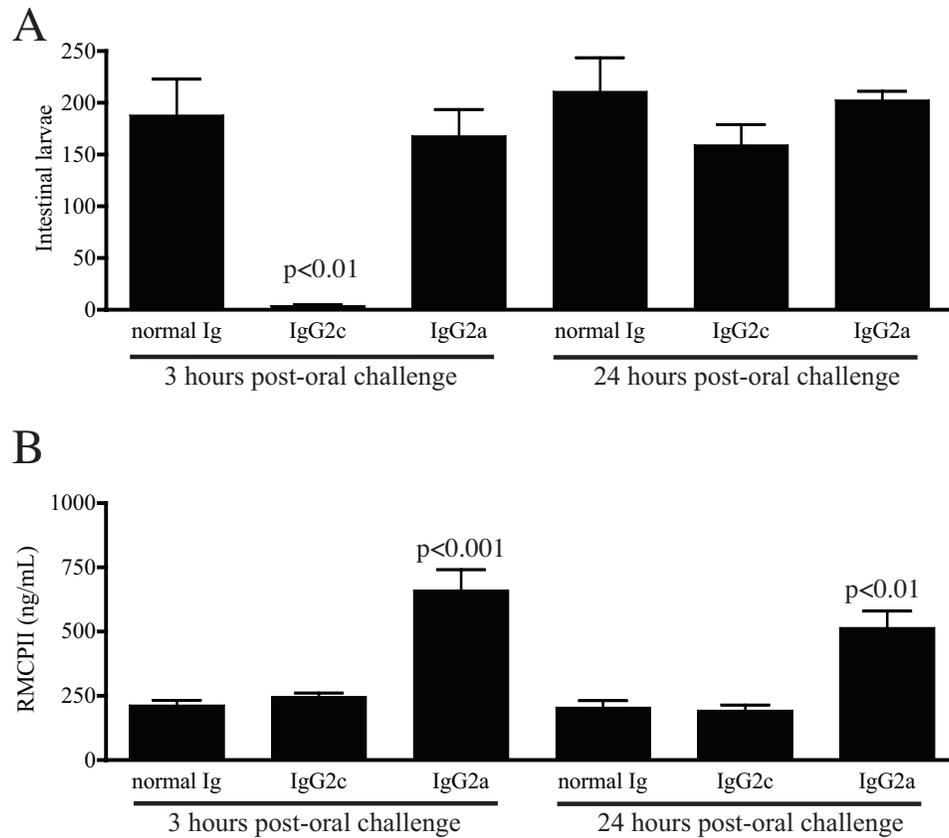
<sup>4</sup>Experiments performed by L.K. Blum and others.

recovery procedure. This effect is reversed at the time of the first molt (25). In the experiment shown in Figure 2.7, we also measured serum RMCPII, and found that the protease was significantly increased in rats treated with IgG2a but not those treated with IgG2c (Figure 2.7B). IgG2a-treated adult rats did not expel the infection, despite the increase in RMCPII (Figure 2.7A). The results confirm that immunity is independent of RMCPII release.

### Discussion

Rapid expulsion is a rare example of an immune response that causes immediate elimination of an intestinal worm. Elucidation of the mechanism of immunity would afford valuable insight applicable to the development of new prophylactic measures that would protect humans and animals against nematode infection. The effort to elucidate the mechanism of immunity in rapid expulsion has been challenged by the combinatorial nature of immune mechanisms in adult rats. Several studies that tested adoptive transfer of lymphocytes or passive immunization with specific antibodies failed to fully replicate rapid expulsion in adult recipients (29, 43, 44), suggesting that innate mediators contribute to immunity. In this report we show that rapid expulsion can be induced by an infection limited to the muscle phase and that immunity is T lymphocyte-dependent. We investigated whether this T-dependent effect involved mucosal mast cells or intestinal mastocytosis.

Previous studies showed a correlation between *T. spiralis* expulsion and mucosal mast cell degranulation, but a specific mediator or causal relationship was not identified (2-4, 45). Rats maintain a resident population of mucosal mast cells in the lamina propria of the small intestine under homeostatic conditions (46). It has been suggested that mast cell activation might be a direct effect of the parasite, and such activation has been demonstrated *in vitro* following exposure of cultured mast cells to tyvelose-bearing *T. spiralis* antigens (47, 48). In contrast, we found that parasite challenge in the absence of specific antibodies did not cause significant RMCPII release. The kinetics of RMCPII release in immune rats was consistent with antibody-dependent activation. In rats displaying rapid expulsion following immunization via muscle



**FIGURE 2.7. RMCPII release in passively immunized adult rats.**<sup>5</sup> Adult rats were injected intraperitoneally with 5 mg of tyvelose-specific IgG2c or IgG2a, or normal serum immunoglobulin (negative control). (A) Intestinal larvae burden and (B) serum RMCPII concentration were determined at 3 and 24 hours post-challenge with 500  $L_1$ .

<sup>5</sup>Experiments performed by L.K. Blum and others.

infection, IgG2a was the isotype most likely to cause degranulation, while in orally infected rats IgE was present in high concentrations. The differences in immune responses induced by intestinal and muscle infection reduces the likelihood of a role for two other innate mediators, goblet cells and eosinophils. Both have been hypothesized to contribute to rapid expulsion.

Throughout the published literature describing rapid expulsion, the most consistent protective effects are attributed to antibodies (49). Rapid expulsion displayed by neonatal rat pups born to infected dams can be reproduced in pups born to naïve dams by passive immunization with tyvelose-specific IgG (21). The protective mechanisms of the antibodies have been documented *in vivo* (21, 24) and confirmed in an *in vitro* model (23). Direct effects of antibodies on *T. spiralis* larvae include entrapment of larvae in mucus as well as exclusion from and encumbrance in the epithelium (23, 24). The potential for mast cells to contribute to antibody-mediated protection has not been investigated previously in neonatal rats. Furthermore, the functional activities of mucosal mast cells in neonatal rats had not been tested in the context of infection.

We found that rat pups born to infected dams demonstrated mast cell activation coincident with rapid expulsion. Passive immunization allowed for the separation of RMCPII release from rapid expulsion. It has been established that passive immunization with tyvelose-specific monoclonal IgG1 or IgG2c confers protection on rat pups, while monoclonal IgG2a and polyclonal IgE are not protective (20, 21, 26). Thus, the isotypes known to cause mast cell degranulation via FcεRI crosslinking (38, 50), and shown to induce release of RMCPII in our experiments, do not protect pups. Our results show that rat mucosal mast cells are weakly activated by IgG1. IgG1 binds the FcεRI (Thrasher and Appleton, unpublished observation) although reports of receptor-mediated activation of mast cells by IgG1 are contradictory (38, 50). It is possible that rat mucosal mast cells are activated by IgG1 via binding to other FcR or by some indirect mechanism, such as complement activation. Experiments with IgE rich serum fractions provided confirmation that release of RMCPII was not sufficient to cause rapid expulsion. Conversely, passive immunization with IgG2c was protective but did not induce

RMCP II release, indicating that expulsion of larvae can occur independently of mast cell activation. RMCP II release caused by IgG2a was not sufficient to provide a protective secondary immune response to adult rats. Overall, our results do not support a role for RMCP II or mast cells in rapid expulsion.

Support for the conclusion that mast cells are not pivotal in rapid expulsion was provided by studies using pharmacological agents that block histamine, serotonin, prostaglandins, and intestinal motility (45). Treatment of infected adult rats with inhibitors of various mast cell effectors did not prevent rapid expulsion. The speed of expulsion would not allow for *de novo* synthesis of mast cell proteins, although cytokines that are pre-stored by mast cells, such as TNF $\alpha$ , may play a role (51). While mMCP-1 promotes adult worm rejection in mice, our results show that release of the equivalent mediator, RMCP II, is not required for rapid expulsion of *T. spiralis* in rat pups, and that release of RMCP II can occur in pups and adults without causing expulsion.

Infection limited to the muscle phase induced rapid expulsion in the absence of intestinal priming and the cellular changes priming induces. While this suggests that systemic immunization against a parasitic nematode produced a mucosal immune response, it must be noted that NBL are efficiently delivered to the lung via intravenous injection and some NBL may reach the intestine via the bloodstream. Efforts to inject NBL directly into the muscles of the hind limbs in numbers sufficient to induce immunity consistently allowed a small proportion of NBL to enter the blood and colonize remote sites (Blum and Appleton, not shown) thus preventing us from determining whether migration through the lung or other tissues was necessary for the induction of immunity. Although we did not detect the obvious cellular changes in the intestines of rats infected by this route, it is possible for stimulation of mucosal associated lymphoid tissue (MALT) in one site to affect another site. An additional source of cellular mediators may derive from the body cavities where cellular expansion during muscle infection has been documented in mice (52, 53). Recirculation of cells from the cavities to the intestinal tract, or direct effects of peritoneal responses on the gut have been shown to be a part of the body's defense against

intestinal pathogens (54). The diaphragm is a preferred site for developing *T. spiralis* larvae and the interaction between cavity cells and the parasite in this location may drive subtle alterations in the intestine. Such alterations may differ from those that are commonly associated with nematode infection such as eosinophilia, goblet cell hyperplasia or mastocytosis.

Taken together, results from parenteral infection of adult rats and passive immunization of neonates and adults demonstrate that both IgE and IgG2a activate mast cells during rapid expulsion. Our data support the conclusion that neither mastocytosis nor release of RMCPII are required for rapid expulsion. Identification of the factor that enables antibodies to protect adult rats requires further investigation. Our results have significance for the development of vaccines against helminths, as they show that protective immunity can be engendered without inducing a potentially dangerous allergic response to antigen.

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### **Disclosures**

The authors have no financial conflicts of interest.

### **References**

1. **Alizadeh, H., and D. Wakelin.** 1982. Comparison of rapid expulsion of *Trichinella spiralis* in mice and rats. *Int J Parasitol* 12:65-73.
2. **Moqbel, R., D. Wakelin, A. J. MacDonald, S. J. King, R. K. Grencis, and A. B. Kay.** 1987. Release of leukotrienes during rapid expulsion of *Trichinella spiralis* from immune rats. *Immunology* 60:425-430.
3. **Tuohy, M., D. A. Lammas, D. Wakelin, J. F. Huntley, G. F. Newlands, and H. R. Miller.** 1990. Functional correlations between mucosal mast cell activity and immunity to

- Trichinella spiralis* in high and low responder mice. *Parasite Immunol* 12:675-685.
4. **Woodbury, R. G., H. R. Miller, J. F. Huntley, G. F. Newlands, A. C. Palliser, and D. Wakelin.** 1984. Mucosal mast cells are functionally active during spontaneous expulsion of intestinal nematode infections in rat. *Nature* 312:450-452.
  5. **Ha, T. Y., N. D. Reed, and P. K. Crowle.** 1983. Delayed expulsion of adult *Trichinella spiralis* by mast cell-deficient W/W<sup>v</sup> mice. *Infect Immun* 41:445-447.
  6. **Kamiya, M., Y. Oku, H. Itayama, and M. Ohbayashi.** 1985. Prolonged expulsion of adult *Trichinella spiralis* and eosinophil infiltration in mast cell-deficient W/W<sup>v</sup> mice. *J Helminthol* 59:233-239.
  7. **Oku, Y., H. Itayama, and M. Kamiya.** 1984. Expulsion of *Trichinella spiralis* from the intestine of W/W<sup>v</sup> mice reconstituted with haematopoietic and lymphopoietic cells and origin of mucosal mast cells. *Immunology* 53:337-344.
  8. **Donaldson, L. E., E. Schmitt, J. F. Huntley, G. F. Newlands, and R. K. Grencis.** 1996. A critical role for stem cell factor and c-kit in host protective immunity to an intestinal helminth. *Int Immunol* 8:559-567.
  9. **Faulkner, H., N. Humphreys, J. C. Renauld, J. Van Snick, and R. Grencis.** 1997. Interleukin-9 is involved in host protective immunity to intestinal nematode infection. *Eur J Immunol* 27:2536-2540.
  10. **Lawrence, C. E., Y. Y. Paterson, S. H. Wright, P. A. Knight, and H. R. Miller.** 2004. Mouse mast cell protease-1 is required for the enteropathy induced by gastrointestinal helminth infection in the mouse. *Gastroenterology* 127:155-165.
  11. **McDermott, J. R., R. E. Bartram, P. A. Knight, H. R. Miller, D. R. Garrod, and R. K. Grencis.** 2003. Mast cells disrupt epithelial barrier function during enteric nematode infection. *Proc Natl Acad Sci U S A* 100:7761-7766.
  12. **Russell, D. A., and G. A. Castro.** 1984. Immune rejection of *Trichinella spiralis* infective larvae in the guinea pig. *J Parasitol* 70:593-595.
  13. **Palmer, J. M., and G. A. Castro.** 1986. Anamnestic stimulus-specific myoelectric

- responses associated with intestinal immunity in the rat. *Am J Physiol* 250:G266-273.
14. **Hessel, J., K. Ramaswamy, and G. A. Castro.** 1982. Reduced hexose transport by enterocytes associated with rapid, noninjurious rejection of *Trichinella spiralis* from immune rats. *J Parasitol* 68:202-207.
  15. **Bell, R. G.** 1992. *Trichinella spiralis*: evidence that mice do not express rapid expulsion. *Exp Parasitol* 74:417-430.
  16. **Grove, D. I., J. Hamburger, and K. S. Warren.** 1977. Kinetics of immunological responses, resistance to reinfection, and pathological reactions to infection with *Trichinella spiralis*. *J Infect Dis* 136:562-570.
  17. **Chernin, J., H. R. Miller, G. F. Newlands, and D. J. McLaren.** 1988. Proteinase phenotypes and fixation properties of rat mast cells in parasitic lesions caused by *Mesocostoides corti*: selective and site-specific recruitment of mast cell subsets. *Parasite Immunol* 10:433-442.
  18. **Gibson, S., A. Mackellar, G. F. Newlands, and H. R. Miller.** 1987. Phenotypic expression of mast cell granule proteinases. Distribution of mast cell proteinases I and II in the rat digestive system. *Immunology* 62:621-627.
  19. **Huntley, J. F., A. Mackellar, G. F. Newlands, J. Irvine, and H. R. Miller.** 1990. Mapping of the rat mast cell granule proteinases RMCPI and II by enzyme-linked immunosorbent assay and paired immunofluorescence. *APMIS* 98:933-944.
  20. **Appleton, J. A., and D. D. McGregor.** 1984. Rapid expulsion of *Trichinella spiralis* in suckling rats. *Science* 226:70-72.
  21. **Appleton, J. A., L. R. Schain, and D. D. McGregor.** 1988. Rapid expulsion of *Trichinella spiralis* in suckling rats: mediation by monoclonal antibodies. *Immunology* 65:487-492.
  22. **Reason, A. J., L. A. Ellis, J. A. Appleton, N. Wisnewski, R. B. Grieve, M. McNeil, D. L. Wassom, H. R. Morris, and A. Dell.** 1994. Novel tyvelose-containing tri- and tetra-antennary N-glycans in the immunodominant antigens of the intracellular parasite

- Trichinella spiralis*. *Glycobiology* 4:593-603.
23. **McVay, C. S., P. Bracken, L. F. Gagliardo, and J. Appleton.** 2000. Antibodies to tyvelose exhibit multiple modes of interference with the epithelial niche of *Trichinella spiralis*. *Infect Immun* 68:1912-1918.
  24. **Carlisle, M. S., D. D. McGregor, and J. A. Appleton.** 1990. The role of mucus in antibody-mediated rapid expulsion of *Trichinella spiralis* in suckling rats. *Immunology* 70:126-132.
  25. **Otubu, O. E., M. S. Carlisle-Nowak, D. D. McGregor, R. H. Jacobson, and J. A. Appleton.** 1993. *Trichinella spiralis*: the effect of specific antibody on muscle larvae in the small intestines of weaned rats. *Exp Parasitol* 76:394-400.
  26. **Appleton, J. A., and D. D. McGregor.** 1987. Characterization of the immune mediator of rapid expulsion of *Trichinella spiralis* in suckling rats. *Immunology* 62:477-484.
  27. **Bell, R. G., J. A. Appleton, D. A. Negrao-Correa, and L. S. Adams.** 1992. Rapid expulsion of *Trichinella spiralis* in adult rats mediated by monoclonal antibodies of distinct IgG isotypes. *Immunology* 75:520-527.
  28. **Harari, Y., and G. A. Castro.** 1989. Simulation of parasite-induced gut hypersensitivity: implications for vaccination. *Immunology* 66:302-307.
  29. **Crum, E. D., D. D. Despommier, and D. D. McGregor.** 1977. Immunity to *Trichinella spiralis*. I. Transfer of resistance by two classes of lymphocytes. *Immunology* 33:787-795.
  30. **Beiting, D. P., L. F. Gagliardo, M. Hesse, S. K. Bliss, D. Meskill, and J. A. Appleton.** 2007. Coordinated control of immunity to muscle stage *Trichinella spiralis* by IL-10, regulatory T cells, and TGF-beta. *J Immunol* 178:1039-1047.
  31. **Carson, D., H. Metzger, and H. Bazin.** 1975. Simple radio-immunoassay for the measurement of human and rat IgE levels by ammonium sulfate precipitation. *J Immunol* 115:561-563.
  32. **Appleton, J. A., and L. Usack.** 1993. Identification of potential antigenic targets for rapid expulsion of *Trichinella spiralis*. *Mol Biochem Parasitol* 58:53-62.

33. **Conrad, D. H., E. Studer, J. Gervasoni, and T. Mohanakumar.** 1983. Properties of two monoclonal antibodies directed against the Fc and Fab' regions of rat IgE. *Int Arch Allergy Appl Immunol* 70:352-360.
34. **Mendoza, G. R., and H. Metzger.** 1976. Disparity of IgE binding between normal and tumor mouse mast cells. *J Immunol* 117:1573-1578.
35. **Despommier, D. D.** 1983. Biology. In *Trichinella and trichinosis*. W. C. Campbell, ed. Plenum Press, New York. 75-151.
36. **Appleton, J. A., and D. D. McGregor.** 1985. Life-phase specific induction and expression of rapid expulsion in rats suckling *Trichinella spiralis*-infected dams. *Immunology* 55:225-232.
37. **Carlisle, M. S., D. D. McGregor, and J. A. Appleton.** 1991. Intestinal mucus entrapment of *Trichinella spiralis* larvae induced by specific antibodies. *Immunology* 74:546-551.
38. **Benhamou, M., E. H. Berenstein, M. H. Jouvin, and R. P. Siraganian.** 1994. The receptor with high affinity for IgE on rat mast cells is a functional receptor for rat IgG2a. *Mol Immunol* 31:1089-1097.
39. **Ruitenbergh, E. J., and A. Elgersma.** 1976. Absence of intestinal mast cell response in congenitally athymic mice during *Trichinella spiralis* infection. *Nature* 264:258-260.
40. **Vos, J. G., E. J. Ruitenbergh, N. Van Basten, J. Buys, A. Elgersma, and W. Kruizinga.** 1983. The athymic nude rat. IV. Immunocytochemical study to detect T-cells, and immunological and histopathological reactions against *Trichinella spiralis*. *Parasite Immunol* 5:195-215.
41. **Peters, P. J., L. F. Gagliardo, E. A. Sabin, A. B. Betchen, K. Ghosh, J. B. Oblak, and J. A. Appleton.** 1999. Dominance of immunoglobulin G2c in the antiphosphorylcholine response of rats infected with *Trichinella spiralis*. *Infect Immun* 67:4661-4667.
42. **Culbertson, J. T.** 1943. Natural Transmission of Immunity against *Trichinella spiralis* from Mother Rats to Their Offspring. *The Journal of Parasitology* 29:114-116.

43. **Bell, R. G., and D. D. McGregor.** 1980. Rapid expulsion of *Trichinella spiralis*: coinduction by using antigenic extracts of larvae and intestinal stimulation with an unrelated parasite. *Infect Immun* 29:194-199.
44. **Love, R. J., B. M. Ogilvie, and D. J. McLaren.** 1976. The immune mechanism which expels the intestinal stage of *Trichinella spiralis* from rats. *Immunology* 30:7-15.
45. **Bell, R. G., D. D. McGregor, and L. S. Adams.** 1982. Studies on the inhibition of rapid expulsion of *Trichinella spiralis* in rats. *Int Arch Allergy Appl Immunol* 69:73-80.
46. **Enerback, L.** 1966. Mast cells in rat gastrointestinal mucosa. I. Effects of fixation. *Acta Pathol Microbiol Scand* 66:289-302.
47. **Bolas-Fernandez, F., and L. D. Corral Bezara.** 2006. TSL-1 antigens of *Trichinella*: an overview of their potential role in parasite invasion, survival and serodiagnosis of trichinellosis. *Res Vet Sci* 81:297-303.
48. **Yepez-Mulia, L., R. Hernandez-Bello, N. Arizmendi-Puga, R. Fonseca-Linan, and G. Ortega-Pierres.** 2007. Contributions to the study of *Trichinella spiralis* TSL-1 antigens in host immunity. *Parasite Immunol* 29:661-670.
49. **Bell, R. G.** 1998. The generation and expression of immunity to *Trichinella spiralis* in laboratory rodents. *Adv Parasitol* 41:149-217.
50. **Philips, J. R., W. Brouwer, M. Edwards, S. Mahler, J. Ruhno, and A. M. Collins.** 1999. The effectiveness of different rat IgG subclasses as IgE-blocking antibodies in the rat basophil leukaemia cell model. *Immunol Cell Biol* 77:121-126.
51. **Gordon, J. R., and S. J. Galli.** 1990. Mast cells as a source of both preformed and immunologically inducible TNF-alpha/cachectin. *Nature* 346:274-276.
52. **Beiting, D. P., S. K. Bliss, D. H. Schlafer, V. L. Roberts, and J. A. Appleton.** 2004. Interleukin-10 limits local and body cavity inflammation during infection with muscle-stage *Trichinella spiralis*. *Infect Immun* 72:3129-3137.
53. **Chang, N. C., S. I. Hung, K. Y. Hwa, I. Kato, J. E. Chen, C. H. Liu, and A. C. Chang.** 2001. A macrophage protein, Ym1, transiently expressed during inflammation is a novel

mammalian lectin. *J Biol Chem* 276:17497-17506.

54. **van der Vliet, A., K. I. van der Poel, and A. Bast.** 1992. Intestinal smooth muscle dysfunction after intraperitoneal injection of zymosan in the rat: are oxygen radicals involved? *Gut* 33:336-341.

## Chapter 3

### Intestinal infection with *Trichinella spiralis* induces distinct, regional immune responses<sup>1</sup>

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<sup>1</sup> Originally in *Veterinary Parasitology*.

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## Abstract

The aim of this study was to evaluate differences between the small and large intestines (SI and LI) with regard to colonization and immunity during infection with *Trichinella spiralis*. In orally infected C57BL/6 mice, the gender ratios of worms differed among the SI, cecum, and LI. Mucosal mastocytosis developed in the SI but not in the LI, consistent with reduced IL-9 and IL-13 production by explants from the LI. Despite these differences, worms were cleared at the same rate from both sites. Furthermore, IL-10 production was reduced in the LI, yet it was instrumental in limiting local inflammation. Finally, passive immunization of rat pups with tyvelose-specific antibodies effectively cleared fist-stage larvae from all intestinal regions. We conclude that despite regional differences in immune responsiveness and colonization, immune mechanisms that clear *T. spiralis* operate effectively throughout the intestinal tract.

## Introduction

Regions of the vertebrate intestine differ with regard to size, physiology, and microbial flora. In addition, the SI and the LI constitute distinct immune environments, with differences in regulatory T cell phenotypes and the production of IL-10 and TGF- $\beta$  (6, 19). Endothelial cells displaying MAdCAM-1 recruit  $\alpha 4\beta 7$ -expressing lymphocytes throughout the intestine, while CCR9-bearing lymphocytes are selectively recruited to the SI by locally produced CCL25/TECK (14). In this way, immune responses can be directed to the SI. Although many parasites are restricted in their distribution in the gastrointestinal tract, *Trichinella spiralis* colonizes both the small and large intestines (10, 21), providing an opportunity to investigate variation in regional immune responses and the impact this may have on infection.

## Materials and Methods

### Animals

C57BL/6<sup>NHsd</sup> and BALB/c<sup>NHsd</sup> were purchased (Harlan, USA) and IL-10 deficient

(B6.129P2-IL10<sup>tm1Cgn</sup>) mice and Albino Oxford (AO) rats were bred and maintained under specific pathogen-free conditions according to the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. Experiments were performed with the approval of the Cornell University Institutional Animal Care and Use Committee.

### **Parasite and infections**

Maintenance of *Trichinella spiralis* (pig strain), L<sub>1</sub> recovery, and intestinal worm burden estimation were carried out as described (9, 13). Adult mice (7 to 10 weeks old) or suckling rat pups (12 to 16 days old) received 400 or 200 L<sub>1</sub> by gavage, respectively. Crude L<sub>1</sub> parasite extract (cAg) was prepared as described (4).

### **Tissue collection, preparation, and evaluation**

Mice were euthanized with CO<sub>2</sub>. The SI and LI were removed and cut longitudinally, prepared as swiss rolls (20), fixed in Carnoy's solution, and sectioned for staining with Alcian Blue (pH 0.4) and Nuclear Fast Red. Alternatively, tissues were fixed in formalin prior to sectioning and staining with Hematoxylin and Eosin. Mast cells in Alcian Blue-stained sections were estimated per crypt-villus unit (CVU) in a minimum of 50 CVU per section. Scoring of enteropathy in H & E-stained sections was as follows: epithelial hyperplasia (0-3), severity of inflammation (0-4); the sum of these two scores was multiplied by a value assigned to the distribution of inflammatory foci (0-3) for a total score ranging from 0 to 21. Severity of inflammation was defined as follows: no significant inflammation - 0; cellular infiltrate within the lamina propria, mild - 1, moderate - 2, severe and extending into the submucosa - 3; severe with crypt abscess, goblet cell depletion, and ulceration - 4. Neutrophil infiltration was given a score from 0 (no infiltration) to 3 (severe infiltration). Microscopy and image capture were performed with an Olympus BX51 microscope and DP25 camera, using Microsuite Basic Edition software.

### **Antibody treatment of rat pups**

Monoclonal, tyvelose-specific IgG1 (clone 9D4) and polyclonal IgG (nIgG) were prepared as described previously (1, 3). Rat pups were treated with 2.5 mg of antibody per 20 g

of body weight by gavage, challenged one hour later, and intestinal parasite burdens estimated after 24 and 48 hours (9).

### **Cytokine Measurement**

Five mm pieces of jejunum, ileum, or LI were weighed prior to processing for explant cultures, as described (11). Explants were cultured with 50 µg/mL of cAg for 16-18 hours at 37°C. Explant supernatants were centrifuged at 138 x g and assayed for IL-4, IL-5, and IL-10 by ELISA as described previously (7). The same protocol was applied to measure IL-9 (BD Biosciences: 2.5 µg/mL capture clone D8402E8, 0.25 µg/mL detection antibody clone D9302C12), IL-13 (Ebioscience: 2 µg/mL capture clone ebio13A, 0.2 µg/mL detection antibody clone eBio1316H), IL-17A (BD Biosciences: 2 µg/mL capture clone TC11-18H10, 0.17 µg/mL detection antibody clone TC11-8H4.1), and IFN-γ (BD Biosciences: 1 µg/mL capture antibody clone AN-18; Ebioscience: 0.125 µg/mL detection antibody clone XMG1.2). Recombinant cytokine standards were purchased (Ebioscience).

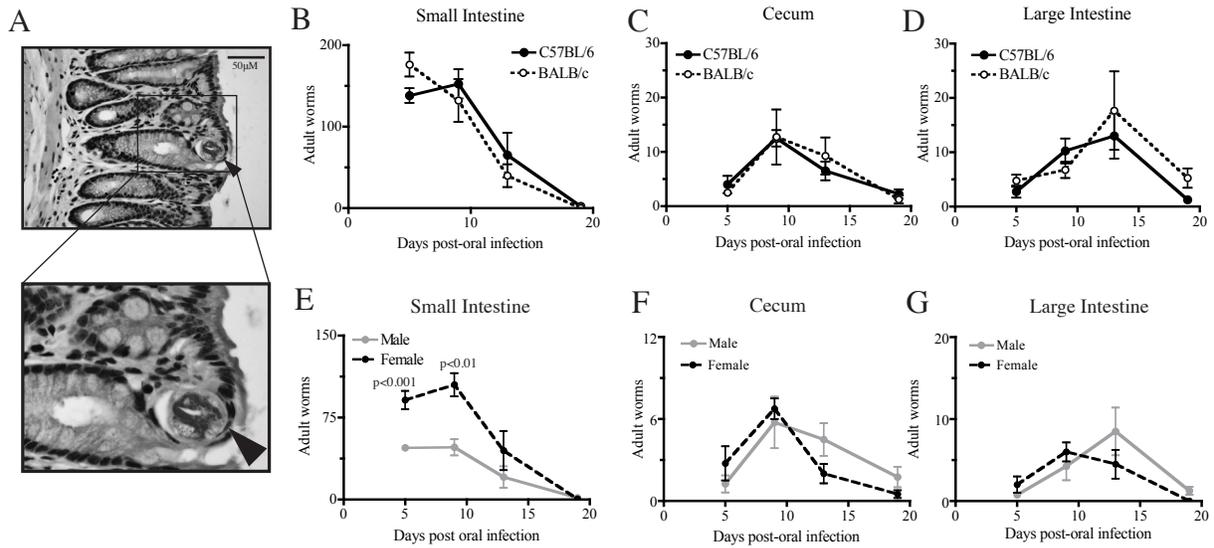
### **Statistical analysis**

Experiments were performed twice and data were evaluated using Student's t test or ANOVA with Tukey's post-hoc test for multiple means. P-values less than 0.05 were considered to be statistically significant.

## **Results**

### **Distribution of *T. spiralis* in the intestinal tract**

Previous reports have documented the presence of *T. spiralis* in the large intestine; however, the location of the worms in the tissue has not been described. Ten days post-infection (dpi), adult worms occupied an epithelial habitat similar to that observed in the small intestine (Figure 3.1A)(23). Peak worm burdens occurred prior to day 5 in the SI, on day 9 in the cecum, and on day 13 in the LI (Figure 3.1B, C, D). Once established, worms were expelled at comparable rates from each site. Results obtained from C57BL/6 and BALB/c mice were



**Figure 3.1: Colonization and expulsion of intestinal *T. spiralis*.** (A) Cross section of adult *T. spiralis* in an H&E stained section of the LI from a C57BL/6 mouse. Arrow indicates the parasite. (B-D) Parasite colonization and expulsion from the three compartments of the intestine in C57BL/6 and BALB/c mice infected orally with 400 L<sub>1</sub>. (E-G) Numbers of male and female worms in C57BL/6 mice. P-values represent differences between the numbers of male and female worms (n = 4 mice per group, means compared using Student's t-test).

indistinguishable.

The ratio of female to male *T. spiralis* colonizing the SI has been reported to be approximately 2:1, shifting to 1:1 as worms are expelled (15). Figure 3.1 (panels E, F, G) shows the expected transition in gender ratio in the SI of C57BL/6 mice, while equal numbers of male and female parasites were observed at all times in the cecum and LI. Similar results were obtained from BALB/c mice (not shown). These results suggest that females are cleared more rapidly from the SI and are less successful than males in colonizing the distal compartments.

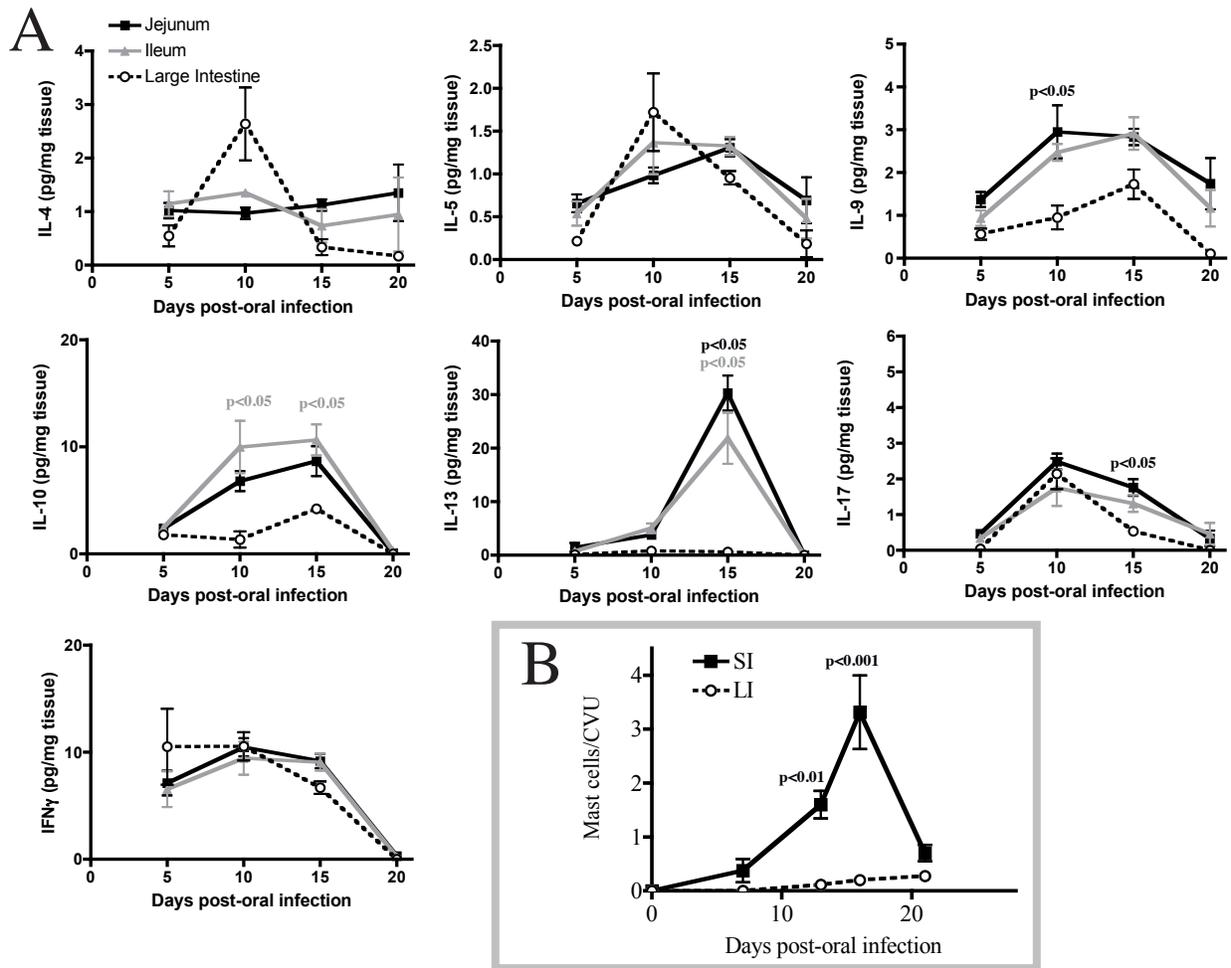
### **Cytokine production and mast cell response**

Assay of cytokines in explant cultures revealed no significant differences in IL-4, IL-5, or IFN- $\gamma$  among the jejunum, ileum, and LI (Figure 3.2A). Although colonization of the sites was not synchronous, the response kinetics were similar for these cytokines across the sites. In contrast, IL-9, IL-10, and IL-13 were significantly lower in LI explant cultures, and the modest output of these cytokines was delayed compared to the SI. IL-17 was reproducibly lower in the LI only at 15 dpi.

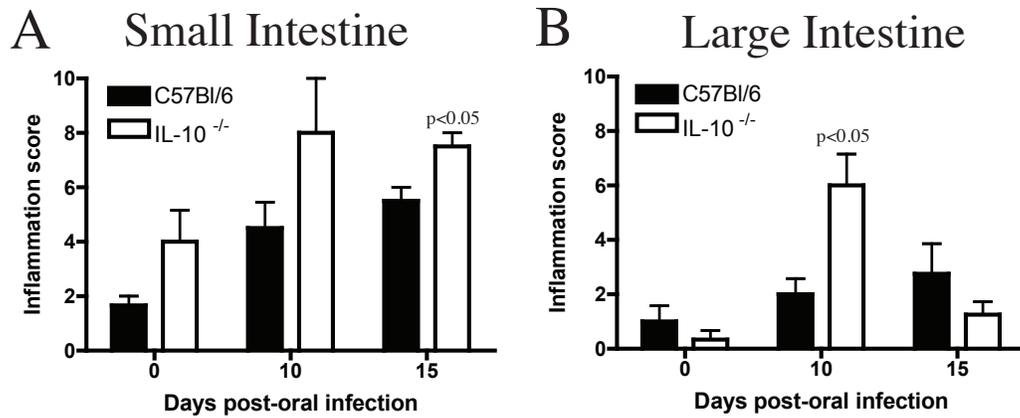
In the context of *T. spiralis* infection, intestinal mastocytosis in the SI of mice is driven by IL-9 and IL-10 (12, 17), and mast cells are believed to be essential to the mechanism of worm expulsion (16, 18). The absence of a significant increase in Alcian Blue positive cells in the LI during infection (Figure 3.2B) correlated with reduced production of IL-9 and IL-10 in that compartment. Despite these deficiencies, worms were expelled at a rate similar to that observed in the SI. Thus, the immune response to *T. spiralis* in the LI is significantly different from that of the SI, and does not feature mastocytosis, yet expulsion occurs in a timely manner.

### **Enteropathy and the influence of IL-10**

In C57BL/6 mice, inflammation in the SI increased dramatically during infection, while no reproducible inflammatory response was detected at any time in the LI (Figure 3.3). In contrast, inflammation increased significantly on day 10 of infection in the LI of IL-10<sup>-/-</sup> mice, and this inflammation resolved by day 15. We concluded that IL-10 is a key regulator of



**Figure 3.2: Cytokine and mast cell responses in the SI and LI.** (A) Cytokines in culture supernatants of tissue explants cultured with crude parasite extract. Values are expressed as picograms of cytokine per milligram of tissue. P-values indicate significant differences between LI and jejunum (black) or LI and ileum (gray) ( $n = 4$  mice per group, means compared using ANOVA with Tukey's post-test). (B) Intestinal mast cells in SI and LI during the course of infection. Values represent the mean number of mast cells per crypt-villus unit (CVU) estimated from a minimum of 50 CVU per mouse. P-values indicate a significant increase in the SI relative to uninfected mice, with no significant changes in the LI ( $n = 3$  mice per group, means compared using ANOVA with Tukey's post-test).



**Figure 3.3: Enteropathy in C57Bl/6 and IL-10<sup>-/-</sup> infected with *T. spiralis*.** H&E preparations were scored using a 21-point scale (see text). (A) SI (B) LI. P-values represent significant differences relative to uninfected mice (n = 4 mice per group, means compared using Student's t-test).

inflammation in the LI during *T. spiralis* infection, and that an IL-10-independent regulatory mechanism functions in the LI to quickly resolve inflammation, while SI enteropathy persists.

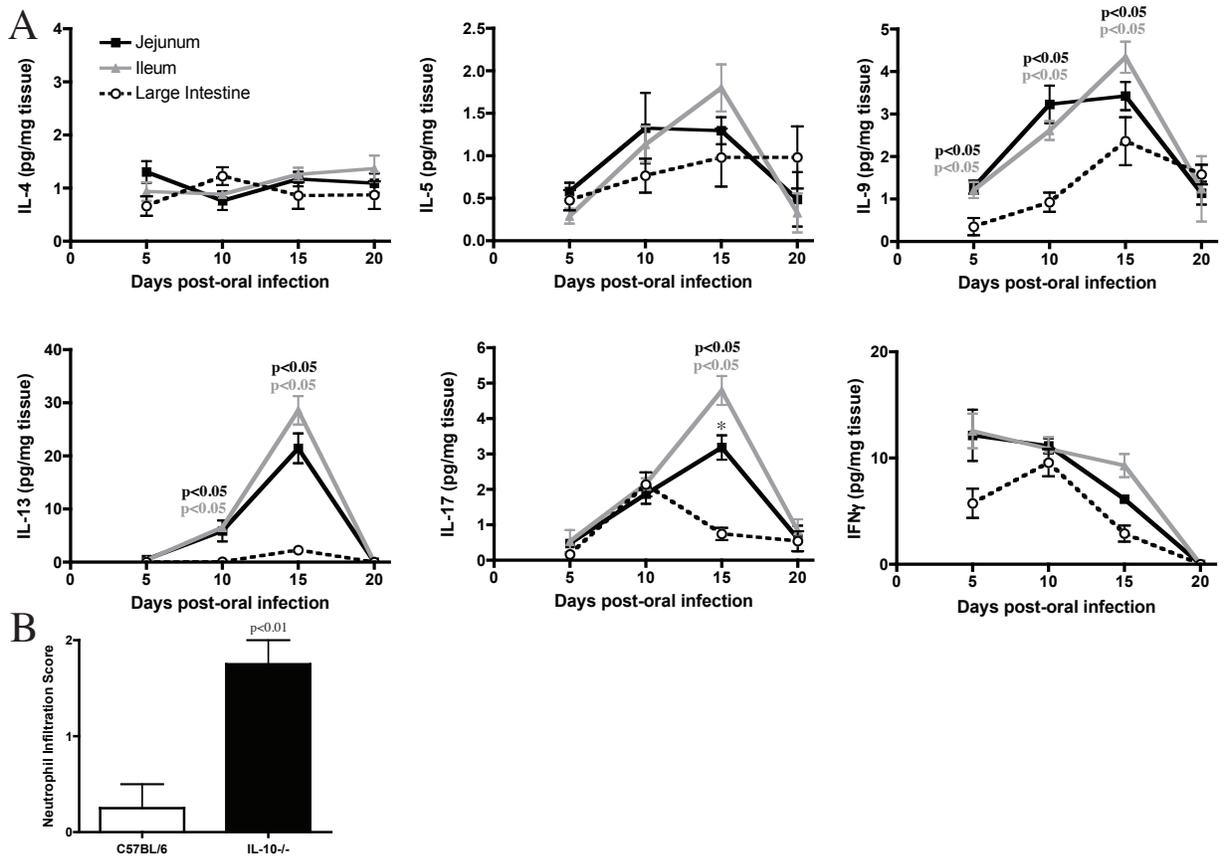
To further evaluate the importance of IL-10 in regulating immunity, we measured the cytokines produced by tissue explants from IL-10<sup>-/-</sup> mice (Figure 3.4A). These mice were tested in parallel with C57BL/6 mice, allowing direct comparison of results graphed in Figures 2 and 4. Explants from IL-10<sup>-/-</sup> and C57BL/6 mice yielded nearly identical cytokine quantities, with the exception that IL-17 production by SI explants was significantly greater in IL-10<sup>-/-</sup> mice. The increase in IL-17 was correlated with a significantly greater influx of neutrophils in IL-10<sup>-/-</sup> mice at day 15 pi (Figure 3.4B). Thus, IL-10 regulated IL-17 production in the SI but not the LI, and the increased inflammation observed in the LI of IL-10<sup>-/-</sup> mice did not correlate with alterations in the production of the cytokines assayed.

### **Protective immunity in the SI and LI**

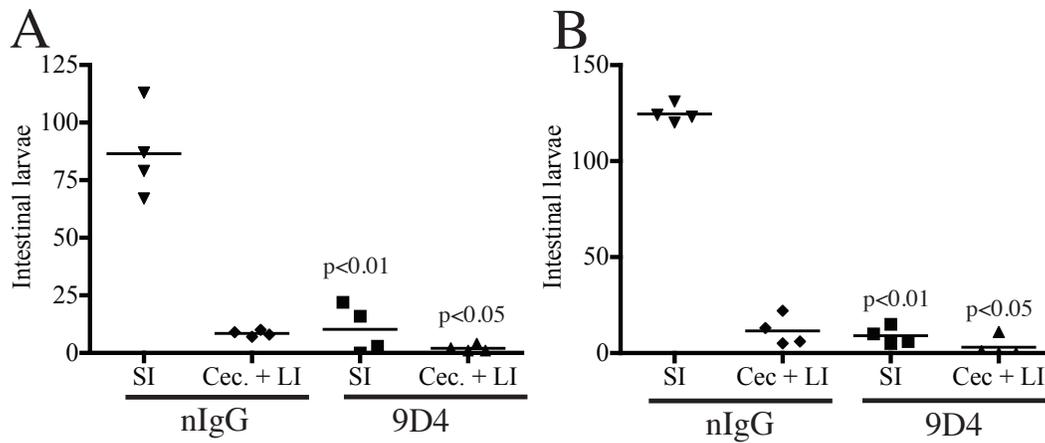
Previously infected rats display a dramatic protective immunity in which 90% of L<sub>1</sub> are cleared from the intestine within an hour of challenge. Suckling rats display this rapid expulsion when passively immunized with antibodies specific for tyvelose (1, 2, 8). In order to determine whether rapid expulsion occurs in the LI, we estimated worm burdens in passively immunized pups after 24 (Figure 3.5A) or 48 (Figure 3.5B) hours. Treatment with specific antibody cleared parasites from all compartments, compatible with the conclusion that antibodies can effectively exclude or eliminate *T. spiralis* from epithelia in the cecum and LI.

### **Discussion**

Immunity to *T. spiralis* is often compared with immunity to *Trichuris muris*, a cecal dwelling nematode. Although the two parasites are closely related, the mechanisms of worm clearance are distinct, with mast cells reported to play a prominent role in expulsion of adult *T. spiralis* while epithelial cell turnover and function appear to be key to clearance of *T. muris* (5, 16). The differences in effector mechanisms may be dictated by properties intrinsic to each parasite, or by their preferred locations in the intestinal tract. Although immunity induced by *T.*



**Figure 3.4: Cytokine response in the SI and LI of IL-10<sup>-/-</sup> mice.** (A) Cytokines in culture supernatants of tissue explants in IL-10<sup>-/-</sup> mice, expressed as picograms of cytokine per milligram of tissue. P-values indicate significant differences between LI and jejunum (black) or LI and ileum (gray), \* represents a difference of p<0.05 between jejunum and ileum (n = 4 mice per group, means compared using ANOVA with Tukey's post-test).



**Figure 3.5: Protective immunity in the LI of suckling rats.** Pups were treated with tyvelose-specific IgG1 (9D4) or naive rat IgG (nIgG) one hour prior to challenge with 200  $L_1$ . (A) Parasite burden in the SI, or combined cecum and LI, 24 hours after challenge. (B) Parasite burden in the SI, or combined cecum and LI, 48 hours after challenge. P-values are shown for nIgG versus corresponding compartment in 9D4-treated pups ( $n = 4$  rats per group, means compared using Student's t-test).

*spiralis* in the SI of mice and rats has been studied in depth, immunity in the LI has been largely overlooked. We set out to evaluate immunity in the small and large intestines within the context of a single worm infection in order to determine whether the mechanisms of immunity vary across intestinal compartments.

We evaluated *T. spiralis* infection in two mouse strains and observed a pattern of rolling colonization and expulsion. Females were more numerous in the SI compared to the cecum and LI. Female *T. spiralis* are much larger than males (3-4 mm versus 1 mm in length), which may increase their susceptibility to physical disruption and nutritional deprivation. The reduced survival of female worms as the parasites migrate distally may necessitate the high female to male ratio that occurs in the SI at the onset of infection.

Adult worms move rapidly within the intestinal epithelium, destroying epithelial cells that they occupy (24). It is possible that as the habitable space in one section of intestine is diminished, the parasites are forced to migrate to more distal regions in order to find healthy cells. Alternatively, the immune response in the SI may make that site less hospitable to the worms, prompting migration to the immunologically naïve cecum and LI. Explant culture assays showed that although IL-4, IL-5, and IFN- $\gamma$  were produced in the LI at early time points, IL-9, IL-10, and IL-13 production was delayed or absent in that compartment. The distal movement of the parasites may occur due to the gradients of these cytokines along the cranial-caudal axis of the intestine. In C57BL/6 mice, inflammation was more robust in the SI than the LI at days 10 and 15 pi, indicating that the LI may be a more permissive region for the parasite.

Our results suggest that lymphocytes induced in the jejunum can home to the ileum and initiate a response there, but do not enter the LI simultaneously. Cytokine responses were different in the two sites, and IL-13 was not detected in the LI at any time. In addition, mucosal mastocytosis was evident in the SI but not in the LI, consistent with weak IL-9 and IL-10 production by LI explants. Mast cells are believed to be essential for the mechanism of worm expulsion from the SI, and their absence in the LI, despite clearance of worms from this site, indicates that the mechanisms of protective immunity may differ between the SI and LI.

Pathologic responses were also regulated distinctly in different sites. IL-10 has been widely studied for its role in suppressing intestinal inflammation. A major function of IL-10 in the intestine is in controlling pathogenic IL-17-producing T cells (22, 25). Consistent with this, IL-17 production and neutrophil infiltration were greater in the jejunum and ileum of IL-10<sup>-/-</sup> mice relative to C57BL/6 controls during *T. spiralis* infection. Nevertheless, it was the LI that demonstrated strong control of enteropathy by IL-10. The interplay between IL-10 and IL-17 in the SI and LI during *T. spiralis* infection merits further attention.

Our results demonstrate that mucosal mastocytosis and the cytokines that influence it are not prominent in the LI during worm expulsion, and that immunopathology is more tightly regulated in the LI than the SI. Thus, mechanisms of immunity and enteropathy vary in different regions of the intestine during helminth infection.

### Acknowledgements

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### References

1. **Appleton, J. A., and D. D. McGregor.** 1987. Characterization of the immune mediator of rapid expulsion of *Trichinella spiralis* in suckling rats. *Immunology* **62**:477-484.
2. **Appleton, J. A., and D. D. McGregor.** 1984. Rapid expulsion of *Trichinella spiralis* in suckling rats. *Science* **226**:70-72.
3. **Appleton, J. A., L. R. Schain, and D. D. McGregor.** 1988. Rapid expulsion of *Trichinella spiralis* in suckling rats: mediation by monoclonal antibodies. *Immunology* **65**:487-492.
4. **Appleton, J. A., and L. Usack.** 1993. Identification of potential antigenic targets for rapid expulsion of *Trichinella spiralis*. *Mol Biochem Parasitol* **58**:53-62.
5. **Artis, D., C. S. Potten, K. J. Else, F. D. Finkelman, and R. K. Grencis.** 1999. *Trichuris*

- muris: host intestinal epithelial cell hyperproliferation during chronic infection is regulated by interferon-gamma. *Exp Parasitol* **92**:144-153.
6. **Autenrieth, I. B., N. Bucheler, E. Bohn, G. Heinze, and I. Horak.** 1997. Cytokine mRNA expression in intestinal tissue of interleukin-2 deficient mice with bowel inflammation. *Gut* **41**:793-800.
  7. **Beiting, D. P., L. F. Gagliardo, M. Hesse, S. K. Bliss, D. Meskill, and J. A. Appleton.** 2007. Coordinated control of immunity to muscle stage *Trichinella spiralis* by IL-10, regulatory T cells, and TGF-beta. *J Immunol* **178**:1039-1047.
  8. **Bell, R. G., J. A. Appleton, D. A. Negrao-Correa, and L. S. Adams.** 1992. Rapid expulsion of *Trichinella spiralis* in adult rats mediated by monoclonal antibodies of distinct IgG isotypes. *Immunology* **75**:520-527.
  9. **Blum, L. K., S. M. Thrasher, L. F. Gagliardo, V. Fabre, and J. A. Appleton.** 2009. Expulsion of secondary *Trichinella spiralis* infection in rats occurs independently of mucosal mast cell release of mast cell protease II. *J Immunol* **183**:5816-5822.
  10. **E. E. Tyzzer, a. J. A. H.** 1916. The effects of radiation on the development of *Trichinella spiralis*. *J Parasitol* **3**:43-56.
  11. **Egan, C. E., K. J. Maurer, S. B. Cohen, M. Mack, K. W. Simpson, and E. Y. Denkers.** 2011. Synergy between intraepithelial lymphocytes and lamina propria T cells drives intestinal inflammation during infection. *Mucosal Immunol*.
  12. **Faulkner, H., N. Humphreys, J. C. Renauld, J. Van Snick, and R. Grencis.** 1997. Interleukin-9 is involved in host protective immunity to intestinal nematode infection. *Eur J Immunol* **27**:2536-2540.
  13. **Gagliardo, L. F., C. S. McVay, and J. A. Appleton.** 2002. Molting, ecdysis, and reproduction of *Trichinella spiralis* are supported in vitro by intestinal epithelial cells. *Infect Immun* **70**:1853-1859.
  14. **Gorfu, G., J. Rivera-Nieves, and K. Ley.** 2009. Role of beta7 integrins in intestinal lymphocyte homing and retention. *Curr Mol Med* **9**:836-850.

15. **Gursch, O. F.** 1949. Intestinal phase of *Trichinella spiralis*, Owen, 1835, Railliet, 1895. *J Parasitol* **35**:19-26.
16. **Ha, T. Y., N. D. Reed, and P. K. Crowle.** 1983. Delayed expulsion of adult *Trichinella spiralis* by mast cell-deficient W/W<sup>v</sup> mice. *Infect Immun* **41**:445-447.
17. **Helmby, H., and R. K. Grencis.** 2003. Contrasting roles for IL-10 in protective immunity to different life cycle stages of intestinal nematode parasites. *Eur J Immunol* **33**:2382-2390.
18. **Knight, P. A., S. H. Wright, C. E. Lawrence, Y. Y. Paterson, and H. R. Miller.** 2000. Delayed expulsion of the nematode *Trichinella spiralis* in mice lacking the mucosal mast cell-specific granule chymase, mouse mast cell protease-1. *J Exp Med* **192**:1849-1856.
19. **Maynard, C. L., L. E. Harrington, K. M. Janowski, J. R. Oliver, C. L. Zindl, A. Y. Rudensky, and C. T. Weaver.** 2007. Regulatory T cells expressing interleukin 10 develop from Foxp3<sup>+</sup> and Foxp3<sup>-</sup> precursor cells in the absence of interleukin 10. *Nat Immunol* **8**:931-941.
20. **Moolenbeek, C., and E. J. Ruitenber.** 1981. The "Swiss roll": a simple technique for histological studies of the rodent intestine. *Lab Anim* **15**:57-59.
21. **Roth, H.** 1938. On the localization of adult *Trichinella spiralis* in the intestinal tract. *J Parasitol* **24**:225-231.
22. **Schaefer, J. S., D. Montufar-Solis, N. Vigneswaran, and J. R. Klein.** 2010. ICOS promotes IL-17 synthesis in colonic intraepithelial lymphocytes in IL-10<sup>-/-</sup> mice. *J Leukoc Biol* **87**:301-308.
23. **Wright, K. A.** 1979. *Trichinella spiralis*: an intracellular parasite in the intestinal phase. *J Parasitol* **65**:441-445.
24. **Wright, K. A., E. Weidman, and H. Hong.** 1987. The distribution of cells killed by *Trichinella spiralis* in the mucosal epithelium of two strains of mice. *J Parasitol* **73**:935-939.
25. **Yen, D., J. Cheung, H. Scheerens, F. Poulet, T. McClanahan, B. McKenzie, M. A.**

**Kleinschek, A. Owyang, J. Mattson, W. Blumenschein, E. Murphy, M. Sathe, D. J. Cua, R. A. Kastelein, and D. Rennick.** 2006. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Invest* **116**:1310-1316.

# Chapter 4

**Epithelial disruption impacts intestinal immunity to *Trichinella spiralis***

## Abstract

*Trichinella spiralis* is a highly destructive parasitic nematode that damages large numbers of epithelial cells during intestinal infection. We set out to investigate the impact of this damage on the adaptive immune response. Although MyD88 was influential in promoting Th2 responses, this was not well correlated with the presence of intestinal bacteria, and MyD88 operated independently of TLR2 and TLR4. Parasite molecules with the potential to function as PAMPs did not activate murine or human TLRs. Of the remaining MyD88-dependent receptors, IL-18 is known to inhibit the Th2 response during *T. spiralis* infection, but the influence of another IL-1 family member, IL-33, has not been described. Mice deficient in ST2<sup>-/-</sup>, the receptor for IL-33, recapitulated the results obtained from MyD88<sup>-/-</sup> mice, supporting a role for IL-33 as an amplifier of the Th2 response. IL-33 was constitutively expressed in intestinal tissue, was not upregulated during infection, and was detected in intestinal leukocytes and epithelial cells where it became concentrated in the nucleus within two days of infection. Array analysis revealed that this translocation was coincident with downregulation of genes targeted by NF-κB in the epithelium of RAG2<sup>-/-</sup> mice, while expression of genes that promote the response to TGF-β were increased. Nuclear IL-33 did not co-localize with NF-κB. Our results implicate IL-33 as an innate mediator of Th2 immunity in the intestine and offer a model that parallels the cellular redistribution of human IL-33 that has been reported in ulcerative colitis.

## Introduction

Despite a prodigious increase in the past 20 years in our understanding of how bacterial and viral pathogens induce Th1-driven immunity, innate drivers of the Th2 response remain poorly understood. *Trichinella spiralis* is a parasitic nematode that is a human pathogen, in addition to naturally infecting other mammalian species, such as rodents. During intestinal infection, *T. spiralis* travels rapidly in a multi-intracellular niche within the epithelium, leaving a path of cellular destruction in its wake (39, 66). Cell injury and death is likely to influence adaptive immunity, yet this aspect of infection has not been investigated.

Receptor recognition of pathogen-associated molecular patterns (PAMPs) by toll-like receptors (TLRs) is a common mechanism of innate defense. Such patterns have been identified in bacterial, viral, protozoan, and helminth pathogens (32, 61, 63). MyD88 is a signaling adaptor molecule that propagates the response to most TLRs as well as responses to IL-1 family members, including IL-18 and IL-33 (1, 35, 42). MyD88 binds to the intracellular TIR domain of these receptors (directly or via a bridging adaptor such as MAL), where it initiates a signaling cascade that ultimately leads to the activation of proinflammatory genes by the transcription factor NF- $\kappa$ B (14, 25).

*T. spiralis* larvae secrete multiple immunogenic glycoproteins as they invade the intestinal epithelium, and the mechanism by which these glycans may be detected by the innate immune system, including the potential dependence on TLRs/MyD88, is not known (15, 22, 49, 51). In addition, the intestinal epithelium is exposed to an enormous number of bacteria (100 trillion bacteria belonging to 1000 different species), yet these bacteria do not induce inflammation under homeostatic conditions (23, 54). Most gut bacteria remain physically separated from the epithelium by a 50  $\mu$ m zone, maintained by the secreted antibacterial lectin RegIII $\gamma$ , and the presence of an intestinal parasite may disturb this protective zone, or alter the composition of the microbiota (31, 62).

One inherent distinction between bacterial, viral, or protozoan pathogens and large parasitic nematodes is the physical destruction that can be caused by the size and mobility of worms (66). Danger-associated molecular patterns (DAMPs), such as IL-33 and HMGB1, can give rise to inflammation when released by necrotic cells (38, 47, 48). In addition to its role in the TLR pathway, MyD88 functions as an adaptor for the IL-1 family of cytokine receptors, including ST2, the receptor for IL-33 (42, 55). IL-33 differs from other IL-1 family cytokines in that it is a so-called 'necrocrine' cytokine, which is released in its active form by necrotic cells (3, 38, 71). IL-33 is mainly expressed in fixed tissue cells such as epithelial and endothelial cells, adipocytes, astrocytes, and airway smooth muscle cells, although it may also be expressed by mast cells, dendritic cells, and monocytes under inflammatory conditions (28, 44, 45, 50, 65, 68,

69).

In addition to its role as a paracrine cytokine, IL-33 also carries out an intracellular signaling mechanism, which is not well-understood (16). In many tissues, IL-33 is constitutively located in the nucleus, where it binds to the histone H2A/H2B acidic pocket, and is released in response to inflammatory stimuli (36, 53). In addition, it was recently reported that nuclear IL-33 binds to NF- $\kappa$ B and prevents it from acting as a transcriptional activator (2); however, many of the studies regarding IL-33 nuclear translocation have been carried out in transfected cell lines, which may not be an accurate model of the *in vivo* functions of nuclear IL-33.

Disruption of the epithelium by *T. spiralis* could promote Th2 immunity by the detection of parasite-derived glycoproteins, by altering the composition or location of the microbiota, or by the release of DAMPs from damaged host cells. We set out to investigate the innate response to epithelial cell destruction in order to identify drivers of Th2 immunity in the small intestine.

## Materials and Methods

### Mice and Rats

MyD88<sup>-/-</sup> mice on a C57BL/6 background developed by Dr. S. Akira (Osaka University, Osaka, Japan) were generated by (♀ +/- by ♂ +/-) or (♀ +/- by ♂ -/-) crosses, and genotyped as previously described (59). MyD88<sup>+/+</sup> littermates served as controls. TLR2/4<sup>-/-</sup> mice on a C57BL/6 background were also generated by Dr. Akira. C57BL/6 mice and RAG2<sup>-/-</sup> mice were purchased from Taconic (USA). Mice were bred and/or maintained at Cornell under specific pathogen-free conditions according to the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. Albino Oxford (AO) rats were used as parasite donors, and were produced and maintained under specific pathogen-free conditions. Animals were infected at 7-10 weeks old. Rodents were euthanized by CO<sub>2</sub> inhalation, and blood was collected by cardiac puncture. Experiments at Cornell were performed with the approval of the Cornell University Institutional Animal Care and Use Committee.

ST2<sup>-/-</sup> mice were originally provided by Dr. Andrew McKenzie (Laboratory of Molecular

Biology, Medical Research Council, Cambridge, U.K.) (12). ST2<sup>-/-</sup> mice were bred and maintained at Trinity College Dublin; animals were housed in a specific pathogen-free facility in individually ventilated and filtered cages under positive pressure. Experiments were performed in compliance with Irish Department of Health and Children regulations and approved by the Trinity College Dublin's BioResources ethical review board.

### **Parasite and infections**

Maintenance of *Trichinella spiralis* (pig strain), L<sub>1</sub> recovery, and intestinal worm burden estimation were carried out as described (11, 26). Mice (7 to 10 weeks old) were infected with 400 L<sub>1</sub> by oral gavage. Crude L<sub>1</sub> parasite extract, ES antigens, and purified phosphorylcholine-bearing proteins were prepared as described (4, 49). Muscle larvae burdens were assessed in whole carcasses as previously described (5).

### **Culture of leukocytes and cytokine ELISA**

Cells from cervical lymph nodes (CLN) or mesenteric lymph nodes (MLN) were prepared, cultured, and supernatants were assayed for IL-4, IL-5, and IL-10 by ELISA as described previously (6). The same protocol was applied to measure IL-13 (Ebioscience: 2 µg/mL capture clone eBio13A, 0.2 µg/mL detection antibody clone eBio1316H), IL-17A (BD Biosciences: 2 µg/mL capture clone TC11-18H10, 0.17 µg/mL detection antibody clone TC11-8H4.1), and IFN-γ (BD Biosciences: 1 µg/mL capture antibody clone AN-18; Ebioscience: 0.125 µg/mL detection antibody clone XMG1.2). Recombinant cytokine standards were purchased (Ebioscience). Serum mouse mast cell protease-1 (mMCP-1) was measured using the MCPT-1 (mMCP-1) Ready-SET-Go! kit (Ebioscience).

### **Histology**

Samples of small intestine were cut longitudinally, prepared as swiss rolls (43), and fixed

in formalin for immunohistochemistry (IHC). For evaluation of skeletal muscle, tongues were fixed in formalin. IHC for iNOS, IL-33 [3 µg/mL polyclonal goat anti-IL-33 (R&D systems) with 0.6 µg/mL biotinylated rabbit anti-goat antibody (Vector Labs)], and mmcp-1 [5 µg/mL rat IgG2b anti-mmcp-1 (R&D systems) with 1 µg/mL biotinylated rabbit anti-rat antibody (Vector Labs)] was performed as described (6). Mast cells in mMCP-1 stained sections were counted per crypt-villus unit (CVU) for a minimum of 50 CVU per section.

For immunofluorescent staining, paraffin sections were prepared as for IHC, and treated with an avidin-biotin blocking kit (Vector Labs). After blocking for one hour with 5% normal rabbit serum in PBS, 5 µg/mL of polyclonal goat anti-IL-33 (R&D systems) and biotinylated mouse IgG1 anti-NF-κB (Imgenex) diluted in 1% BSA/PBS were added and slides incubated for one hour. After washing three times with PBS, Alexa 488-rabbit F(ab)<sub>2</sub> anti-goat and Alexa 594-Streptavidin (Invitrogen - Molecular Probes) in 1% BSA/PBS were added and slides incubated for one hour, followed by three washes in PBS. All incubations were carried out at room temperature. Images were collected with a Nikon TE300 fluorescent microscope, using a Hamamatsu OrcaER charge-coupled-device camera, with different labels collected sequentially as separate channels. Images were analyzed using SimplePCI (Hamamatsu, Sewickley, PA). Paraffin sections from mice infected with *Toxoplasma gondii*, provided by Dr. C. Egan (Cornell) were used as a positive control for NF-κB staining.

### **Flow cytometry**

Single cell suspensions were prepared from spleen, and eosinophils were stained using phycoerythrin-labeled Siglec-F (BD Pharmingen) using methods previously described (5).

### **TLR Transactivation Assay**

HEK293 cells were transfected with a plasmid encoding the indicated TLR, as previously described (18). Cells were cultured overnight with crude parasite extract, ES antigens, or purified

phosphorylcholine-bearing proteins. Data were analyzed by determining the fold induction relative to the activation of a constitutive  $\beta$ -galactosidase reporter construct.

### **Antibiotic Treatment**

C57Bl/6 mice were provided with drinking water containing 0.5 mg/mL Bacitracin and Streptomycin (Sigma Aldrich), *ad libitum*, starting one week prior to *T. spiralis* infection. This protocol has been previously characterized as clearing all culturable bacteria (20). We evaluated bacterial clearance by culturing fecal samples on LB agar plates under aerobic conditions.

### **Quantitative PCR**

Peyer's patches were removed, and total RNA was isolated from the remaining whole intestinal tissue using the TRIZOL reagent (Invitrogen) with a PowerGen handheld homogenizer (Fischer Scientific). cDNA was prepared using the SuperScript III First-Strand cDNA Synthesis System (Invitrogen). A sample lacking reverse transcriptase served as negative control. IL-33 expression was measured with TaqMan qRT-PCR ( $\Delta\Delta$ CT method) using an ABI PRISM 7500 Sequence Detection System with HPRT as the endogenous control. HPRT expression was not affected by infection (not shown). Primers and probes for IL-33 and HPRT were purchased from Applied Biosystems.

### **Invasion Assay**

An *in vitro* assay of epithelial cell invasion by *T. spiralis* was performed in 100 mm<sup>2</sup> petri dishes as described previously (15, 39). Briefly, DLD-1 cells (ATCC #CCL-221) were plated at  $1.8 \times 10^5$  cells per dish, and incubated overnight in 8% CO<sub>2</sub> at 37°C. Confluent monolayers ( $2 \times 10^7$  cells) were inoculated with 3000 *T. spiralis* L<sub>1</sub> in warm agarose and larvae were allowed to invade the monolayer for 2 hours prior to removal of the parasites and agarose. Cell collection and separation of cytoplasmic contents were performed using the Nuclear Extract Kit (Active

Motif) according to the manufacture's directions. After removal of the cytoplasmic fraction, total (soluble and insoluble) nuclear proteins were resuspended in a volume of Nuclear Extract Kit lysis buffer equal to the volume of the cytoplasmic fraction.

### **Western Blotting**

Samples were denatured in sample preparation buffer with 2-mercaptoethanol by boiling for 5 minutes, resolved by SDS-PAGE, and transferred to nitrocellulose as described (21). Blocking was carried out for 4 hours at room temperature in Tris-buffered saline (TBS) with 0.05% Tween-20 and 5% nonfat dry milk. Antibodies were diluted in blocking solution, and blots were washed with TBS containing 0.05% Tween-20. Blots were incubated with primary goat anti-human IL-33 (0.1  $\mu\text{g}/\text{mL}$ , R&D Systems), mouse anti-Lamin B1 (0.2  $\mu\text{g}/\text{mL}$ , Santa Cruz Biotechnology), or biotinylated anti-Tubulin- $\alpha$  (0.5  $\mu\text{g}/\text{mL}$ , BioLegend) for 2 hours at room temperature. Streptavidin-HRP or the appropriate secondary antibodies were diluted 1/2000 and incubated for 1 hour at room temperature, and the binding detected using ECL chemiluminescence reagent (Pearce, USA) with x-ray film. The results were quantified using ImageJ software.

### **Intestinal Epithelial Cell (IEC) Isolation**

IECs from RAG2<sup>-/-</sup> mice (uninfected or two days after infection with 400L<sub>1</sub>) were isolated using a protocol based on previously described methods (9, 52). A 10-cm piece of jejunum was flushed with 10 mL of 37°C Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free Hank's Balanced Salt Solution (CMF-HBSS, Gibco, USA). The portal vein was cut to allow an outlet for perfusion fluid. Mice were perfused via the left ventricle of the heart with 60 mL of warm CMF-HBSS containing 24 mM EDTA (Ambion). The rinsed segment of intestine was immediately removed, inverted, placed in 5 mL of ice cold CMF-HBSS, and shaken vigorously for 20 seconds. Cells were allowed to settle on ice for 5 minutes, and the supernatant was removed. An aliquot of cells was taken for histology,

cold TRIZol reagent was added to the remaining cells, and cells in TRIZol were frozen at  $-80^{\circ}\text{C}$ . In order to confirm the purity of IECs, the ( $\sim 30\ \mu\text{L}$ ) aliquot of cells for histology was mixed into  $100\ \mu\text{L}$  of melted 3.5% SeaPlaque Agarose (Lonza, USA) dissolved in Dulbecco's Phosphate Buffered Saline (DPBS) and briefly allowed to cool in a 96-well plate. The hardened agarose plug was frozen in OCT medium. OCT blocks were stored at  $-80^{\circ}\text{C}$  prior to being sectioned with a cryostat and stained with Hematoxylin and Eosin.

### **Microarray**

RNA was isolated by chloroform extraction from epithelial cells frozen in TRIZol reagent, and concentrated using an RNeasy kit (Qiagen, USA). Prior to hybridization, RNA quality was assessed with a BioAnalyzer (Agilent Technologies, USA). RNA integrity numbers (RIN) were between 8.4 and 9.6. cDNA synthesis, labeling, hybridization, and microarray analysis were performed by the Genomics Core Facility at Cornell University. Generation of double-stranded cDNA, preparation and labeling of cRNA, hybridization to GeneChip® Mouse Genome 430 2.0 Arrays (Affymetrix, CA), and washing were performed according to the standard Affymetrix protocol.

### **Data Analysis**

Affymetrix array results were compared between uninfected and infected mice ( $n = 3$  mice per group). Data were analyzed through the use of Ingenuity Pathway Analysis (Ingenuity® Systems [www.ingenuity.com](http://www.ingenuity.com)). Canonical pathways analysis identified the pathways from the Ingenuity Pathways Analysis library of canonical pathways that were most significant to the data set. Molecules from the data set that met the  $p < 0.05$  cutoff and were associated with a canonical pathway in Ingenuity's Knowledge Base were considered for the analysis. The significance of the association between the data set and the canonical pathway was measured in 2 ways: 1) A ratio of the number of molecules from the data set that map to the pathway divided by

the total number of molecules that map to the canonical pathway is displayed. 2) Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

A network/My Pathways is a graphical representation of the molecular relationships between molecules. Molecules are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least 1 reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base. The intensity of the node color indicates the degree of up- (red) or down- (green) regulation. Nodes are displayed using various shapes that represent the functional class of the gene product.

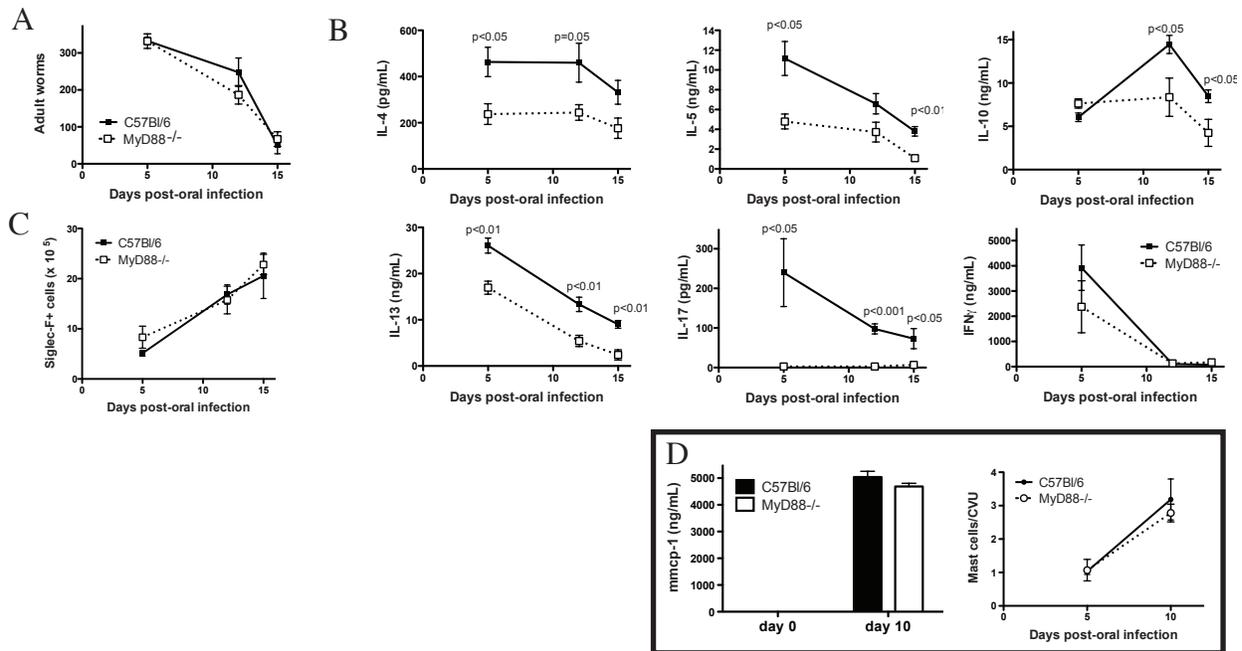
### **Statistical Analysis**

Experiments were performed twice and data were evaluated using Student's t test or ANOVA with Tukey's post-hoc test for multiple means. P-values less than 0.05 were considered to be statistically significant. All experiments were performed twice, with n = 3 to 8 mice per group. Array data were analyzed as described above.

## **Results**

### **MyD88 is required for efficient amplification of the Th2 cytokine response to infection.**

To investigate a role for TLRs in the innate response to *T. spiralis* infection, we evaluated mice deficient in MyD88, the signaling adaptor for most TLRs. Worm expulsion was similar in MyD88<sup>-/-</sup> mice and littermate controls (Figure 4.1A); however, in MLN cultures from MyD88<sup>-/-</sup> mice the canonical Th2 cytokines IL-4, IL-5, IL-10, and IL-13 were significantly diminished, while there was no difference in IFN-g production (Figure 4.1B). Despite these differences, there was no difference in the number of eosinophils (Siglec-F<sup>+</sup> cells), intestinal



**Figure 4.1: Role of MyD88 in immunity to *T. spiralis*.** Mice were infected orally with 400 L<sub>1</sub>. (A) Parasite expulsion kinetic in MyD88<sup>-/-</sup> mice compared to C57Bl/6 littermates. (B) ELISA measurement of Th2 cytokines in cAg-restimulated MLN cultures from MyD88-deficient mice and C57BL/6 controls. (C) Number of eosinophils (Siglec-F<sup>+</sup> cells) in the spleen of infected MyD88<sup>-/-</sup> mice and controls. (D) Serum mmcp-1 concentration and results of mmcp-1 IHC staining in sections of the jejunum.

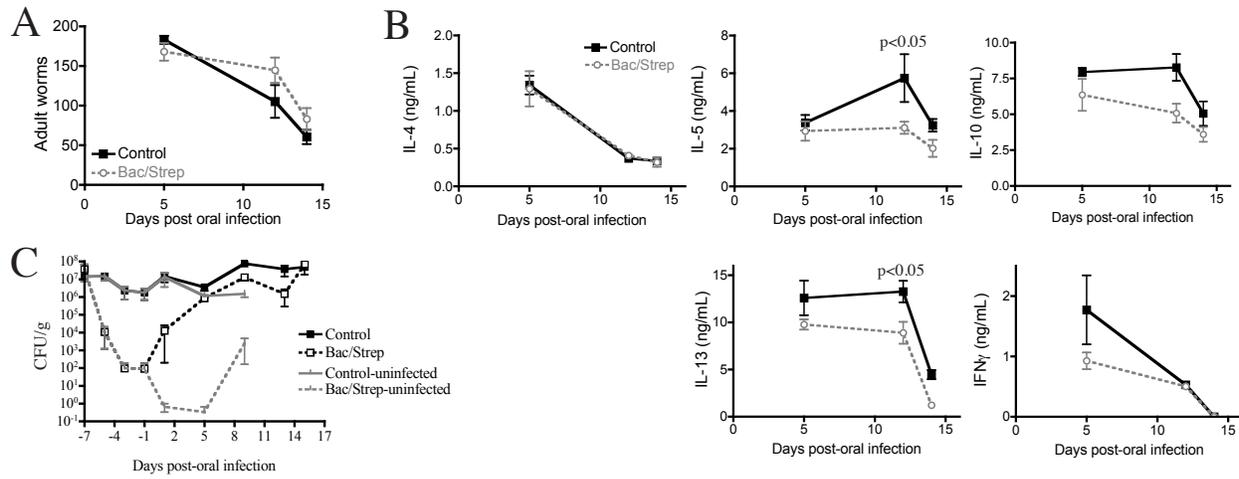
mastocytosis, or serum mMCP-1 (Figure 4.1C, D). No differences were observed in the parasite burden in muscle, and in contrast to the MLN response, the CLN cytokine response to muscle infection was not affected by MyD88 deficiency (not shown).

The involvement of MyD88 in amplification of the Th2 response may indicate that TLRs are directly activated by parasite products in a way that induces Th2 immunity, that parasite destruction of the epithelium leads to TLR activation via endogenous ligands or microbiota, or a role for IL-1 family cytokines that utilize this receptor.

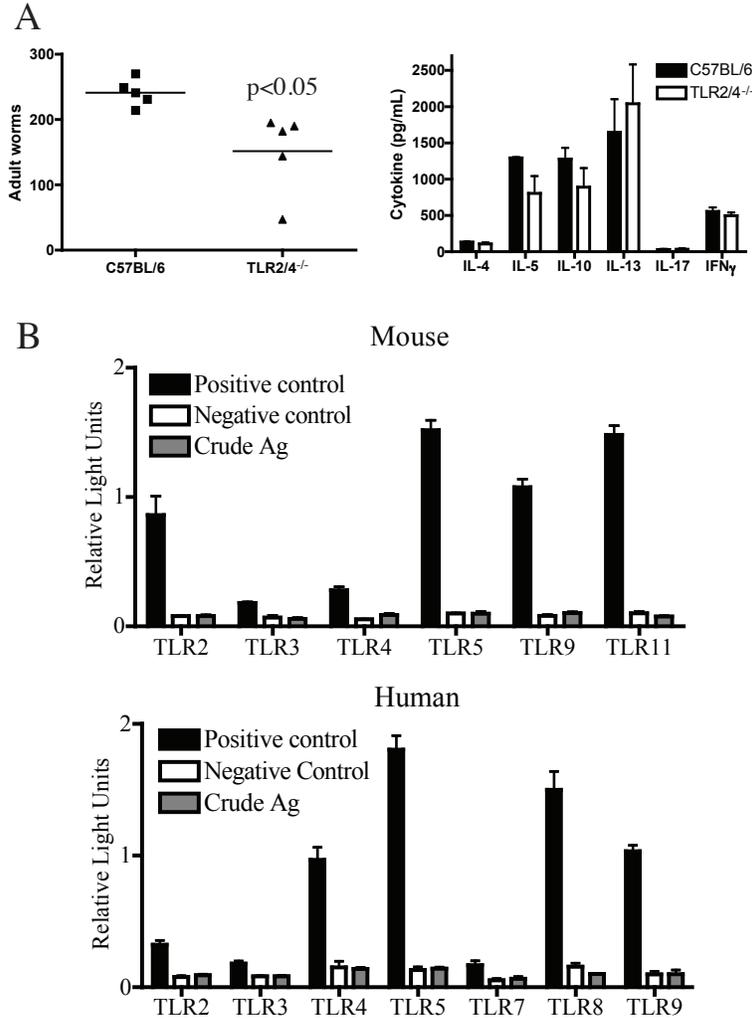
### ***T. spiralis does not directly activate TLRs in vitro or in vivo***

To assess the contribution of the microbiota to *T. spiralis* immunity, we evaluated mice that were treated with antibiotics starting one week prior to infection, and continuously during infection, using a treatment regimen that was reported to eliminate all culturable bacteria from the intestine (20). Antibiotic treatment had no effect on parasite expulsion (Figure 4.2A) or IL-4 production in MLN cultures, although IL-5 and IL-13 were modestly reduced at a single time point (Figure 4.2B). These results are consistent with a modest contribution of the microbiota towards inducing the Th2 response to *T. spiralis*. Although bacteria were easily cleared after one week of antibiotic treatment, *T. spiralis* infection induced an overgrowth of antibiotic-resistant bacteria (Figure 4.2C). The same infection-induced bacterial overgrowth was observed with enrofloxacin (baytril) treatment (not shown). To clarify the results of antibiotic treatment, we measured Th2 cytokines in infected TLR2/4<sup>-/-</sup> mice (Figure 4.3A). These mice had slightly accelerated parasite expulsion but no difference in the MLN cytokine response, providing additional evidence that intestinal bacteria and their products do not play a significant role in amplifying the Th2 response.

Using a luciferase reporter assay to measure NF- $\kappa$ B activation, we investigated the possibility that *T. spiralis* products directly activate TLRs. HEK293 cells were transfected with plasmids encoding human or mouse TLRs, an NF- $\kappa$ B-driven luciferase gene, and a constitutively active  $\beta$ -galactosidase gene to control for transfection. Cells were stimulated overnight with *T.*



**Figure 4.2: Effect of the microbiota on Th2-mediated immunity.** C57Bl/6 mice were treated with bacitracin and streptomycin for 7 days, prior to infection with 400 L<sub>1</sub>. (A) Parasite burden in the small intestine of antibiotic treated mice and controls. (B) Cytokine response in cAg-restimulated MLN cells from antibiotic-treated mice and controls. (C) Quantification of fecal bacteria during treatment and infection.



**Figure 4.3: TLR response to *T. spiralis* in vitro and in vivo** (A) Immunity to *T. spiralis* in TLR2/4<sup>-/-</sup> mice. Mice were infected orally with 400 L<sub>1</sub> and parasite expulsion and MLN cytokine response were measured 12 days after infection. (B) Activation of mouse and human TLRs by *T. spiralis* crude antigen or the appropriate positive control for each TLR in an HEK293 cell luciferase reporter assay. All positive controls were significantly different from the negative control (p<0.05 or less).

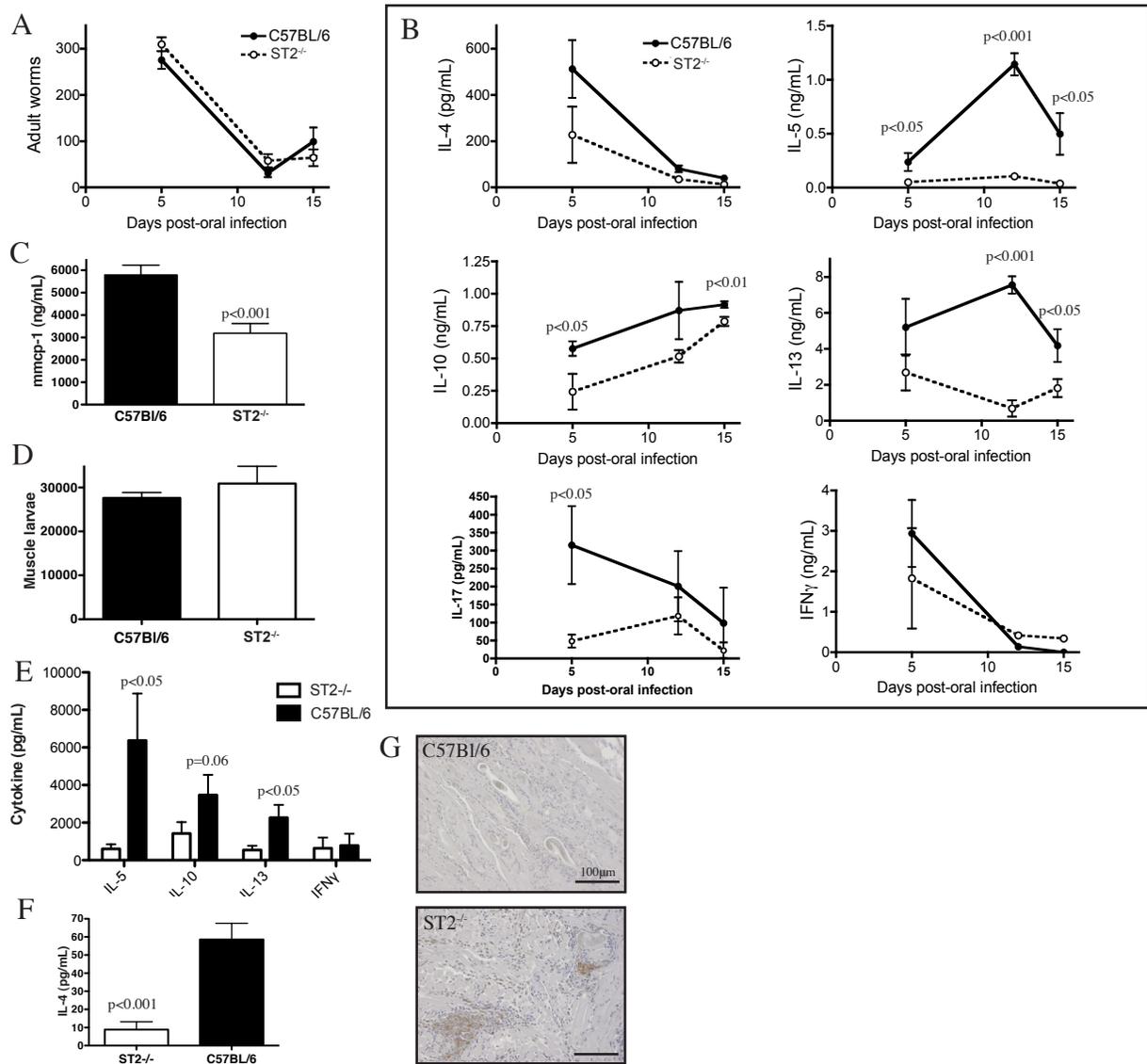
*spiralis* crude parasite extract, medium control, or the appropriate positive control ligand for each TLR. No reporter activity was seen for any of the human or mouse TLRs tested (Figure 4.3B). We also tested *T. spiralis* excretory-secretory proteins and the purified phosphorylcholine-bearing proteins from the parasite with negative results (not shown). Overall, we have found no evidence that TLRs promote the Th2 response to *T. spiralis*.

### **ST2<sup>-/-</sup> mice have impaired Th2 immunity**

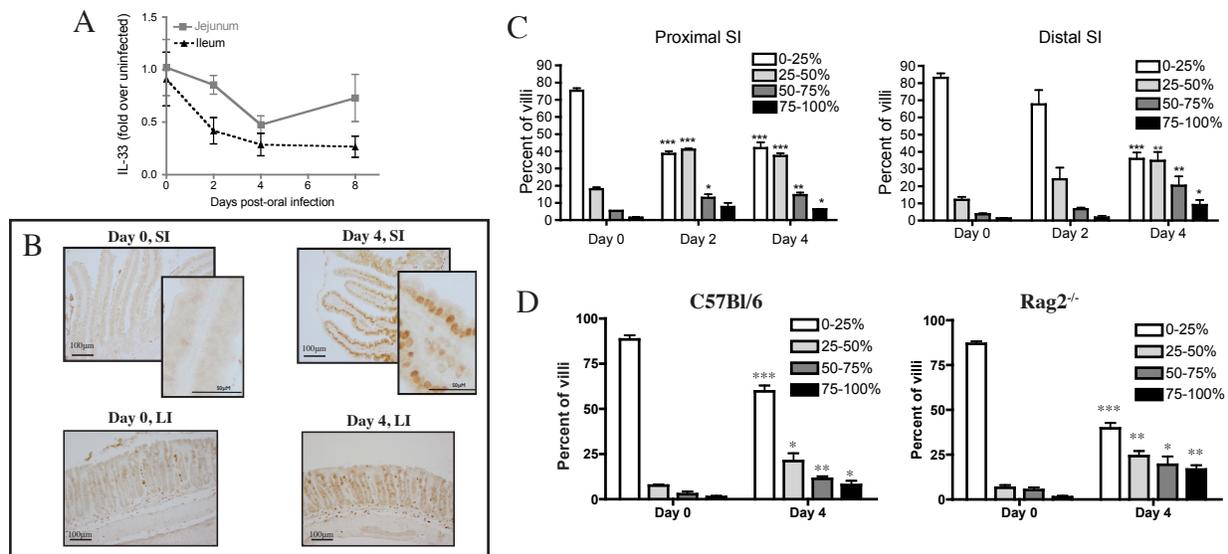
In the absence of compelling evidence that TLRs induce Th2 immunity to *T. spiralis*, we turned our attention to another receptor that employs MyD88, the IL-33 receptor ST2 (35, 55). Similar to MyD88<sup>-/-</sup> mice, ST2<sup>-/-</sup> mice displayed normal parasite expulsion but a deficient Th2 cytokine response in the MLN (Figures 4.4A, B). The reduction in Th2 cytokines was more robust in ST2<sup>-/-</sup> mice than in MyD88<sup>-/-</sup> mice, particularly for IL-5 and IL-13. Unlike MyD88<sup>-/-</sup> mice, sera from ST2<sup>-/-</sup> mice also had significantly lower concentrations of mMCP-1 (Figure 4C). MyD88<sup>-/-</sup> and ST2<sup>-/-</sup> mice also differed during the chronic (muscle) phase of infection. ST2<sup>-/-</sup> mice had normal muscle burdens at 28 dpi (Figure 4.4D), but displayed a strongly diminished Th2 cytokine response in CLN cells restimulated with parasite extract (Figure 4.4E, F). Despite the decreased Th2 response, histology of the tongue (muscle) during infection showed a robust cellular infiltration of the nurse cells (not shown), with numerous iNOS<sup>+</sup> cells detected, compatible with an enhanced Th1 response (Figure 4.4G).

### **IL-33 localization is altered during infection**

Transcription of IL-33 was not upregulated in the intestine following infection (Figure 4.5A); however, the location of IL-33 protein within epithelial cells changed dramatically within two days of infection (Figure 4.5B). Prior to infection, IL-33 was diffusely distributed in the cytoplasm of IECs, as well as some leukocytes in the lamina propria. Following infection, IL-33 was concentrated in the nuclei of IECs in the SI. Although IL-33 did not translocate to the



**Figure 4.4: Infection of ST2<sup>-/-</sup> mice.** (A) Intestinal parasite expulsion kinetics in ST2<sup>-/-</sup> mice compared to C57BL/6 controls. (B) Cytokine response in parasite antigen-restimulated MLN cultures of infected ST2<sup>-/-</sup> mice and controls. (C) Serum mmcp-1 concentration on day 15 post-oral infection, measured by ELISA. (D) Muscle burden in ST2-deficient mice and controls at 28 dpi. (E-F) CLN cytokine response at 17 dpi. (G) iNOS immunohistochemical staining in the tongue (muscle) at 17 dpi.



**Figure 4.5: IL-33 mRNA and protein expression during *T. spiralis* infection.** (A) IL-33 mRNA expression. RNA was isolated from jejunum and ileum of C57Bl/6 mice infected orally with 400 L<sub>1</sub>, and IL-33 was measured using TaqMan qRT-PCR with HPRT as the endogenous control. (B) Immunohistochemistry images of intestinal IL-33 staining showing nuclear translocation of IL-33 during infection of the SI but not the LI. (C) Quantification of IL-33 nuclear translocation in C57Bl/6 mice during *T. spiralis* infection. (D) Quantification of IL-33 nuclear translocation in Rag2<sup>-/-</sup> mice and controls. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 (versus uninfected)

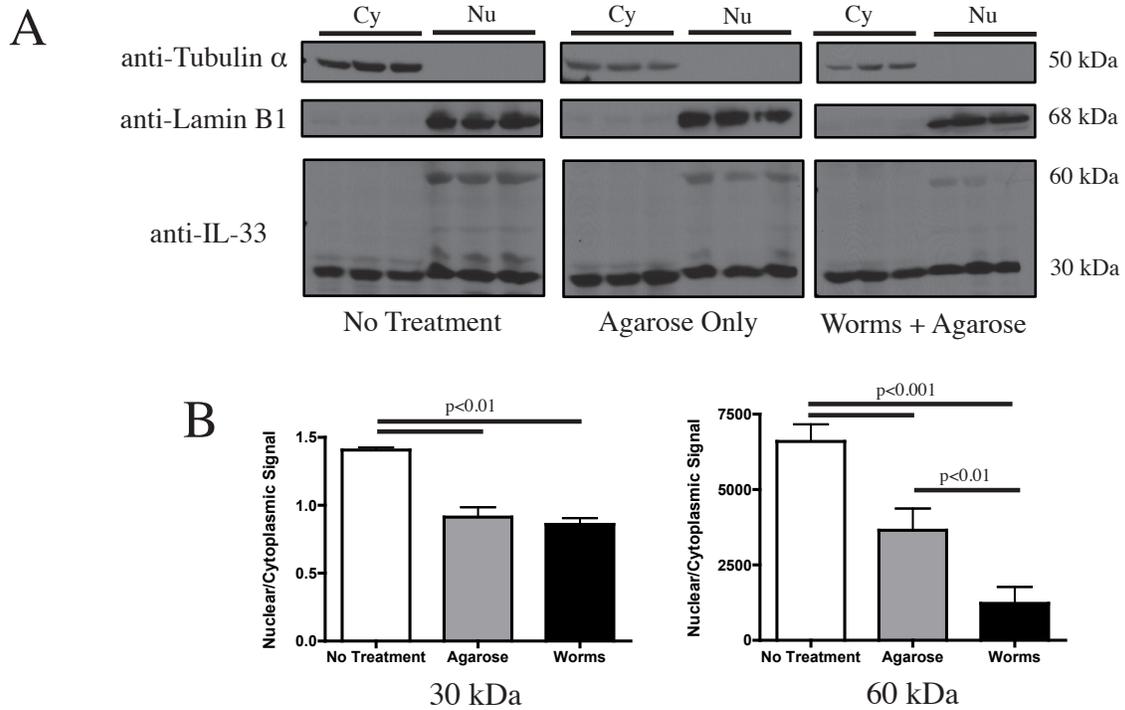
nucleus during infection of the LI, detection of cytoplasmic IL-33 staining increased in this site during infection. The change in the numbers of cells that demonstrated nuclear localization was statistically significant in the proximal SI by day 2 and in the distal SI by day 4 (Figure 4.5C), consistent with the distal migration of worms during the course of infection (58). Translocation occurred in IECs of RAG2<sup>-/-</sup> mice, confirming the process to be driven by innate, rather than adaptive, immune mechanisms (Figure 4.5D). Translocation occurred independently of MyD88 (not shown).

### **IL-33 translocation does not occur *in vitro***

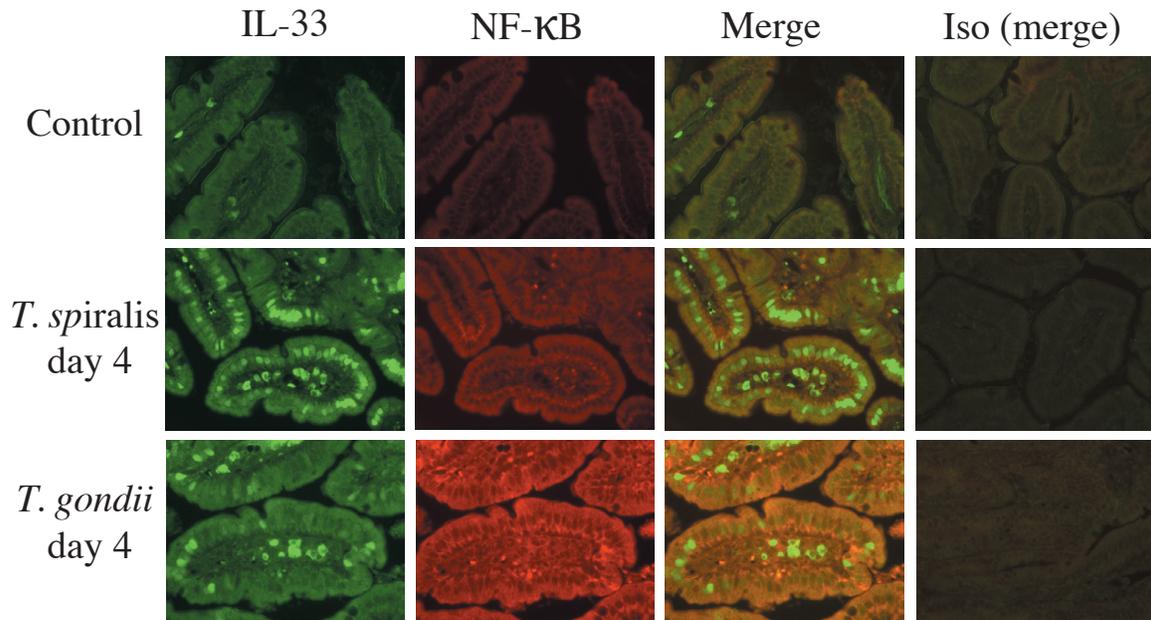
We evaluated IL-33 nuclear translocation in an *in vitro* parasite invasion assay using DLD-1 human colon carcinoma cells, which constitutively express IL-33. Monolayers of DLD-1 cells were overlaid with *T. spiralis* parasites in warm agarose, warm agarose alone, or no treatment, incubated for 2 hours, and nuclear and cytoplasmic fractions were evaluated for IL-33. Although the cellular distributions of monomeric (30 kDa) and dimeric (60 kDa) species of IL-33 were not affected by infection (Figure 4.6A, B), both parasite inoculation and warm agarose alone contributed to a significant reduction in the amount of dimeric (60 kDa) IL-33 detected. Failure to induce IL-33 nuclear translocation in this assay suggests that the signal for translocation may not be intrinsic to IECs, or that human LI-derived DLD-1 cells behave like murine LI IECs *in vivo* and do not translocate.

### **Association of IL-33 with NF- $\kappa$ B**

It was recently reported that nuclear IL-33 can bind to NF- $\kappa$ B and suppress the activity NF- $\kappa$ B as a transcriptional activator (2). We used immunofluorescent staining to visualize the locations of NF- $\kappa$ B and IL-33 in IECs during infection (Figure 4.7). IECs in uninfected mice did not show NF- $\kappa$ B staining, and although NF- $\kappa$ B staining increased slightly at day 4 of *T. spiralis* infection, NF- $\kappa$ B remained in the cytoplasm with an apical distribution and



**Figure 4.6: IL-33 localization in DLD-1 human colon carcinoma cells.** DLD-1 cells were collected after no treatment (control), warm agarose only, or parasite invasion, and nuclear and cytoplasmic fractions were separated. (A) Western blots showing Tubulin- $\alpha$  (cytoplasmic marker), Lamin B1 (nuclear marker), and IL-33 content in the nucleus and cytoplasm of the cells. (B) Quantitative analysis of the same blots, reported as the ratio of nuclear/cytoplasmic signal.

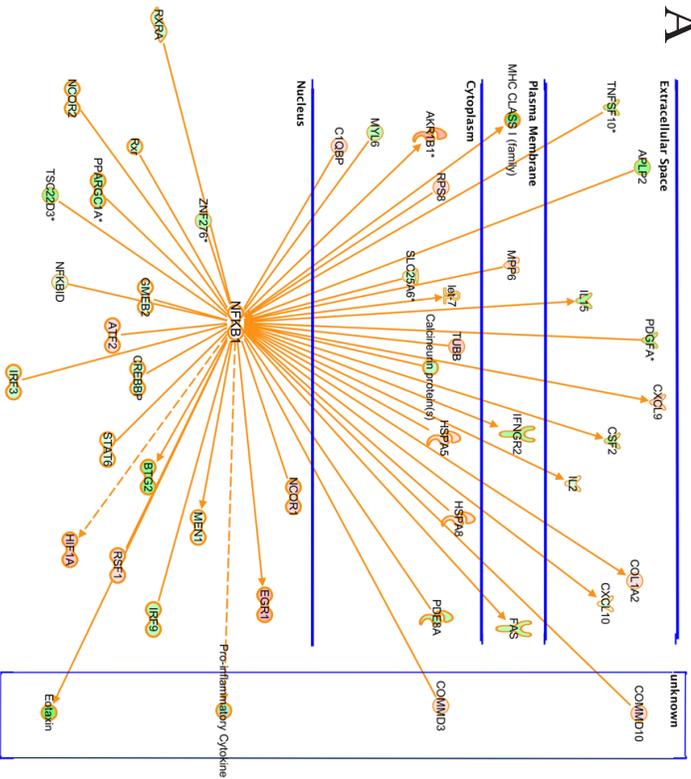


**Figure 4.7: Location of IL-33 and NF-κB in IECs during infection.** Sections of intestine from *T. spiralis*-infected (day 4) and uninfected mice were stained for IL-33 (green) and NF-κB (red). Sections of intestine from C57BL/6 mice infected orally with 100 *T. gondii* (Me49) cysts were used as a positive control for NF-κB staining. Iso indicates staining with isotype-matched control antibodies for each treatment condition. Slides were analyzed for 3 mice per group and representative images are shown.

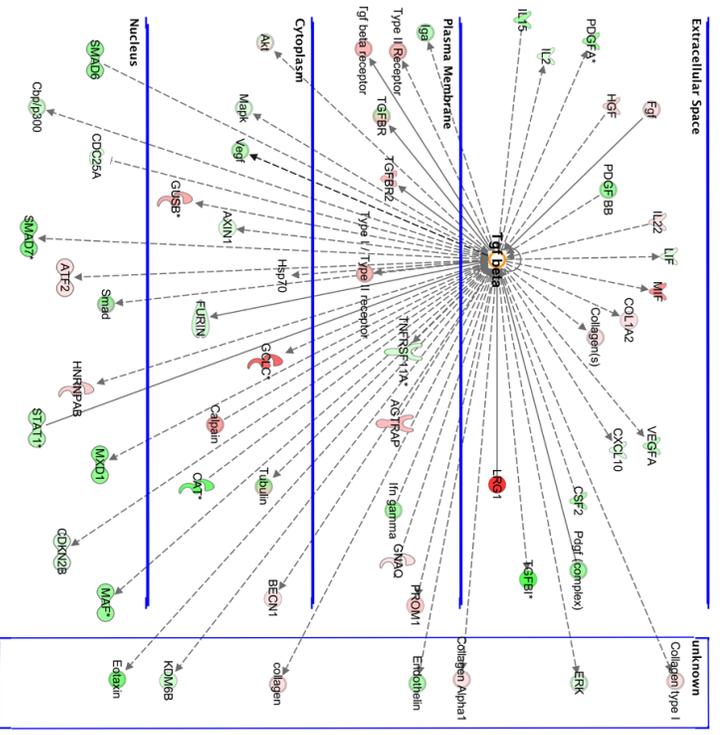
did not co-localize with IL-33 in the nuclei of the cells. IECs of mice infected orally with 100 *Toxoplasma gondii* (Me49) cysts demonstrated bright NF- $\kappa$ B staining. We concluded that IL-33 does not sequester nuclear NF- $\kappa$ B in IECs during *T. spiralis* infection.

### Gene expression microarray analysis of IECs

To further investigate the changes that occur in the intestinal epithelium at a time coincident with IL-33 translocation, but prior to the robust inflammatory response, we performed genomic microarrays comparing purified IECs from uninfected mice to mice at day 2 pi. To reduce the influence of contaminating immune cells, the array samples were collected from RAG2<sup>-/-</sup> mice. Of 26,447 informative (detected) genes, 483 were significantly altered by infection ( $p < 0.05$ ). We analyzed the differential genes using Ingenuity Pathways Analysis (IPA). Based on the analysis, the ‘top network’ that was altered in the dataset was that of ‘antimicrobial response, inflammatory response, and cell death’. The network view of this pathway revealed that many genes downstream of NF- $\kappa$ B were downregulated in IECs from infected mice. When the expression values from the dataset were overlaid onto the complete NF- $\kappa$ B interaction network, we found that 36 genes downstream of NF- $\kappa$ B were significantly diminished during infection (Figure 4.8A). In addition, the second ‘top network’ that was altered in the array was that of lipid metabolism, which revealed that many genes interacting with TGF- $\beta$  were influenced by *T. spiralis* infection of IECs. Figure 4.8B shows the overlay of the differentially expressed genes onto the TGF- $\beta$  interaction network. Genes downstream of the stress-responsive transcription factor NRF2 were highly induced by infection, as were genes such as *Lrg1*, *Capg*, *Gsta2*, *Fdps*, *Ero11*, and hexokinase 1, which carry out pro-survival functions (13, 19, 33, 46, 64, 67). The infected and uninfected samples that were compared in the array were collected and processed in an identical manner, so it is not likely that the observed increase in NRF2-mediated stress response was an artifact of tissue collection. Nine members of the cytochrome p450 family of oxidases, mainly in the CYP2 and CYP3 families, were significantly decreased during infection. Appendix A lists the genes that were most highly altered in the epithelium during infection.

**A**

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**B**

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**Figure 4.8: Changes revealed by array analysis of NF-κB and TGF-β interaction networks.** Purified IECs from uninfected or infected (2 dpi) RAG2<sup>-/-</sup> mice were compared using genomic microarrays. (A) Array expression values overlaid onto the NF-κB interaction network. (B) Array expression values overlaid onto the TGF-β interaction network. Green indicates a decrease in expression during infection, while red indicates an increase in expression. Color intensity represents the degree of up- or down-regulation of the gene. Lines represent canonical or experimentally validated direct (solid line) or indirect (dashed line) interactions, and the direction of the arrow represents the directionality of the reported interaction. The shape of each molecule represents the type of molecule.

## Discussion

We evaluated the mechanisms by which a Th2 response to *T. spiralis* is induced in the intestine and the behavior of IL-33 during infection. Although MyD88<sup>-/-</sup> mice mounted a diminished Th2 response, TLRs did not contribute in an obvious way to this effect. During antibiotic treatment, we observed a reproducible outgrowth of antibiotic-resistant bacteria in infected mice. Dysbiosis induced by *T. spiralis* infection has not been described previously, and it may occur in mice with normal gut microbiota. Dysbiosis has been implicated in the etiology of IBD (10, 60). Gene expression array data confirmed that genes downstream of the TLR-MyD88 or TLR-TRIF activation pathways were not upregulated in IECs, providing confirmation that these mediators are not significant in the epithelial cell response. Mice treated to attain complete bacterial clearance, gnotobiotic mice, or mice that cannot sense commensal bacteria may prove to have a stronger reduction in Th2 immunity, as we observed a modest effect despite achieving only transient bacterial clearance.

In addition to its role in potentiating TLR signaling, MyD88 also functions in the response to IL-33 binding of ST2 (35, 55). The weak production of IL-5 and IL-13 observed in ST2<sup>-/-</sup> mice is consistent with previous reports that IL-33 has a greater influence on IL-5 and IL-13, than on IL-4 (8, 37, 55, 56, 70). The defect in Th2 immunity was more pronounced in ST2<sup>-/-</sup> mice than in MyD88<sup>-/-</sup> mice, as ST2<sup>-/-</sup> mice had a greater reduction in IL-5 and IL-13 and lower serum mMCP-1 concentrations. IL-18 signaling, which is MyD88-dependent, suppresses mastocytosis and the Th2 cytokine response to *T. spiralis*, and the involvement of other IL-1 family members is not clear (1, 27). Thus, the results observed in MyD88<sup>-/-</sup> mice may represent the conflicting influences of molecules that suppress (e.g., IL-18) and promote (e.g., IL-33) the Th2 response.

Cytokine production by mast cells is an important part of their function during *T. spiralis* infection (30). Our results support the inductive role of mast cells (rather than a direct effector

function), because intestinal expulsion was not delayed in ST2<sup>-/-</sup> mice despite a diminished mMCP-1 response. Similarly, mice fully deficient in mMCP-1 have only a moderately delayed expulsion (34). Taken together with our results, these observations suggest that there is a threshold level of mMCP-1 that is sufficient to promote parasite clearance from the intestine.

Th2 immunity to chronic (muscle) infection with *T. spiralis* was strongly dependent on ST2, although there was no difference in muscle burden in ST2<sup>-/-</sup> mice. We have previously shown that a Th1 response is protective against the muscle phase of infection (24). Eosinophils execute a novel function during chronic *T. spiralis* infection, in which they promote survival of the parasite, rather than protection from it (24). In the absence of eosinophils, muscle larvae are killed by NO (24). It has been shown in other models that eosinophils express ST2 (17, 57). Our results suggest that the role of eosinophils in protecting the parasite during chronic infection is likely to be independent of ST2, as iNOS production in ST2<sup>-/-</sup> mice did not correlate with parasite killing.

We observed constitutive expression of IL-33 in mouse small and large intestines, mainly localized to IECs and lamina propria leukocytes. In contrast to *Trichuris muris* infection, IL-33 mRNA expression did not increase during infection (29); however, following epithelial cell invasion by the parasite, IL-33 became concentrated in the nuclei of IECs. A similar cellular distribution of IL-33 has been observed in the human intestine: IL-33 is present at a low level in the cytoplasm of IECs in healthy individuals, and is concentrated in the nucleus during ulcerative colitis (7). Further studies are warranted to shed light on the nuclear functions of IL-33 in the intestine, and whether nuclear IL-33 contributes to a defensive response in the mucosa. Translocation was not dependent on the adaptive immune response. It did not occur in the mouse large intestine or in human DLD-1 colon carcinoma cells, indicating that differences in the behavior of IL-33 during infection with *T. muris* and *T. spiralis* may relate to the different habitats of these parasites. *T. muris* colonizes the cecum, while *T. spiralis* predominantly inhabits the SI. Factors intrinsic to the SI may be required to transmit the signal that induces IL-33 translocation. Translocation was often observed along the length of an entire villus, although

the parasite is mainly found at the crypt-villus junction. Myofibroblasts along the basement membrane transmit information between disparate locations in the epithelium, making this cell type a likely candidate for disseminating the signal that induces translocation.

In the endothelia of certain tissues, IL-33 is constitutively located in the nucleus, where it binds to the histone H2A/H2B acidic pocket, and is released in response to inflammatory stimuli (36, 53). *In vitro*, nuclear translocation was associated with reduced expression of a Gal4 reporter gene, and was proposed as a mechanism for controlling proliferation in pancreatic stellate cells (16, 40). In addition, it was recently reported that nuclear IL-33 binds to NF- $\kappa$ B and prevents it from acting as a transcriptional activator in transfected cells (2). Although our analysis of gene expression in IECs revealed that targets downstream of NF- $\kappa$ B were diminished at 2 days post-infection, we did not observe colocalization of NF- $\kappa$ B with IL-33 in the nuclei of IECs. Suppression of NF- $\kappa$ B may occur due to an IL-33-independent mechanism, an indirect interaction between IL-33 and NF- $\kappa$ B, or by IL-33 binding to NF- $\kappa$ B in the cytoplasm of the cells. In other models, activation of TLRs causes an increase in expression of genes activated by NF- $\kappa$ B, so our array findings highlight the suppression (or lack of activation) of TLRs in this system.

In order to limit the comparison to genes directly altered in the epithelium, expression array analysis was performed using purified IECs from RAG2<sup>-/-</sup> mice collected at 2 dpi. This design eliminated lymphocytes and avoided the enteropathy initiated by NK cell-derived IL-13 that occurs in immunodeficient mice (41).

In addition to revealing the absence of NF- $\kappa$ B activation, other changes in gene expression were evident at a time when IL-33 is present in the nucleus. Genes downstream of the stress-responsive transcription factor NRF2 were highly induced by infection. Upregulation of pro-survival genes and downregulation of the cytochrome p450 oxidase family indicate that the epithelium becomes altered in a way that allows it to combat oxidative stress while maintaining the function of the mucosal barrier. IPA analysis revealed that lipid metabolism pathways were altered during infection, particularly molecules in the TGF- $\beta$  network. Proteins that promote the

response to TGF- $\beta$ , such as MIF, ATF-2 and several TGF- $\beta$  receptors, were strongly upregulated, while the negative regulators SMAD6 and SMAD7 were decreased during infection. These changes suggest that the sensitivity to TGF- $\beta$  is increased in the epithelium during infection.

Overall, our findings suggest that a rapid response is induced in the intestinal epithelium, characterized by the suppression of NF- $\kappa$ B activation, induction of a pro-survival program, and an increase in the sensitivity to TGF- $\beta$ . These changes occur at a time coincident with IL-33 nuclear translocation, and are independent of the adaptive immune response. In the absence of such changes, dysbiosis induced by infection could lead to the production of TNF- $\alpha$  via NF- $\kappa$ B, promoting a non-protective and destructive Th1 response. Epithelial cell death (rather than survival) would allow the microbiota access into the lamina propria, which would also likely contribute to pathology, rather than protective immunity. In contrast, TGF- $\beta$  helps regulate inflammation and protects the epithelial barrier. Even in the absence of an adaptive immune response, innate mediators in the epithelium respond to infection in a manner appropriate for a physically destructive intestinal pathogen. Furthermore, release of the endogenous danger signal IL-33 provides an innate mechanism for recognizing epithelial damage, and leads to robust induction of the Th2 response.

### **Acknowledgements**

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## References

1. **Adachi, O., T. Kawai, K. Takeda, M. Matsumoto, H. Tsutsui, M. Sakagami, K. Nakanishi, and S. Akira.** 1998. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* **9**:143-150.
2. **Ali, S., A. Mohs, M. Thomas, J. Klare, R. Ross, M. L. Schmitz, and M. U. Martin.** 2011. The dual function cytokine IL-33 interacts with the transcription factor NF-kappaB to dampen NF-kappaB-stimulated gene transcription. *Journal of immunology* **187**:1609-1616.
3. **Ali, S., D. Q. Nguyen, W. Falk, and M. U. Martin.** 2010. Caspase 3 inactivates biologically active full length interleukin-33 as a classical cytokine but does not prohibit nuclear translocation. *Biochem Biophys Res Commun* **391**:1512-1516.
4. **Appleton, J. A., and L. Usack.** 1993. Identification of potential antigenic targets for rapid expulsion of *Trichinella spiralis*. *Mol Biochem Parasitol* **58**:53-62.
5. **Beiting, D. P., S. K. Bliss, D. H. Schlafer, V. L. Roberts, and J. A. Appleton.** 2004. Interleukin-10 limits local and body cavity inflammation during infection with muscle-stage *Trichinella spiralis*. *Infection and immunity* **72**:3129-3137.
6. **Beiting, D. P., L. F. Gagliardo, M. Hesse, S. K. Bliss, D. Meskill, and J. A. Appleton.** 2007. Coordinated control of immunity to muscle stage *Trichinella spiralis* by IL-10, regulatory T cells, and TGF-beta. *J Immunol* **178**:1039-1047.
7. **Beltran, C. J., L. E. Nunez, D. Diaz-Jimenez, N. Farfan, E. Candia, C. Heine, F. Lopez, M. J. Gonzalez, R. Quera, and M. A. Hermoso.** 2010. Characterization of the novel ST2/IL-33 system in patients with inflammatory bowel disease. *Inflammatory bowel diseases* **16**:1097-1107.
8. **Besnard, A. G., D. Togbe, N. Guillou, F. Erard, V. Quesniaux, and B. Ryffel.** 2011. IL-33-activated dendritic cells are critical for allergic airway inflammation. *European journal of immunology* **41**:1675-1686.
9. **Bjerknes, M., and H. Cheng.** 1981. Methods for the isolation of intact epithelium from

- the mouse intestine. *Anat Rec* **199**:565-574.
10. **Blaser, M. J., and S. Falkow.** 2009. What are the consequences of the disappearing human microbiota? *Nat Rev Microbiol* **7**:887-894.
  11. **Blum, L. K., S. M. Thrasher, L. F. Gagliardo, V. Fabre, and J. A. Appleton.** 2009. Expulsion of secondary *Trichinella spiralis* infection in rats occurs independently of mucosal mast cell release of mast cell protease II. *J Immunol* **183**:5816-5822.
  12. **Brint, E. K., D. Xu, H. Liu, A. Dunne, A. N. McKenzie, L. A. O'Neill, and F. Y. Liew.** 2004. ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance. *Nature immunology* **5**:373-379.
  13. **Bryson, J. M., P. E. Coy, K. Gottlob, N. Hay, and R. B. Robey.** 2002. Increased hexokinase activity, of either ectopic or endogenous origin, protects renal epithelial cells against acute oxidant-induced cell death. *The Journal of biological chemistry* **277**:11392-11400.
  14. **Burns, K., F. Martinon, C. Esslinger, H. Pahl, P. Schneider, J. L. Bodmer, F. Di Marco, L. French, and J. Tschopp.** 1998. MyD88, an adapter protein involved in interleukin-1 signaling. *The Journal of biological chemistry* **273**:12203-12209.
  15. **Butcher, B. A., L. F. Gagliardo, T. ManWarren, and J. A. Appleton.** 2000. Larvae-induced plasma membrane wounds and glycoprotein deposition are insufficient for *Trichinella spiralis* invasion of epithelial cells. *Molecular and biochemical parasitology* **107**:207-218.
  16. **Carriere, V., L. Roussel, N. Ortega, D. A. Lacorre, L. Americh, L. Aguilar, G. Bouche, and J. P. Girard.** 2007. IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **104**:282-287.
  17. **Cherry, W. B., J. Yoon, K. R. Bartemes, K. Iijima, and H. Kita.** 2008. A novel IL-1 family cytokine, IL-33, potently activates human eosinophils. *The Journal of allergy and clinical immunology* **121**:1484-1490.

18. **Chockalingam, A., J. C. Brooks, J. L. Cameron, L. K. Blum, and C. A. Leifer.** 2009. TLR9 traffics through the Golgi complex to localize to endolysosomes and respond to CpG DNA. *Immunology and cell biology* **87**:209-217.
19. **Codina, R., A. Vanasse, A. Kelekar, V. Vezys, and R. Jemmerson.** 2010. Cytochrome c-induced lymphocyte death from the outside in: inhibition by serum leucine-rich alpha-2-glycoprotein-1. *Apoptosis* **15**:139-152.
20. **Croswell, A., E. Amir, P. Teggtatz, M. Barman, and N. H. Salzman.** 2009. Prolonged impact of antibiotics on intestinal microbial ecology and susceptibility to enteric Salmonella infection. *Infection and immunity* **77**:2741-2753.
21. **Daley, L. P., L. F. Gagliardo, M. S. Duffy, M. C. Smith, and J. A. Appleton.** 2005. Application of monoclonal antibodies in functional and comparative investigations of heavy-chain immunoglobulins in new world camelids. *Clin Diagn Lab Immunol* **12**:380-386.
22. **Denkers, E. Y., D. L. Wassom, and C. E. Hayes.** 1990. Characterization of *Trichinella spiralis* antigens sharing an immunodominant, carbohydrate-associated determinant distinct from phosphorylcholine. *Molecular and biochemical parasitology* **41**:241-249.
23. **Eckburg, P. B., E. M. Bik, C. N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S. R. Gill, K. E. Nelson, and D. A. Relman.** 2005. Diversity of the human intestinal microbial flora. *Science* **308**:1635-1638.
24. **Fabre, V., D. P. Beiting, S. K. Bliss, N. G. Gebreselassie, L. F. Gagliardo, N. A. Lee, J. J. Lee, and J. A. Appleton.** 2009. Eosinophil deficiency compromises parasite survival in chronic nematode infection. *J Immunol* **182**:1577-1583.
25. **Fitzgerald, K. A., E. M. Palsson-McDermott, A. G. Bowie, C. A. Jefferies, A. S. Mansell, G. Brady, E. Brint, A. Dunne, P. Gray, M. T. Harte, D. McMurray, D. E. Smith, J. E. Sims, T. A. Bird, and L. A. O'Neill.** 2001. Mal (MyD88-adaptor-like) is required for Toll-like receptor-4 signal transduction. *Nature* **413**:78-83.
26. **Gagliardo, L. F., C. S. McVay, and J. A. Appleton.** 2002. Molting, ecdysis, and

- reproduction of *Trichinella spiralis* are supported in vitro by intestinal epithelial cells. *Infect Immun* **70**:1853-1859.
27. **Helmby, H., and R. K. Grencis.** 2002. IL-18 regulates intestinal mastocytosis and Th2 cytokine production independently of IFN-gamma during *Trichinella spiralis* infection. *J Immunol* **169**:2553-2560.
  28. **Hsu, C. L., C. V. Neilsen, and P. J. Bryce.** 2010. IL-33 is produced by mast cells and regulates IgE-dependent inflammation. *PLoS One* **5**:e11944.
  29. **Humphreys, N. E., D. Xu, M. R. Hepworth, F. Y. Liew, and R. K. Grencis.** 2008. IL-33, a potent inducer of adaptive immunity to intestinal nematodes. *J Immunol* **180**:2443-2449.
  30. **Ierna, M. X., H. E. Scales, K. L. Saunders, and C. E. Lawrence.** 2008. Mast cell production of IL-4 and TNF may be required for protective and pathological responses in gastrointestinal helminth infection. *Mucosal Immunol* **1**:147-155.
  31. **Johansson, M. E., M. Phillipson, J. Petersson, A. Velcich, L. Holm, and G. C. Hansson.** 2008. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proceedings of the National Academy of Sciences of the United States of America* **105**:15064-15069.
  32. **Kawai, T., and S. Akira.** 2011. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* **34**:637-650.
  33. **Khoo, C., J. Yang, G. Rajpal, Y. Wang, J. Liu, P. Arvan, and D. A. Stoffers.** 2011. Endoplasmic reticulum oxidoreductin-1-like beta (ERO1beta) regulates susceptibility to endoplasmic reticulum stress and is induced by insulin flux in beta-cells. *Endocrinology* **152**:2599-2608.
  34. **Knight, P. A., S. H. Wright, C. E. Lawrence, Y. Y. Paterson, and H. R. Miller.** 2000. Delayed expulsion of the nematode *Trichinella spiralis* in mice lacking the mucosal mast cell-specific granule chymase, mouse mast cell protease-1. *The Journal of experimental medicine* **192**:1849-1856.

35. **Kroeger, K. M., B. M. Sullivan, and R. M. Locksley.** 2009. IL-18 and IL-33 elicit Th2 cytokines from basophils via a MyD88- and p38alpha-dependent pathway. *J Leukoc Biol* **86**:769-778.
36. **Kuchler, A. M., J. Pollheimer, J. Balogh, J. Sponheim, L. Manley, D. R. Sorensen, P. M. De Angelis, H. Scott, and G. Haraldsen.** 2008. Nuclear interleukin-33 is generally expressed in resting endothelium but rapidly lost upon angiogenic or proinflammatory activation. *The American journal of pathology* **173**:1229-1242.
37. **Kurowska-Stolarska, M., P. Kewin, G. Murphy, R. C. Russo, B. Stolarski, C. C. Garcia, M. Komai-Koma, N. Pitman, Y. Li, W. Niedbala, A. N. McKenzie, M. M. Teixeira, F. Y. Liew, and D. Xu.** 2008. IL-33 induces antigen-specific IL-5+ T cells and promotes allergic-induced airway inflammation independent of IL-4. *Journal of immunology* **181**:4780-4790.
38. **Luthi, A. U., S. P. Cullen, E. A. McNeela, P. J. Duriez, I. S. Afonina, C. Sheridan, G. Brumatti, R. C. Taylor, K. Kersse, P. Vandenabeele, E. C. Lavelle, and S. J. Martin.** 2009. Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases. *Immunity* **31**:84-98.
39. **ManWarren, T., L. Gagliardo, J. Geyer, C. McVay, S. Pearce-Kelling, and J. Appleton.** 1997. Invasion of intestinal epithelia in vitro by the parasitic nematode *Trichinella spiralis*. *Infect Immun* **65**:4806-4812.
40. **Masamune, A., T. Watanabe, K. Kikuta, K. Satoh, A. Kanno, and T. Shimosegawa.** 2010. Nuclear expression of interleukin-33 in pancreatic stellate cells. *American journal of physiology. Gastrointestinal and liver physiology* **299**:G821-832.
41. **McDermott, J. R., N. E. Humphreys, S. P. Forman, D. D. Donaldson, and R. K. Grencis.** 2005. Intraepithelial NK cell-derived IL-13 induces intestinal pathology associated with nematode infection. *Journal of immunology* **175**:3207-3213.
42. **Medzhitov, R., P. Preston-Hurlburt, E. Kopp, A. Stadlen, C. Chen, S. Ghosh, and C. A. Janeway, Jr.** 1998. MyD88 is an adaptor protein in the hToll/IL-1 receptor family

- signaling pathways. *Mol Cell* **2**:253-258.
43. **Moolenbeek, C., and E. J. Ruitenber**. 1981. The “Swiss roll”: a simple technique for histological studies of the rodent intestine. *Lab Anim* **15**:57-59.
  44. **Moussion, C., N. Ortega, and J. P. Girard**. 2008. The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel ‘alarmin’? *PLoS One* **3**:e3331.
  45. **Nile, C. J., E. Barksby, P. Jitprasertwong, P. M. Preshaw, and J. J. Taylor**. 2010. Expression and regulation of interleukin-33 in human monocytes. *Immunology* **130**:172-180.
  46. **Notarnicola, M., C. Messa, A. Cavallini, M. Bifulco, M. F. Tecce, D. Eletto, A. Di Leo, S. Montemurro, C. Laezza, and M. G. Caruso**. 2004. Higher farnesyl diphosphate synthase activity in human colorectal cancer inhibition of cellular apoptosis. *Oncology (Williston Park)* **67**:351-358.
  47. **Oboki, K., T. Ohno, N. Kajiwara, K. Arae, H. Morita, A. Ishii, A. Nambu, T. Abe, H. Kiyonari, K. Matsumoto, K. Sudo, K. Okumura, H. Saito, and S. Nakae**. 2010. IL-33 is a crucial amplifier of innate rather than acquired immunity. *Proceedings of the National Academy of Sciences of the United States of America* **107**:18581-18586.
  48. **Park, J. S., J. Arcaroli, H. K. Yum, H. Yang, H. Wang, K. Y. Yang, K. H. Choe, D. Strassheim, T. M. Pitts, K. J. Tracey, and E. Abraham**. 2003. Activation of gene expression in human neutrophils by high mobility group box 1 protein. *Am J Physiol Cell Physiol* **284**:C870-879.
  49. **Peters, P. J., L. F. Gagliardo, E. A. Sabin, A. B. Betchen, K. Ghosh, J. B. Oblak, and J. A. Appleton**. 1999. Dominance of immunoglobulin G2c in the antiphosphorylcholine response of rats infected with *Trichinella spiralis*. *Infect Immun* **67**:4661-4667.
  50. **Prefontaine, D., S. Lajoie-Kadoch, S. Foley, S. Audusseau, R. Olivenstein, A. J. Halayko, C. Lemiere, J. G. Martin, and Q. Hamid**. 2009. Increased expression of IL-33 in severe asthma: evidence of expression by airway smooth muscle cells. *Journal of*

- immunology **183**:5094-5103.
51. **Reason, A. J., L. A. Ellis, J. A. Appleton, N. Wisnewski, R. B. Grieve, M. McNeil, D. L. Wassom, H. R. Morris, and A. Dell.** 1994. Novel tyvelose-containing tri- and tetra-antennary N-glycans in the immunodominant antigens of the intracellular parasite *Trichinella spiralis*. *Glycobiology* **4**:593-603.
  52. **Rosbottom, A., P. A. Knight, G. McLachlan, E. M. Thornton, S. W. Wright, H. R. Miller, and C. L. Scudamore.** 2002. Chemokine and cytokine expression in murine intestinal epithelium following *Nippostrongylus brasiliensis* infection. *Parasite immunology* **24**:67-75.
  53. **Roussel, L., M. Erard, C. Cayrol, and J. P. Girard.** 2008. Molecular mimicry between IL-33 and KSHV for attachment to chromatin through the H2A-H2B acidic pocket. *EMBO Rep* **9**:1006-1012.
  54. **Savage, D. C.** 1977. Microbial ecology of the gastrointestinal tract. *Annu Rev Microbiol* **31**:107-133.
  55. **Schmitz, J., A. Owyang, E. Oldham, Y. Song, E. Murphy, T. K. McClanahan, G. Zurawski, M. Moshrefi, J. Qin, X. Li, D. M. Gorman, J. F. Bazan, and R. A. Kastelein.** 2005. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* **23**:479-490.
  56. **Smithgall, M. D., M. R. Comeau, B. R. Yoon, D. Kaufman, R. Armitage, and D. E. Smith.** 2008. IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. *International immunology* **20**:1019-1030.
  57. **Stolarski, B., M. Kurowska-Stolarska, P. Kewin, D. Xu, and F. Y. Liew.** 2010. IL-33 exacerbates eosinophil-mediated airway inflammation. *Journal of immunology* **185**:3472-3480.
  58. **Sukhdeo, M. V., and N. A. Croll.** 1981. The location of parasites within their hosts:

- factors affecting longitudinal distribution of *Trichinella spiralis* in the small intestine of mice. *International journal for parasitology* **11**:163-168.
59. **Sukhumavasi, W., C. E. Egan, A. L. Warren, G. A. Taylor, B. A. Fox, D. J. Bzik, and E. Y. Denkers.** 2008. TLR adaptor MyD88 is essential for pathogen control during oral *Toxoplasma gondii* infection but not adaptive immunity induced by a vaccine strain of the parasite. *Journal of immunology* **181**:3464-3473.
60. **Swidsinski, A., J. Weber, V. Loening-Baucke, L. P. Hale, and H. Lochs.** 2005. Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease. *J Clin Microbiol* **43**:3380-3389.
61. **Thomas, P. G., M. R. Carter, O. Atochina, A. A. Da'Dara, D. Piskorska, E. McGuire, and D. A. Harn.** 2003. Maturation of dendritic cell 2 phenotype by a helminth glycan uses a Toll-like receptor 4-dependent mechanism. *Journal of immunology* **171**:5837-5841.
62. **Vaishnav, S., M. Yamamoto, K. M. Severson, K. A. Ruhn, X. Yu, O. Koren, R. Ley, E. K. Wakeland, and L. V. Hooper.** 2011. The antibacterial lectin RegIII $\gamma$  promotes the spatial segregation of microbiota and host in the intestine. *Science* **334**:255-258.
63. **van der Kleij, D., E. Latz, J. F. Brouwers, Y. C. Kruize, M. Schmitz, E. A. Kurt-Jones, T. Espevik, E. C. de Jong, M. L. Kapsenberg, D. T. Golenbock, A. G. Tielens, and M. Yazdanbakhsh.** 2002. A novel host-parasite lipid cross-talk. Schistosomal lysophosphatidylserine activates toll-like receptor 2 and affects immune polarization. *The Journal of biological chemistry* **277**:48122-48129.
64. **Watari, A., K. Takaki, S. Higashiyama, Y. Li, Y. Satomi, T. Takao, A. Tanemura, Y. Yamaguchi, I. Katayama, M. Shimakage, I. Miyashiro, K. Takami, K. Kodama, and M. Yutsudo.** 2006. Suppression of tumorigenicity, but not anchorage independence, of human cancer cells by new candidate tumor suppressor gene CapG. *Oncogene* **25**:7373-7380.

65. **Wood, I. S., B. Wang, and P. Trayhurn.** 2009. IL-33, a recently identified interleukin-1 gene family member, is expressed in human adipocytes. *Biochem Biophys Res Commun* **384**:105-109.
66. **Wright, K. A., E. Weidman, and H. Hong.** 1987. The distribution of cells killed by *Trichinella spiralis* in the mucosal epithelium of two strains of mice. *J Parasitol* **73**:935-939.
67. **Xie, J., K. Shults, L. Flye, F. Jiang, D. R. Head, and R. C. Briggs.** 2005. Overexpression of GSTA2 protects against cell cycle arrest and apoptosis induced by the DNA inter-strand crosslinking nitrogen mustard, mechlorethamine. *J Cell Biochem* **95**:339-351.
68. **Yanagawa, Y., M. Suzuki, M. Matsumoto, and H. Togashi.** 2011. Prostaglandin E(2) enhances IL-33 production by dendritic cells. *Immunology letters*.
69. **Yasuoka, S., J. Kawanokuchi, B. Parajuli, S. Jin, Y. Doi, M. Noda, Y. Sonobe, H. Takeuchi, T. Mizuno, and A. Suzumura.** 2011. Production and functions of IL-33 in the central nervous system. *Brain Res* **1385**:8-17.
70. **Yin, H., X. Y. Li, X. B. Jin, B. B. Zhang, Q. Gong, H. Yang, F. Zheng, F. L. Gong, and J. Y. Zhu.** 2010. IL-33 prolongs murine cardiac allograft survival through induction of TH2-type immune deviation. *Transplantation* **89**:1189-1197.
71. **Zhao, W., and Z. Hu.** 2010. The enigmatic processing and secretion of interleukin-33. *Cell Mol Immunol* **7**:260-262.

# **Chapter 5**

## **Summary and Conclusions**

Intestinal helminths infect over 2 billion people worldwide, and the mechanisms by which immunity to these pathogens is induced remain poorly understood (27). We investigated the role of mast cells in protective secondary immunity, differences in protection and pathology between different regions of the intestine, and the contribution of innate mediators in initiating the Th2 response to infection. Secondary immunity to *Trichinella spiralis* involves a novel immune mechanism, in which the systemic antibody response is crucial to the expression of intestinal immunity (2). In rats, 90% of parasites are cleared from the intestine within one hour of a challenge infection. Understanding this mechanism may aid in the development of vaccines, which often aim to deliver antigen systemically in order to protect a mucosal surface.

Although tyvelose-specific antibodies are sufficient to confer protection on suckling rats, adult rats require an additional inflammatory mediator, such as adoptive transfer of T cells or a heterologous infection (2, 4, 6). We showed that intestinal priming is not the factor required to enable antibody-mediated protection in adult rats, as infection limited to the muscle phase induces protective immunity. In addition, our results contradict the dogma that mast cell degranulation directly contributes to the mechanism of parasite expulsion. However, mast cell deficient rats have compromised expulsion, and mast cell-derived cytokines contribute to protective immunity in mice during primary infection (16, 25). We also hypothesized that peritoneal mast cells constitute the link between systemic and intestinal sites of infection, but transfer of these cells, in combination with antibody, did not confer protection (not shown). Taken together these facts suggest that the crucial function of mast cells during rapid expulsion may be during the initiation of immunity, rather than the effector phase.

Immunity to *T. spiralis* is often compared with immunity to *Trichuris muris*, a cecal dwelling nematode. Although the two parasites are closely related, the mechanisms of worm clearance are distinct, with mast cells reported to play a prominent role in expulsion of adult *T. spiralis*, while epithelial cell turnover and function appear to be key to the clearance of *T. muris* (5, 14). The differences in effector mechanisms may be dictated by properties intrinsic to each

parasite, or by their preferred locations in the intestinal tract. Similarly, differences between two common types of inflammatory bowel disease (IBD), Crohn's disease and ulcerative colitis, may be caused by mechanisms intrinsic to the etiology of each disease state or to differences in the affected sites.

During intestinal colonization and expulsion, *T. spiralis* ultimately passes through every region of the rodent intestine, and these sites vary greatly with regard to morphology, physiology, and immunology. We observed differences in the quality of the Th2 cytokine response between the small and large intestines (SI and LI, Chapter 3). In addition, a potent mastocytosis occurs in the SI, but was not present in the LI. Enteropathy in the LI was tightly regulated by IL-10, while infection-induced pathology occurred in the SI even in the presence of IL-10. However, IL-10-deficient mice produced significantly more IL-17 in the SI than C57BL/6 controls, and IL-17 production correlated with an increase in neutrophil infiltration. Our results demonstrate that mucosal mastocytosis and the cytokines that influence it are not prominent in the LI during worm expulsion, and that immunopathology is more tightly regulated in the LI than the SI. Thus, the mechanisms of immunity and enteropathy vary in different regions of the intestine during helminth infection. The results presented in this chapter support the hypothesis that infection of distinct regions of the intestine contributes to differences between the mechanisms of immunity to *T. spiralis* and *Trichuris muris*. Further work should utilize intra-cecal injection of parasites, to discern if infections limited to the LI can promote SI immunity. Evaluation and adoptive transfer of CCR9+ lymphocytes isolated from the SI would further clarify the presence of a pathogenic IL-17-producing population, and whether these cells can home to the SI or LI after transfer.

We went on to investigate the influence of innate mediators in inducing the Th2 response to infection. We collected evidence suggesting that MyD88 is influential in promoting Th2 responses (Chapter 4). To further dissect the role of MyD88, we showed that mice treated to alter the gut microbiota had a modest decrease in Th2 cytokine production. However, TLR2/4<sup>-/-</sup> mice did not recapitulate this reduction, and we did not observe activation of TLRs by *T. spiralis* products *in vitro*. Dampened immunity in MyD88<sup>-/-</sup> mice did not correspond with impaired

parasite expulsion. Although mice completely deficient in Th2 cytokines have impaired parasite clearance, it is possible that a threshold level of IL-4 is sufficient to promote expulsion (22). In some cases, the mechanism of TLR-dependent modulation of the immune response by helminth-derived molecules is to dampen the response of TLRs to Th1-driving ligands, such as LPS (9, 12, 13, 26). Thus, although a more complex mechanism may be at play between *T. spiralis*, TLRs, and non-parasite TLR stimuli (i.e., microbiota), our results do not support a role for direct activation of TLRs by parasite-derived molecules in the MyD88-dependent induction of Th2 immunity. Stimulation of TLRs (other than TLR2/4) by endogenous DAMPs released from necrotic cells, such as host nucleic acids, remains a possible mechanism of immune induction during *T. spiralis* infection.

In addition to its role in TLR signaling, MyD88 also perpetuates the response to IL-1 family members, including IL-18 and IL-33 (1, 17, 19). IL-18 is known to inhibit the Th2 response during *T. spiralis* infection, but the influence of other IL-1 family members has not been reported. We investigated the importance of ST2, the receptor for IL-33. ST2<sup>-/-</sup> mice displayed a strong reduction in the Th2 cytokine response, particularly for IL-5 and IL-13. These results fit with previous studies that have shown that IL-33 has a stronger influence in inducing IL-5 and IL-13, relative to IL-4 (8, 18, 23, 24, 28). Although many papers cite the importance of IL-33 in helminth infection, the previous report (during *T. muris* infection) only encompassed the effects of administering recombinant IL-33 (15). Use of a mouse strain with targeted deletion of ST2 allows for a stronger evaluation of the role of IL-33 during helminth infection.

In addition to its role as a paracrine cytokine, IL-33 also carries out an intracellular signaling mechanism, which is not well-understood (11). We found that IL-33 is constitutively expressed in intestinal tissue, and is not upregulated during infection. IL-33 was observed in intestinal leukocytes and in epithelial cells, where it concentrated in the nucleus within two days of infection. The behavior of IL-33 that we observed in mice follows a similar pattern to what has recently been reported for humans: IL-33 was present at a low level in the cytoplasm of IECs in healthy controls, and became concentrated in the nucleus during ulcerative colitis (7). Given

this parallel, and the necessity for a better understanding of the behavior of the immune system during IBD, further study is warranted to clarify the function of IL-33 nuclear translocation during Th2-mediated intestinal inflammation. In addition, elucidation of the mediators that trigger translocation may provide a useful clinical target for reducing inflammation and promoting epithelial healing during IBD.

In addition to binding histones, nuclear IL-33 binds to NF- $\kappa$ B and prevents it from acting as a transcriptional activator (3, 21). We observed that targets downstream of NF- $\kappa$ B were diminished in infected epithelial cells. However, immunofluorescent staining did not reveal nuclear NF- $\kappa$ B in cells with nuclear IL-33. Downregulation of NF- $\kappa$ B targets may occur due to an IL-33-independent mechanism, an indirect interaction between IL-33 and NF- $\kappa$ B, or by IL-33 binding to NF- $\kappa$ B in the cytoplasm of the cells. Our results highlight the importance of evaluating the behavior of IL-33 *in vivo*, as we did not see the same pattern that was reported in a transfected cell line *in vitro*.

Suppression of epithelial NF- $\kappa$ B activation during *T. spiralis* infection differs from what has been observed in other models of intestinal infection: NF- $\kappa$ B DNA binding activity in the epithelium is induced by *T. muris* infection, and mice with epithelial-specific inhibition of NF- $\kappa$ B have a Th1 response, which is pathologic, rather than protective (29). Similarly, infection with *Salmonella enterica* induces NF- $\kappa$ B activation in IECs (10). However, the specific habitats of these pathogens differ from that of *T. spiralis*. *T. muris* lives imbedded in the epithelium of the cecum, and remains within a limited tunnel of dead cells (reviewed in (20)). *Salmonella* invades epithelial cells of the distal ileum and LI, without causing these cells to undergo necrosis, and ultimately infects macrophages in the lamina propria (reviewed in (30)). These differences are consistent with the hypothesis that physical disruption of the epithelium during *T. spiralis* infection contributes to the suppression of NF- $\kappa$ B in our model. In addition, we observed differences in the behavior of IL-33 between the SI and LI, which may correlate with the differences in behavior of NF- $\kappa$ B between *T. muris* and *T. spiralis*.

Four hundred and eighty-three genes were differentially regulated in our genomic

microarray. Rather than subjectively choosing genes of interest, we used Ingenuity Pathways Analysis (IPA) to identify pathways that are strongly altered in intestinal epithelial cells (IECs) during infection. This type of analysis provides an objectively ranked list of the most highly altered pathways, as well as the ability to identify pathways in which many genes undergo a low level of regulation. In addition to revealing diminished NF- $\kappa$ B activation, we observed other global changes in infected IECs, at a time when IL-33 is present in the nucleus. Genes downstream of the stress-responsive transcription factor NRF2 were highly induced by infection. NRF2 responds to oxidative stress by activating the transcription of antioxidant enzymes. NRF2 signaling has been widely studied, and it would be of interest to measure the behavior of molecules in this pathway during epithelial invasion *in vitro*. The infected and uninfected samples that were compared in the array were collected and processed in an identical manner, so it is not likely that the observed increase in NRF2-mediated stress response was an artifact of tissue collection.

IPA analysis also revealed that lipid metabolism pathways were altered during infection, particularly molecules in the TGF- $\beta$  network. Proteins that promote the response to TGF- $\beta$ , such as MIF, ATF-2 and several TGF- $\beta$  receptors, were strongly upregulated, while the negative regulators SMAD6 and SMAD7 were decreased during infection. Overall, the results suggest that a global response is induced in the epithelium, which leads to a reduction in the NF- $\kappa$ B-mediated pathologic response, protection of the mucosal barrier by TGF- $\beta$ , and the induction of a pro-survival program. These changes occur at a time coincident with IL-33 nuclear translocation, and prior to initiation of the adaptive response. Further work is needed to determine if nuclear IL-33 is required for the induction of protective changes in the epithelium during infection. Our results support a model in which TLR-independent activation by the microbiota, in combination with epithelial tissue damage and release of IL-33 (by IECs or leukocytes) promote Th2 immunity during *T. spiralis* infection.

Taken together, these three studies clarify the interactions by which a potent Th2 response to *T. spiralis* is induced. We reported that mast cells are likely to play an inductive,

rather than effector, role in protective immunity; that the regulation and mechanisms of inflammation induced by a single parasite differ between distinct intestinal locations within the host; that innate mediators such as IL-33 contribute to the adaptive response; and that nuclear translocation of IL-33 coincides with dramatic changes that occur in the epithelium within two days of infection. These results allow us to conclude that a complex interaction between innate mediators and adaptive immunity is required for both protective and pathologic responses.

### References

1. **Adachi, O., T. Kawai, K. Takeda, M. Matsumoto, H. Tsutsui, M. Sakagami, K. Nakanishi, and S. Akira.** 1998. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* **9**:143-150.
2. **Ahmad, A., C. H. Wang, M. Korenaga, R. G. Bell, and L. S. Adams.** 1990. Synergistic interaction between immune serum and thoracic duct cells in the adoptive transfer of rapid expulsion of *Trichinella spiralis* in adult rats. *Exp Parasitol* **71**:90-99.
3. **Ali, S., A. Mohs, M. Thomas, J. Klare, R. Ross, M. L. Schmitz, and M. U. Martin.** 2011. The dual function cytokine IL-33 interacts with the transcription factor NF-kappaB to dampen NF-kappaB-stimulated gene transcription. *Journal of immunology* **187**:1609-1616.
4. **Appleton, J. A., L. R. Schain, and D. D. McGregor.** 1988. Rapid expulsion of *Trichinella spiralis* in suckling rats: mediation by monoclonal antibodies. *Immunology* **65**:487-492.
5. **Artis, D., C. S. Potten, K. J. Else, F. D. Finkelman, and R. K. Grencis.** 1999. *Trichuris muris*: host intestinal epithelial cell hyperproliferation during chronic infection is regulated by interferon-gamma. *Exp Parasitol* **92**:144-153.
6. **Bell, R. G., J. A. Appleton, D. A. Negrao-Correa, and L. S. Adams.** 1992. Rapid expulsion of *Trichinella spiralis* in adult rats mediated by monoclonal antibodies of

- distinct IgG isotypes. *Immunology* **75**:520-527.
7. **Beltran, C. J., L. E. Nunez, D. Diaz-Jimenez, N. Farfan, E. Candia, C. Heine, F. Lopez, M. J. Gonzalez, R. Quera, and M. A. Hermoso.** 2010. Characterization of the novel ST2/IL-33 system in patients with inflammatory bowel disease. *Inflammatory bowel diseases* **16**:1097-1107.
  8. **Besnard, A. G., D. Togbe, N. Guillou, F. Erard, V. Quesniaux, and B. Ryffel.** 2011. IL-33-activated dendritic cells are critical for allergic airway inflammation. *European journal of immunology* **41**:1675-1686.
  9. **Brannstrom, K., M. E. Sellin, P. Holmfeldt, M. Brattsand, and M. Gullberg.** 2009. The *Schistosoma mansoni* protein Sm16/SmSLP/SmSPO-1 assembles into a nine-subunit oligomer with potential To inhibit Toll-like receptor signaling. *Infection and immunity* **77**:1144-1154.
  10. **Bruno, V. M., S. Hannemann, M. Lara-Tejero, R. A. Flavell, S. H. Kleinstein, and J. E. Galan.** 2009. Salmonella Typhimurium type III secretion effectors stimulate innate immune responses in cultured epithelial cells. *PLoS Pathog* **5**:e1000538.
  11. **Carriere, V., L. Roussel, N. Ortega, D. A. Lacorre, L. Americh, L. Aguilar, G. Bouche, and J. P. Girard.** 2007. IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **104**:282-287.
  12. **Goodridge, H. S., F. A. Marshall, K. J. Else, K. M. Houston, C. Egan, L. Al-Riyami, F. Y. Liew, W. Harnett, and M. M. Harnett.** 2005. Immunomodulation via novel use of TLR4 by the filarial nematode phosphorylcholine-containing secreted product, ES-62. *Journal of immunology* **174**:284-293.
  13. **Goodridge, H. S., S. McGuinness, K. M. Houston, C. A. Egan, L. Al-Riyami, M. J. Alcocer, M. M. Harnett, and W. Harnett.** 2007. Phosphorylcholine mimics the effects of ES-62 on macrophages and dendritic cells. *Parasite immunology* **29**:127-137.
  14. **Ha, T. Y., N. D. Reed, and P. K. Crowle.** 1983. Delayed expulsion of adult *Trichinella*

- spiralis by mast cell-deficient W/W<sup>v</sup> mice. *Infect Immun* **41**:445-447.
15. **Humphreys, N. E., D. Xu, M. R. Hepworth, F. Y. Liew, and R. K. Grencis.** 2008. IL-33, a potent inducer of adaptive immunity to intestinal nematodes. *J Immunol* **180**:2443-2449.
  16. **Ierna, M. X., H. E. Scales, K. L. Saunders, and C. E. Lawrence.** 2008. Mast cell production of IL-4 and TNF may be required for protective and pathological responses in gastrointestinal helminth infection. *Mucosal Immunol* **1**:147-155.
  17. **Kroeger, K. M., B. M. Sullivan, and R. M. Locksley.** 2009. IL-18 and IL-33 elicit Th2 cytokines from basophils via a MyD88- and p38alpha-dependent pathway. *J Leukoc Biol* **86**:769-778.
  18. **Kurowska-Stolarska, M., P. Kewin, G. Murphy, R. C. Russo, B. Stolarski, C. C. Garcia, M. Komai-Koma, N. Pitman, Y. Li, W. Niedbala, A. N. McKenzie, M. M. Teixeira, F. Y. Liew, and D. Xu.** 2008. IL-33 induces antigen-specific IL-5<sup>+</sup> T cells and promotes allergic-induced airway inflammation independent of IL-4. *Journal of immunology* **181**:4780-4790.
  19. **Medzhitov, R., P. Preston-Hurlburt, E. Kopp, A. Stadlen, C. Chen, S. Ghosh, and C. A. Janeway, Jr.** 1998. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Mol Cell* **2**:253-258.
  20. **Patel, N., T. Kreider, J. F. Urban, Jr., and W. C. Gause.** 2009. Characterisation of effector mechanisms at the host:parasite interface during the immune response to tissue-dwelling intestinal nematode parasites. *International journal for parasitology* **39**:13-21.
  21. **Roussel, L., M. Erard, C. Cayrol, and J. P. Girard.** 2008. Molecular mimicry between IL-33 and KSHV for attachment to chromatin through the H2A-H2B acidic pocket. *EMBO Rep* **9**:1006-1012.
  22. **Scales, H. E., M. X. Ierna, and C. E. Lawrence.** 2007. The role of IL-4, IL-13 and IL-4Ralpha in the development of protective and pathological responses to *Trichinella spiralis*. *Parasite Immunol* **29**:81-91.

23. **Schmitz, J., A. Owyang, E. Oldham, Y. Song, E. Murphy, T. K. McClanahan, G. Zurawski, M. Moshrefi, J. Qin, X. Li, D. M. Gorman, J. F. Bazan, and R. A. Kastelein.** 2005. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* **23**:479-490.
24. **Smithgall, M. D., M. R. Comeau, B. R. Yoon, D. Kaufman, R. Armitage, and D. E. Smith.** 2008. IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. *International immunology* **20**:1019-1030.
25. **Suzuki, T., T. Sasaki, H. Takagi, K. Sato, and K. Ueda.** 2008. The effectors responsible for gastrointestinal nematode parasites, *Trichinella spiralis*, expulsion in rats. *Parasitology research* **103**:1289-1295.
26. **van Liempt, E., S. J. van Vliet, A. Engering, J. J. Garcia Vallejo, C. M. Bank, M. Sanchez-Hernandez, Y. van Kooyk, and I. van Die.** 2007. *Schistosoma mansoni* soluble egg antigens are internalized by human dendritic cells through multiple C-type lectins and suppress TLR-induced dendritic cell activation. *Molecular immunology* **44**:2605-2615.
27. **WHO** 2010, posting date. PCT databank. [Online.]
28. **Yin, H., X. Y. Li, X. B. Jin, B. B. Zhang, Q. Gong, H. Yang, F. Zheng, F. L. Gong, and J. Y. Zhu.** 2010. IL-33 prolongs murine cardiac allograft survival through induction of TH2-type immune deviation. *Transplantation* **89**:1189-1197.
29. **Zaph, C., A. E. Troy, B. C. Taylor, L. D. Berman-Booty, K. J. Guild, Y. Du, E. A. Yost, A. D. Gruber, M. J. May, F. R. Greten, L. Eckmann, M. Karin, and D. Artis.** 2007. Epithelial-cell-intrinsic IKK-beta expression regulates intestinal immune homeostasis. *Nature* **446**:552-556.
30. **Zhou, D., and J. Galan.** 2001. Salmonella entry into host cells: the work in concert of type III secreted effector proteins. *Microbes and infection / Institut Pasteur* **3**:1293-1298.

# Appendix A

**Most highly altered genes in the murine epithelium during *T. spiralis* infection**

## Upregulated Genes

ID	Gene Symbol	Gene Title	RefSeq ID	mean.I	mean.U	log2(I/U)	p(I/U)
1417290_at	Lrg1	leucine-rich alpha-2-glycoprotein 1	NM_029796	2.214904	-3.47768	5.69257876	0.002079
1434862_at	Fut2	fucosyltransferase 2	NM_018876	2.467319	-2.92168	5.38900435	0.000156
1427912_at	Chr3	carbonyl reductase 3	NM_173047	2.211733	-2.61046	4.82219266	0.000164
1418368_at	Retnlb	resistin like beta	NM_023881	1.778031	-2.72516	4.5031901	0.009946
1448894_at	Akr1b8	aldo-keto reductase family 1, member B8	NM_008012	1.885915	-2.39124	4.27715117	0.001019
1449426_a_at	Anxa10	annexin A10	NM_011922	2.58174	-1.30503	3.88677067	0.014871
1423436_at	Gsta3	glutathione S-transferase, alpha 3	NM_010356	1.749687	-1.57075	3.32044152	0.000809
1416930_at	Ly6d	lymphocyte antigen 6 complex, locus D	NM_010742	1.776846	-1.44574	3.2225865	0.008456
1450355_a_at	Capp	capping protein (actin filament), gelsolin-like	NM_007599	1.51125	-1.4503	2.96155347	0.00895
1435323_a_at	Oact1	O-acyltransferase (membrane bound) domain containing 1	NM_153546	1.555977	-1.30783	2.86381027	0.000483
1451385_at	2310056P07Rik	RIKEN cDNA 2310056P07 gene	NM_027342	1.507869	-1.33072	2.83858784	2.05E-05
1415822_at	Scd2	stearoyl-Coenzyme A desaturase 2	NM_009128	1.602811	-1.20454	2.8073463	0.00261
1429298_at	Ddah1	dimethylarginine dimethylaminohydrolyase 1	NM_026993	1.348061	-1.42746	2.77552599	0.002004
1421040_a_at	Gsta2	glutathione S-transferase, alpha 2 (Yc2) similar to 3(20)alpha-hydroxysteroid/dihydrodiol/indanol dehydrogenase	NM_008182	1.481117	-1.2661	2.747722047	0.0042
1455454_at	LOC432720		XM_484209	1.277091	-1.46757	2.74465674	0.001789
1450387_s_at				1.25094	-1.32445	2.57539015	0.000246
1436216_s_at	2610204M08Rik	RIKEN cDNA 2610204M08 gene	NM_198411	1.009326	-1.54791	2.55723274	0.008098
1449071_at	MyI7	myosin, light polypeptide 7, regulatory	NM_022879	1.136152	-1.39022	2.52636822	0.004103
1449989_at	Mcp2t2	mast cell protease 2	NM_008571	1.08921	-1.40663	2.49583555	0.003886
1422352_at	Mcpt1	mast cell protease 1	NM_008570	0.873915	-1.57243	2.4463479	0.006933
1448330_at	Gstm1	glutathione S-transferase, mu 1	NM_010358	1.145585	-1.27552	2.42110106	0.000151
1416632_at	Mod1	malic enzyme, supernatant	NM_008615	1.429168	-0.9429	2.37206658	0.011268
1450107_a_at	Renbp	renin binding protein	NM_023132	1.493817	-0.85683	2.35064385	0.006697

ID	Gene Symbol	Gene Title	RefSeq ID	mean.I	mean.U	log2(I/U)	p(I/U)
1416416_x_at	Gstm1	glutathione S-transferase, mu 1	NM_010358	1.124017	-1.19292	2.31694166	4.09E-05
1430307_a_at	Mod1	malic enzyme, supernatant	NM_008615	1.043423	-1.2031	2.24652767	0.002884
1455400_at	Ddah1	dimethylarginine dimethylaminohydrolase 1	NM_026993	1.246352	-0.95225	2.19860002	0.006927
1424296_at	Gclc	glutamate-cysteine ligase, catalytic subunit	NM_010295	0.973383	-1.16794	2.1413236	0.001156
1455019_x_at	Ckap4	cytoskeleton-associated protein 4	XM_125808	1.075769	-1.02463	2.10040203	0.003772
1423418_at	Fdps	farnesyl diphosphate synthetase	NM_134469	0.756155	-1.31676	2.07291591	0.004561
1439435_x_at	Pgk1	phosphoglycerate kinase 1	NM_008828	0.79334	-1.26192	2.05525692	0.02788
1417803_at	1110032A04Rik	RIKEN cDNA 1110032A04 gene	NM_133675	0.84833	-1.19555	2.04387583	0.006268
1421375_a_at	S100a6	S100 calcium binding protein A6 (calcyclin)	NM_011313	1.0927	-0.93132	2.02401451	0.00695
1425627_x_at	Gstm1	glutathione S-transferase, mu 1	NM_010358	0.977062	-1.03267	2.00972721	0.002202
1451122_at	Idi1	isopentenyl-diphosphate delta isomerase	NM_145360	1.223132	-0.76426	1.98738926	0.03499
1424574_at	Tmed5	transmembrane emp24 protein transport domain containing 5	NM_028876	1.449332	-0.53564	1.98497388	0.041424
1419029_at	Ero1l	ERO1-like (S. cerevisiae)	NM_015774	0.930475	-1.04736	1.97783506	0.000372
1437453_s_at	Pcsk9	proprotein convertase subtilisin/kexin type 9	NM_153565	0.827672	-1.14278	1.97044942	0.002397
1451557_at	Tat	tyrosine aminotransferase	NM_146214	0.887583	-1.07524	1.96282238	0.001899
1421178_at	913041117Rik	RIKEN cDNA 913041117 gene	NM_026243	0.93384	-0.99413	1.92797099	0.013433
1454995_at	Ddah1	dimethylarginine dimethylaminohydrolase 1	NM_026993	0.850489	-1.06932	1.91980437	0.002597
1422567_at				0.887715	-1.01365	1.90136689	0.000338
1417061_at	Slc40a1	transporter), member 1	NM_016917	0.985143	-0.87237	1.8575088	0.036295

ID	Gene Symbol	Gene Title	RefSeq ID	mean.L	mean.U	log2(I/U)	p(I/U)
1437974_a_at	Hk1	hexokinase 1	NM_010438	1.10664	-0.73838	1.84501996	0.00151
1427747_a_at	Lcn2	lipocalin 2	NM_008491	0.74938	-1.09314	1.84252448	0.00719
1429001_at	Pir	pirin	NM_027153	1.091573	-0.7472	1.83877462	0.002743
1426980_s_at	E130012A19Rik	RIKEN cDNA E130012A19 gene	NM_175332	0.98309	-0.85361	1.83669759	0.016264
1451382_at	1810008K03Rik	RIKEN cDNA 1810008K03 gene	NM_026929	0.752649	-1.08122	1.83387175	0.017954
1421830_at				1.101856	-0.7169	1.81875567	0.001687
1450981_at	Cnn2	calponin 2	NM_007725	0.895615	-0.89459	1.79020899	0.000142
1420641_a_at	Sqrd1	sulfide quinone reductase-like (yeast)	NM_021507	0.952196	-0.83019	1.78238679	0.000766

## Downregulated Genes

ID	Gene Symbol	Gene Title	RefSeq ID	mean.L	mean.U	log2(I/U)	p(I/U)
1416809_at	Cyp3a11	cytochrome P450, family 3, subfamily a,	NM_007818	-1.98374	1.71074	-3.6944844	0.000995
1417133_at	Pmp22	peripheral myelin protein	NM_008885	-2.35657	1.28085	-3.6374153	0.031975
1448723_at	Rdh7	retinol dehydrogenase 7	NM_017473	-1.59115	1.7949	-3.3860558	0.001704
1417070_at	Cyp4v3	polypeptide 3	NM_133969	-1.7829	1.52569	-3.3085944	0.011037
1426990_at	Cubn	cubilin (intrinsic factor-cobalamin receptor)	XM_130038	-1.41114	1.8842	-3.2953408	0.020306
1417751_at	Stk10	serine/threonine kinase 10	NM_009288	-1.82401	1.25695	-3.0809599	0.049355
1421637_at	Slc5a4a	solute carrier family 5, member 4a	NM_133184	-1.12539	1.76401	-2.8893978	0.015732
1419582_at	Cyp2c55	cytochrome P450, family 2, subfamily c,	NM_028089	-1.22861	1.64978	-2.8783874	0.00643
1452270_s_at	Cubn	cubilin (intrinsic factor-cobalamin receptor)	XM_130038	-0.98009	1.87312	-2.8532059	0.035707

ID	Gene Symbol	Gene Title	RefSeq ID	mean.I	mean.U	log2(I/U)	p(I/U)
1417071_s_at	Cyp4v3	cytochrome P450, family 4, subfamily v, polypeptide 3	NM_133969	-2.09066	0.69803	-2.7886836	0.036964
1416203_at	Aqp1	aquaporin 1	NM_007472	-1.19094	1.56677	-2.7577102	0.001791
1451681_at	CRAD-L	dehydrogenase-like	NM_145424	-1.0546	1.66188	-2.7164785	0.002746
1426399_at	4932416A11Rik	RIKEN cDNA 4932416A11 gene	NM_147776	-1.96729	0.69341	-2.6607018	0.024182
1424973_at	Cyp3a25	cytochrome P450, family 3, subfamily a, polypeptide 25	NM_019792	-1.21727	1.42779	-2.6450605	0.000861
1451787_at	Cyp2b20	cytochrome P450, family 2, subfamily b, polypeptide 20	NM_009999	-1.54377	1.07867	-2.6224429	0.003152
1424671_at	Plekhf1	family F (with FYVE domain) member 1	NM_024413	-1.59518	1.01598	-2.6111573	0.004747
1417991_at	Dio1	deiodinase, iodothyronine, type I	NM_007860	-1.84995	0.75454	-2.604496	0.012756
1428752_at	Slc5a11	solute carrier family 5 (sodium/glucose cotransporter), member 11	NM_146198	-1.53747	1.00928	-2.546745	0.003177
1427746_x_at	H2-K1	histocompatibility 2, K1, K region	NM_001001	-1.53683	1.00424	-2.5410751	0.002998
1422257_s_at	Cyp2b20	polypeptide 10 /// cytochrome P450, family	NM_009999	-1.36606	1.17085	-2.5369155	0.000197
1416316_at				-1.14728	1.38106	-2.528339	0.006397
1416645_a_at	Afp	alpha fetoprotein	NM_007423	-1.23309	1.28376	-2.5168426	0.000114
1425645_s_at	Cyp2b10 /// Cyp2b20	cytochrome P450, family 2, subfamily b, polypeptide 10 /// cytochrome P450, family 2, subfamily b, polypeptide 20	NM_009998 NM_009999	-1.42127	1.01453	-2.4358082	0.000898
1421912_at	Slc23a1	solute carrier family 23 (nucleobase transporters), member 1	NM_011397	-1.3021	1.13204	-2.4341417	0.02478
1426278_at	Ifi27	interferon, alpha-inducible protein 27	NM_029803	-1.1389	1.23738	-2.3762745	0.000303
1449375_at	Ces6	carboxylesterase 6	NM_133960	-1.47784	0.89044	-2.3682886	0.008566
1450133_at	2010321J07Rik	RIKEN cDNA 2010321J07 gene	NM_028094	-0.99315	1.37292	-2.3660728	0.005122
1439479_at	Lct	lactase	XM_129479	-1.84674	0.47441	-2.3211555	0.040498

ID	Gene Symbol	Gene Title	RefSeq ID	mean.I	mean.U	log2(I/U)	p(I/U)
1418283_at	Cldn4	claudin 4	NM_009903	-1.52052	0.68246	-2.2029786	0.048912
18SRNAMur/X				-0.92702	1.26083	-2.1878536	0.03084
1417590_at	Cyp27a1	cytochrome P450, family 27, subfamily a, polypeptide 1	NM_024264	-1.32043	0.86149	-2.1819257	0.01143
1425120_x_at	1810023F06RIK	RIKEN cDNA 1810023F06 gene	NM_145449	-1.55017	0.57475	-2.1249216	0.018221
1448123_s_at				-1.39514	0.71873	-2.1138657	0.024285
1415871_at				-1.42313	0.6835	-2.1066307	0.020371
1448470_at	Fbp1	fructose biphosphatase 1	NM_019395	-0.87856	1.19448	-2.0730438	0.003984
	///H2-L///	histocompatibility 2, K1, K region ///	892				
1424948_x_at	LOC56628	histocompatibility 2, D region ///	NM_010380	-1.23534	0.80078	-2.0361199	0.014681
1448683_at	Cyp2d26	polypeptide 26	NM_029562	-1.53988	0.45894	-1.998816	0.035611
1433817_at	Agpat3	acyltransferase 3	NM_053014	-1.03494	0.95922	-1.9941572	0.001181
1417961_a_at	Trim30	tripartite motif protein 30	NM_009099	-0.93838	1.05305	-1.9914289	4.23E-05
1448786_at	1100001H23RIK	RIKEN cDNA 1100001H23 gene	NM_025806	-1.06729	0.87287	-1.9401619	0.021174
1425119_at	Oas1b	2'-5' oligoadenylate synthetase 1B	NM_011853	-1.10068	0.83935	-1.9400304	0.017179
1429076_a_at	Gdppd2	glycerophosphodiester phosphodiesterase domain containing 2	NM_023608	-1.1312	0.80481	-1.9360108	0.016986
1443671_x_at	2010001J22RIK	RIKEN cDNA 2010001J22 gene	XM_128172	-1.4826	0.44886	-1.9314557	0.037626
1433819_s_at				-0.73375	1.19213	-1.9258771	0.003535
1416646_at	Afp	alpha fetoprotein	NM_007423	-1.11568	0.80635	-1.9220307	0.002225
1457271_at	Gm131	gene model 131, (NCBI)	XM_131138	-0.96465	0.9374	-1.9020511	0.005672
1435697_a_at	Pscdbp	pleckstrin homology, Sec7 and coiled-coil domains, binding protein	NM_139200	-0.70661	1.19445	-1.9010545	0.011689

<b>ID</b>	<b>Gene Symbol</b>	<b>Gene Title</b>	<b>RefSeq ID</b>	<b>mean.I</b>	<b>mean.U</b>	<b>log2(I/U)</b>	<b>p(I/U)</b>
1427221_at	MGI:2143217	X transporter protein 3 similar 1 gene	NM_139142	-0.69291	1.20556	-1.8984749	0.042024
1434457_at	Sp100	nuclear antigen Sp100	NM_013673	-1.13903	0.74019	-1.8792177	0.006135